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Properties of a herpes simplex virus multiple immediate-early gene-deleted recombinant as a vaccine vector

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Abstract

Herpes simplex virus (HSV) recombinants induce durable immune responses in rhesus macaques and mice and have induced partial protection in rhesus macaques against mucosal challenge with virulent simian immunodeficiency virus (SIV). In this study, we evaluated the properties of a new generation HSV vaccine vector, an HSV-1 multiple immediate-early (IE) gene deletion mutant virus, *d*106, which contains deletions in the *ICP4*, *ICP27*, *ICP22*, and *ICP47* genes. Because several of the HSV IE genes have been implicated in immune evasion, inactivation of the genes encoding these proteins was expected to result in enhanced immunogenicity. The *d*106 virus expresses few HSV gene products and shows minimal cytopathic effect in cultured cells. When *d*106 was inoculated into mice, viral DNA accumulated at high levels in draining lymph nodes, consistent with an ability to transduce dendritic cells and activate their maturation and movement to lymph nodes. A *d*106 recombinant expressing *Escherichia coli* β-galactosidase induced durable β-gal-specific IgG and $CD8^+$ T cell responses in naive and HSV-immune mice. Finally, *d*106-based recombinants have been constructed that express simian immunodeficiency virus (SIV) gag, env, or a rev-tat-nef fusion protein for several days in cultured cells. Thus, *d*106 shows many of the properties desirable in a vaccine vector: limited expression of HSV gene products and cytopathogenicity, high level expression of transgenes, ability to induce durable immune responses, and an ability to transduce dendritic cells and induce their maturation and migration to lymph nodes.

Keywords: Replication-defective virus; Vaccine vector; SIV

Introduction

Viral recombinant strains expressing heterologous proteins have been tested widely as vaccine vectors against a number of virus infections (Murphy and Chanock, 2001), and a number of viruses, including pox viruses, adenoviruses, parvoviruses, alpha viruses, and herpes viruses have been utilized. Despite the extensive testing of various vaccine strategies as AIDS vaccines, none have been very effective against the pathogenic simian immunodeficiency virus (SIV) strains in nonhuman primates in the infection models that most closely approximate human immunodeficiency virus (HIV) infection of humans. Thus, there is a continuing dire need to design and test novel vaccine approaches as candidate AIDS vaccines.

We have previously used herpes simplex virus 1 recombinants as vaccine vectors expressing SIV envelope protein and have shown that these recombinants could induce partial protection against mucosal SIVmac239 infection in rhesus macaques (Murphy et al., 2000). One of these vectors, HSV-1 *d*27, was a replication-defective mutant strain that was defective for the *UL54* (*ICP27*) gene. In this study, we have attempted to

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improve upon that first-generation vaccine vector by the use of recombinant HSV-1 strains that contain additional mutations beyond the *ICP27* gene mutation.

Herpes simplex virus productive infection involves the expression of a series of kinetically regulated groups of viral genes (Roizman and Knipe, 2001). Viral immediate-early (IE) genes are transcribed immediately upon viral entry without prior viral protein synthesis. Three of the IE proteins, ICP4, ICP0, and ICP27, stimulate later viral gene expression. The next set of genes expressed, the early genes, encode viral proteins mostly involved in viral DNA replication. Following viral DNA replication, the late viral genes are expressed optimally. HSV infection shuts down host transcription, RNA splicing, and protein synthesis. Inhibition of host cell protein synthesis requires both the virion host shutoff (vhs) function and the IE ICP27 protein (Smiley, 2004; Song et al., 2001). Inhibition of host transcription requires both ICP4 and ICP27 (Spencer et al., 1997) whereas inhibition of RNA splicing requires ICP27 (Hardy and Sandri-Goldin, 1994).

HSV recombinants have a number of properties that would be advantageous for a vaccine vector. HSV can infect a wide range of tissues and host species and can generate immune responses by various routes of inoculation, including mucosal (intranasal or intravaginal) administration. HSV induces durable immune responses, in part due to latent infection and reactivation, but replication-defective mutant HSV strains induce equally durable immune responses (Morrison and Knipe, 1994). Herpesviral recombinants can accommodate sizeable inserts of heterologous DNA for vectoring purposes (Knipe et al., 1978) and induce Th1-biased cellular responses (Brubaker et al., 1996; Nguyen et al., 1994), which are desirable properties for a vaccine vector. HSV-1 activates TLR2 (Kurt-Jones et al., 2004, 2005) to induce a pro-inflammatory cytokines as well as activating TLR9 to induce type I interferons (Krug et al., 2004; Lund et al., 2003). Also, an HSV replication-defective vector expressing β-galactosidase showed full immunogenicity in the presence of pre-existing anti-HSV immunity (Brockman and Knipe, 2002).

However, HSV recombinants express a large number of HSV proteins that could either evade or blunt the host immune response or compete for immunogenicity with a vector-expressed heterologous antigen. Therefore, mutating the *ICP4* or *ICP27* genes could improve the immunogenicity of the transgene product by reducing the number of HSV gene products expressed. Similarly, ICP4 and ICP27 inhibit the transcription of host genes, and this could decrease antigenic presentation by down-regulating MHC class I in infected cells, for example. HSV ICP47 has been shown to block TAP transport of peptides into the ER for loading onto MHC class I (York et al., 1994) whereas ICP22 has been reported to inhibit MHC class II presentation (Barcy and Corey, 2001). Thus, mutational inactivation of these genes would be expected to increase the immunogenicity of a vaccine vector. An additional approach that addresses these issues is the use of HSV amplicon or replicon strains (Spaete and Frenkel, 1982) as vaccine vectors. HSV-1 amplicons expressing HIV envelope are immunogenic in mice (Gorantla et al., 2005; Hocknell et al., 2002) but no information is available about immunogenicity in primates. Production issues may limit this approach using current technology.

Samaniego et al. (1998) studied the properties of a number of recombinant HSV-1 strains that are defective for various permutations of IE genes. A recombinant virus in which all IE genes were inactivated showed the lowest cytopathic effect on host cells but very low expression of the GFP transgene. The *d*106 recombinant strain, which expresses only one IE protein, ICP0, showed the highest GFP transgene expression balanced with less cytopathogenicity than many of the other strains. The *d*106 virus contains deletions of the *ICP4* and *ICP27* ORFs and contains deletions of the promoter/enhancers of the *ICP22* and *ICP47* genes (Samaniego et al., 1998). The *d*106 virus can be grown in cell lines that contain the *ICP4* and *ICP27* genes, which are activated upon viral infection and complement the defects in those genes. Expression of ICP4 enhances expression of ICP22, allowing enhanced growth of the virus in the cells. Because of the potential for expression of the transgene and limited cytopathogenicity, we have chosen the *d*106 strain for study as a second-generation HSV-1 replication-defective vaccine vector.

Results

Properties of d106 virus as a vaccine vector

We have previously used an HSV-1 replication-defective *ICP27* (*UL54*) gene mutant, HSV-1 *d*27, as a vaccine vector for expression of SIV proteins and immunization of rhesus macaques, resulting in protection of some of the monkeys against mucosal challenge infection with pathogenic SIV mac239 virus (Murphy et al., 2000). To try to improve upon the *d*27 vector, we tested a virus with additional IE gene mutations, the *d*106 mutant strain (Samaniego et al., 1998). HSV-1 *d*106 (Fig. 1A) contains deletions inactivating four of the five IE genes (*ICP4*, *ICP27*, *ICP22*, and *ICP47*) and has an expression cassette containing the green fluorescent protein (GFP) gene under the control of the human cytomegalovirus (HCMV) IE promoter-enhancer inserted into the *ICP27* gene locus as a model transgene (Fig. 1A). ICP4 is the major transactivator of E and L viral genes; therefore, deletion of the *ICP4* gene would decrease HSV gene expression, reducing antigenic competition with the transgene products. ICP47 binds to the TAP peptide transporter and blocks peptide transport into the endoplasmic reticulum and loading of MHC class I, thereby blocking MHC class I presentation (York et al., 1994). ICP22 has also been reported to inhibit MHC class II presentation (Barcy and Corey, 2001); therefore, its lack of expression should also improve immunogenicity of the vector.

Samaniego et al. (2001) compared a number of HSV-1 strains that were mutated for varying numbers of HSV IE genes. They found that *d*106 showed more GFP transgene expression than other mutant strains. We therefore chose this strain for testing as a vaccine vector. Because Samaniego et al. (2001) had observed that *d*106 infection caused a reduction in colony formation in infected Vero cells, we first examined the cytopathic effect and host protein synthesis shutoff induced

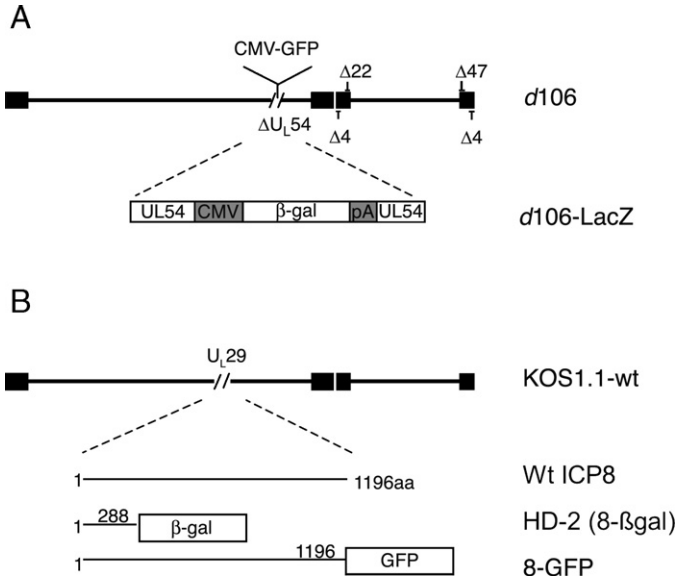

Fig. 1. Genomic structures of HSV-1 recombinants used in this study. (A) Genomic maps of *d*106 and *d*106-LacZ recombinants. A β-galactosidase expression cassette was inserted into the *UL54* gene by homologous recombination in the *d*106-LacZ virus. CMV, promoter/enhancer sequences of the CMV IE gene; PA, polyadenylation signal. (B) HSV-1 wt strain KOS1.1 and replication-defective KOS1.1-derived recombinant HD-2 and replication-competent recombinant 8-GFP. HD-2 encodes a truncated ICP8 protein fused to *E. coli* β-galactosidase (β-gal). The 8-GFP virus encodes a full-length ICP8 protein fused to green fluorescent protein (GFP).

by *d*106 infection over a shorter time frame. For comparison, we used a replication-competent HSV-1 strain, 8GFP, which is derived from HSV-1 strain KOS1.1 and expresses GFP fused to the viral ICP8 protein (Fig. 1B). HSV-1 vhs and ICP27 are both required for shutoff of host protein synthesis (Song et al., 2001). Because of the *ICP27* gene defect in *d*106, we expected that there would be reduced host shutoff in *d*106-infected cells.

To study the effects of *d*106 on its host cells, we infected Vero cells (MOI = 1 PFU/cell) with either *d*106 or 8GFP. First, we examined the morphological changes occurring in infected cells. At 22 h post infection (hpi), cells were fixed and examined by phase-contrast and fluorescence microscopy (Fig. 2A). GFP was expressed in cells infected with each of the viruses. Cells infected with HSV-1 8GFP showed swelling and rounding, characteristic of the typical morphological changes seen in HSV infection. The *d*106-infected Vero cells did not show any remarkable morphological changes through at least 22 hpi.

Second, we examined host protein synthesis to determine the effects of *d*106 virus infection. Vero cells were infected (MOI = 1) with *d*106, 8GFP, or wt HSV-1. At the times indicated in Fig. 2B, cells were pulse-labeled with ^{35}S-methionine for 30 min. After labeling, the cells were lysed in SDS sample buffer and analyzed by SDS–PAGE. In 8GFP- and WT virus-infected cell lysates, viral protein synthesis and the shutoff of host cell protein synthesis were observed. In contrast, in *d*106-infected cell lysate, viral gene expression was restricted in that only ICP0, ICP6 (an early viral protein induced by ICP0) and GFP were observed. Host protein synthesis was maintained through at least 12 h following *d*106 infection (Fig. 2B).HSV-1 infection has been shown to inhibit the maturation of dendritic cells (Salio et al., 1999). Because *d*106 shows little to no host shutoff, we expected that *d*106 would not inhibit dendritic cell maturation. To determine the effect of *d*106 on maturation and lymph node migration of dendritic cells in vivo, we measured the amount of viral DNA in the draining lymph nodes (LNs) of

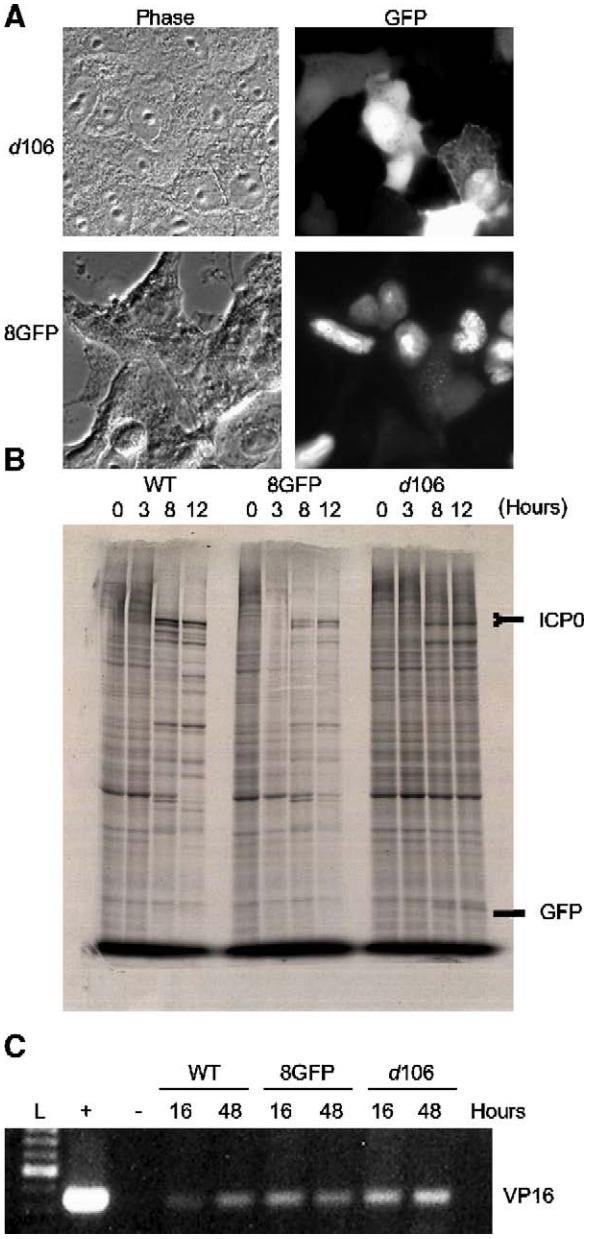

Fig. 2. Properties of the multiple IE gene-deleted HSV-1 d106 recombinant. (A) Comparison of morphological changes between *d*106- and 8GFP-infected cells (MOI = 1) at 20 h post infection. Phase contrast and fluorescence images are shown. (B) Analysis of infected cell protein synthesis. Vero cells were infected at MOI = 1 with wild-type HSV strain KOS1.1, *d*106 or 8GFP. At the indicated times, cells were pulse-labeled with ^{35}S-methionine for 30 min. Infected-cell lysates were resolved by SDS–PAGE. (C) Detection of viral DNA in the draining lymph nodes. BALB/cJ mice were infected by subcutaneous infection into the right flank with 2×10^6 PFU of KOS1.1, *d*106, or 8GFP. At the indicated times, inguinal lymph nodes were removed and viral DNA was detected by PCR in 50 ng of total DNA using primers specific for the *VP16* gene.

mice after viral inoculation. Balb/cJ mice were infected by subcutaneous (s.c.) infection into the right flank with $2x10^6$ PFU of KOS1.1, 8GFP, or *d*106. At the indicated times, the mice were sacrificed, inguinal LNs were removed, and DNA was extracted. HSV DNA was detected by PCR amplification of *VP16* gene sequences and agarose gel electrophoresis, using 50 ng of total DNA (Fig. 2C). WT HSV-1 DNA was detected at only low levels at 16 hpi but higher amounts of 8GFP and high amounts of *d*106 DNA were detected at that time (Fig. 2C). The highest amounts of viral DNA were detected in lymph nodes from *d*106 virus-infected mice, indicating the ability of this virus to transduce cells that migrate to the lymph nodes.

Taken together, these experiments showed that *d*106 has several properties desired in a vaccine vector: limited cytopathogenicity, high-level expression of transgenes, and the ability to transduce dendritic cells and induce their maturation.

A d106 recombinant virus expressing β-galactosidase as a model antigen

To measure immune responses against a model antigen, β-galactosidase (β-gal), we replaced the GFP expression cassette in *d*106 with a *lac*Z expression cassette to generate the *d*106-LacZ virus (Fig. 1A), as described in Materials and methods. To test for expression of β-gal, Vero cells were infected with either *d*106-LacZ or with HD-2 (Fig. 1B), an HSV-1 strain that expresses β-gal fused to the N-terminal 282 residues of the viral ICP8 protein (Gao and Knipe, 1989). At the indicated times (Fig. 3A), infected cells were harvested, and western blotting was performed with an anti-β-gal antibody. β-Gal was detected in *d*106-LacZ-infected Vero cell lysates by at least 6 hpi and increased until 48 hpi. In contrast, β-gal expression peaked at a lower level by 12 hpi in cells infected with HD-2 (Fig. 3A).

As an alternative quantitative assay of β-gal expression, we measured enzymatic activity in infected cell lysates (Fig. 3B). At 12 hpi, the amount of β-gal activity in *d*106-LacZ-infected Vero cells was more than five times greater than that of HD-2-infected cells, and at 24 h post infection, *d*106-LacZ-infected cells still showed high expression of β-gal. These data indicated that *d*106-LacZ can express high amounts of β-gal upon infection of cultured cells.

Immunization with the d106-LacZ virus induced robust and durable IgG antibody responses in mice

To examine the β-gal-specific immune responses induced by *d*106 or other HSV vectors, we immunized mice and examined humoral and cellular responses. Groups of eight BALB/cJ mice were inoculated s.c. in the flank with 2×10^6 PFU of *d*106-LacZ or HD-2 virus or mock-infected with uninfected Vero cell lysate. Booster immunizations were performed at 3 and 6 weeks. Serum samples were collected from the mice at the indicated times, and β-gal-specific IgG antibody titers were measured by ELISA. We observed that the β-gal antibody responses in *d*106-LacZ-infected mice were several fold higher than those in HD-2-inoculated mice at early times (Fig. 4A). We also observed that β-gal-specific antibody responses in *d*106-LacZ mice plateaued at approximately 12 weeks and were maintained at that level for at least 40 weeks, approximately 40% higher than titers than those of HD-2 virus-inoculated mice (Fig. 4B). Therefore, *d*106 has the ability to generate a durable IgG response against a transgene product.

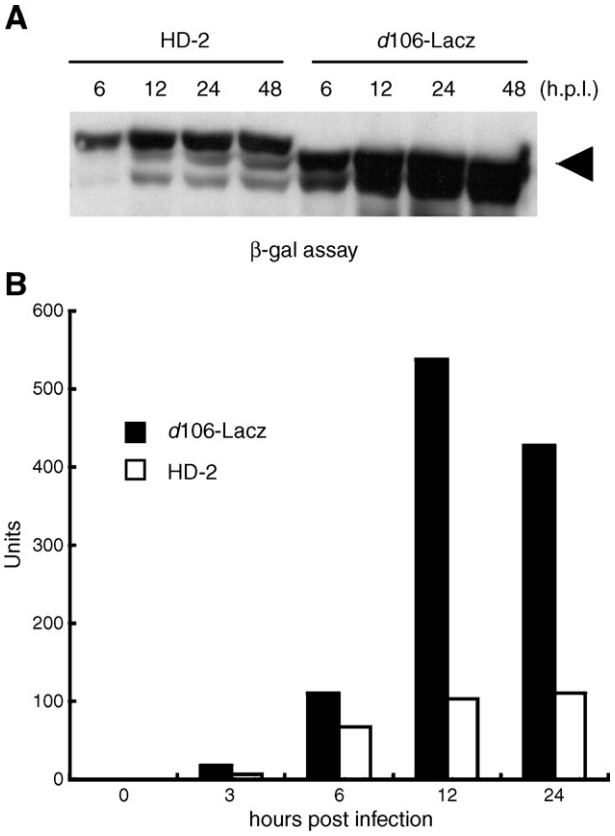

Fig. 3. Expression of β-galactosidase in recombinant virus-infected cells. (A) Vero cells were infected with HD-2 or *d*106-LacZ and harvested at indicated times. Proteins were separated by SDS–PAGE and electrophoretically transferred to PVDF membranes. Western blotting was performed using anti-β-gal antibody. (B) Measurement of β-gal enzymatic activity. Vero cells were infected with *d*106-LacZ or HD-2. At the times indicated, cells were lysed in reporter lysis buffer and enzymatic activity was measured as described in Materials and methods.

Pre-existing anti-HSV immunity does not diminish antibody responses to HSV vector

We observed previously that preexisting host anti-HSV immunity does not diminish the efficacy of an HSV replication-defective virus vector (Brockman and Knipe, 2002). To test if *d*106 shared this property, we first infected mice with 2×10^6 PFU of wild-type HSV-1 or mock-infected mice with Vero cell lysate at 0 and 3 weeks and then inoculated the mice with 2×10^6 PFU of *d*106-LacZ or HD-2 virus at 6, 9, and 12 weeks. We used cell-free virus stocks of the latter two viruses to reduce the levels of soluble β-gal protein in the virus stock, which could induce antibody responses independently of virus infection. Sera were collected at weeks 3, 6, 9, 12, and 15, and β-gal-specific IgG antibody titers were measured by ELISA. Despite the increased level of anti-HSV immunity

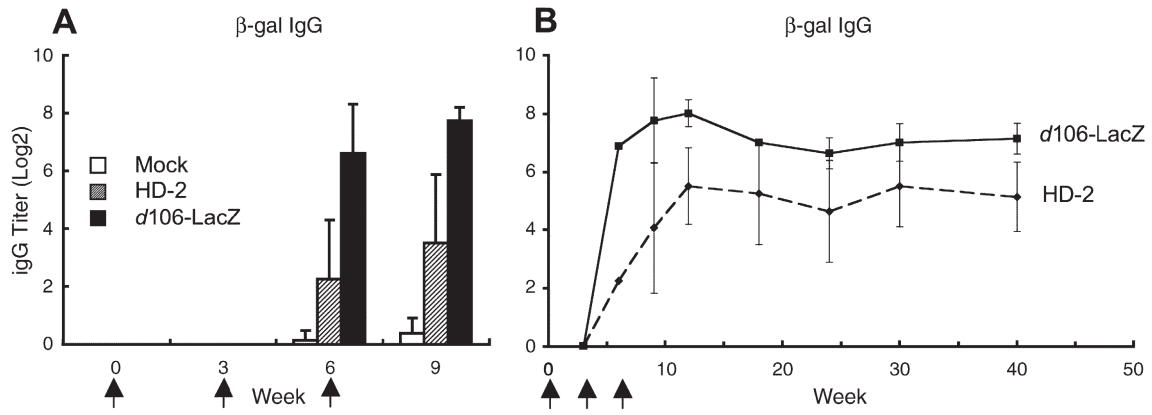

Fig. 4. Induction and durability of IgG responses in mice. The induction of IgG antibody specific for β-galactosidase (A) and the durability of the β-gal IgG responses (B) are shown. Groups of six mice were immunized with 2×10^6 PFU of *d*106-LacZ or HD-2 at weeks 0, 3, and 6 (arrows). Serum samples were collected at the indicated times, and β-gal-specific IgG antibody titers were determined by ELISA. Results are shown as the mean titer ($\log_2$)±standard deviation.

elicited by prior WT HSV-1 infection (mean Log_2 IgG titer=7.75 at week 6, data not shown), HD-2 virus (Fig. 5A) or the *d*106-LacZ virus (Fig. 5B) induced β-gal-specific IgG antibody responses that were equivalent in mock-treated versus immune animals. The IgG titers induced by *d*106-LacZ were more than 4 times greater than those by HD-2 in both naive and immunized mice. Therefore, *d*106-LacZ recombinant virus elicited robust β-gal-specific antibody responses even in mice having preexisting anti-HSV-1 antibody.

Antibody responses to β-galactosidase were diminished by UV irradiation of the vector

The results of ELISA assay showed that both infected cell lysate and supernatant stocks of *d*106-LacZ induced β-gal-specific antibody responses in mice. To further confirm that the β-gal-specific antibody responses that we observed were the result of vector-expressed antigen, we examined IgG responses in mice immunized with virus in cell lysates and cell supernatant virus preparations either UV-inactivated or not treated. UV treatment resulted in a 2000-fold reduction in the infectivity of the viruses (results not shown). Groups of six mice were inoculated s.c. with 2×10^6 PFU (doses of UV-treated viruses were based on titers prior to UV treatment) of lysate or cell-free *d*106-LacZ or cell-free wt HSV-1 virus at 0 and 3 weeks. Serum samples were collected from each mouse prior to infection (week 0) and at 3 and 5 weeks after infection. Both β-gal- and HSV-specific IgG antibody responses were analyzed by ELISA. Wt virus-immunized mice showed high levels of HSV-1 IgG at both 3 and 5 weeks (Fig. 6A). The *d*106-LacZ virus-immunized mice also showed anti-HSV-1 IgG immune responses but these responses were lower than those of WT virus-infected mice. UV treatment caused a limited reduction in the HSV-specific IgG response against HSV-1 in WT or *d*106-LacZ immunized mice. On the other hand, UV inactivation reduced the β-gal-specific IgG responses in *d*106-LacZ virus-

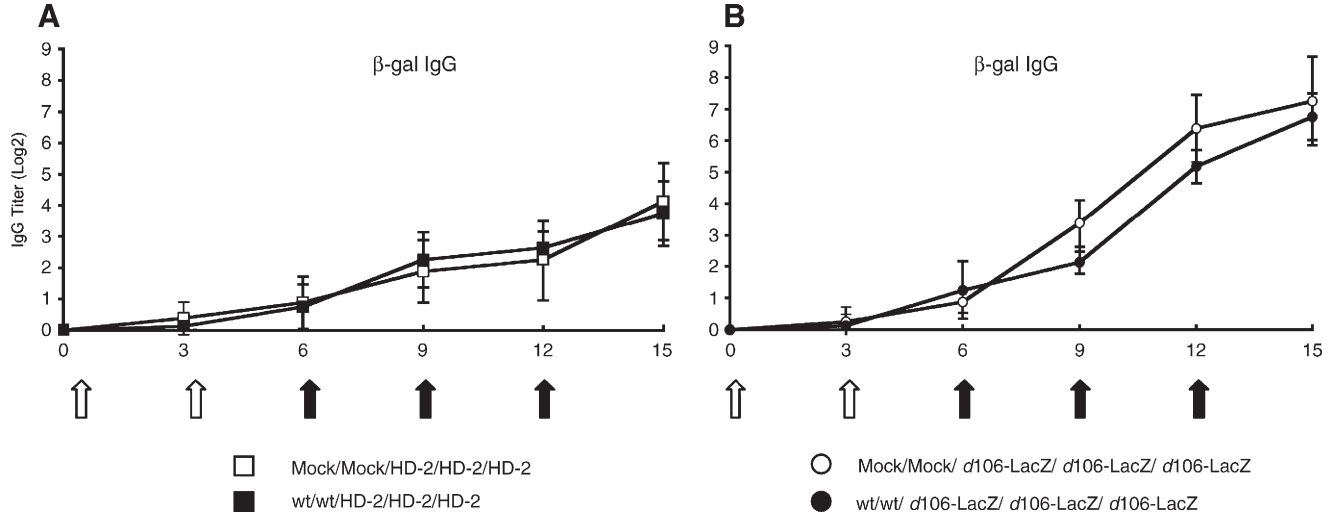

Fig. 5. Induction of β-galactosidase IgG after prior HSV-1 infection. Titers of IgG specific for β-gal induced by HD-2 (A) and *d*106-LacZ (B) are shown. Groups of six mice were immunized twice, at weeks 0 and 3 (open arrows), with 2×10^6 PFU of wt HSV-1 or mock infected. At weeks 6, 9, and 12, all mice were inoculated with 2×10^6 PFU of cell-free HD-2 or *d*106-LacZ virus (solid arrows). Serum samples were collected at the indicated times, and IgG was detected by ELISA. Results are shown as the mean reciprocal dilution ($\log_2$)±standard deviation.

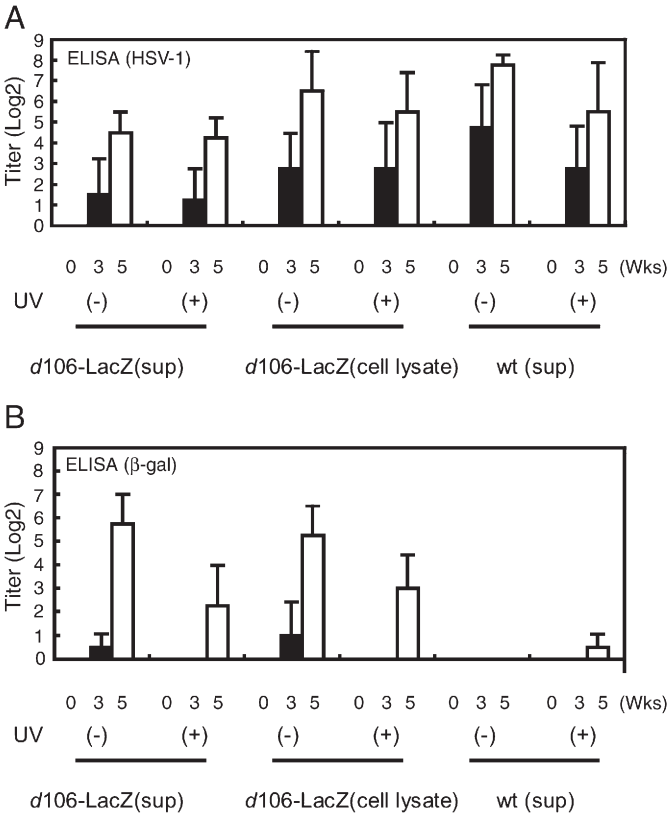

Fig. 6. Effect of UV inactivation of the recombinant viruses on IgG responses. Groups of six mice were immunized twice with cell-free *d*106-LacZ, cell-lysate *d*106-LacZ, or purified WT KOS1.1 virus with or without UV irradiation (2×10^6 PFU prior to treatment, 1×10^3 PFU following treatment), at weeks 0 and 3. Serum samples were collected from each mouse prior to infection (week 0) and at 3 weeks and 5 weeks after infection. HSV-1-specific (A) and β-galactosidase-specific (B) IgG antibody titers were determined by ELISA, and results are shown as the mean reciprocal dilution ($\log_2$)±standard deviation.

immunized mice. We concluded that the de novo expression of β-galactosidase in infected mice was necessary for generation of robust β-gal-specific IgG responses.

CD8$^+$ T cell responses to HSV gB and β-gal epitopes

To measure the CD8$^+$ T cell responses induced by *d*106-LacZ virus immunization, we performed ELISPOT assays to detect HSV-1 glycoprotein B- (gB-1) or β-gal-specific CD8$^+$ T cell responses. Groups of fifteen C57BL/6 mice were immunized by s.c. inoculation with 2×10^6 PFU of either cell-free *d*106-LacZ or HD-2 virus or mock infected. Booster immunizations were performed at days 14 and 42. ELISPOT assays were performed on splenocytes from mice at days 3, 7, 14, 21, and 63 (three mice per group per time point). HD-2 virus elicited a strong cellular response to gB-1 (Fig. 7A) at 7 days with a response of 1200 spot-forming cells (SFC) per 10^6 splenocytes. By day 63, the response had leveled off at 300–400 IFNγ SFC per 10^6 splenocytes (Fig. 7A). In contrast, recombinant *d*106-LacZ virus induced only a weak T cell response specific for the gB-1 epitope (Fig. 7A) consistent with its low level of viral protein expression. β-gal-specific cellular immune responses induced by *d*106-LacZ virus were observed by 7 days and continued to increase through at least 20 weeks to 400 SFC/106 splenocytes (Fig. 7B). HD-2 also elicited cellular immunity to β-gal, but the numbers of SFC by HD-2 were lower than those by *d*106-LacZ throughout the study (Fig. 7B). Therefore, *d*106-LacZ virus induced specific cellular immune responses against the transgene, β-galactosidase, without inducing significant HSV-1 gB-specific CD8$^+$ T cell immune responses.

Construction of d106 vectors expressing simian immunodeficiency virus proteins

The studies described above indicated that *d*106 is a good candidate for recombinant vaccine vector. As the first step in the application of *d*106 to viral vaccine vectors, we constructed *d*106-based recombinants expressing SIV gag, env, and rev-tat-nef (RTN) fusion proteins (Fig. 8A) for further studies in nonhuman primates. We inserted RNA-optimized ORFs encoding SIV p55 gag protein (Rosati et al., 2005) and SIV env gp160 protein (Von Gegerfelt and Felber, 1997) into pCIΔAflII expression vectors (Murphy et al., 2000) and demonstrated that the cassettes expressed the appropriate

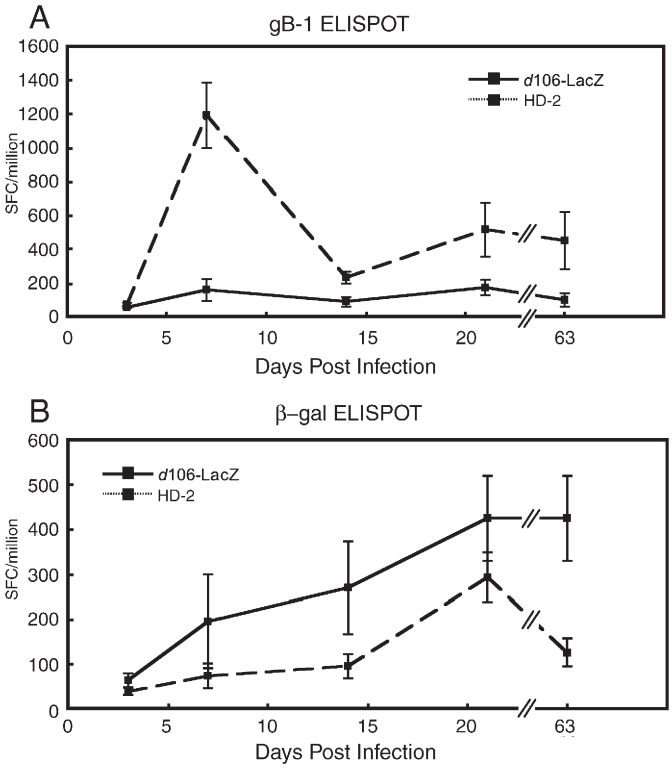

Fig. 7. Detection of IFN-γ-producing cells by ELISPOT assay. Groups of fifteen mice were immunized with 2×10^6 PFU of cell-free *d*106-LacZ or HD-2 at days 0, 14, and 42. At days 3, 7, 14, 21, and 63, splenocytes were collected from each recombinant-infected mice (three mice per group per time point) and were stimulated with an MHC-class I (H-2Kb)-specific peptide for HSV-1 gB (A) or β-galactosidase (B). Results are shown as the mean number of spot forming cells (SFC)/10^6 splenocytes±standard deviation.

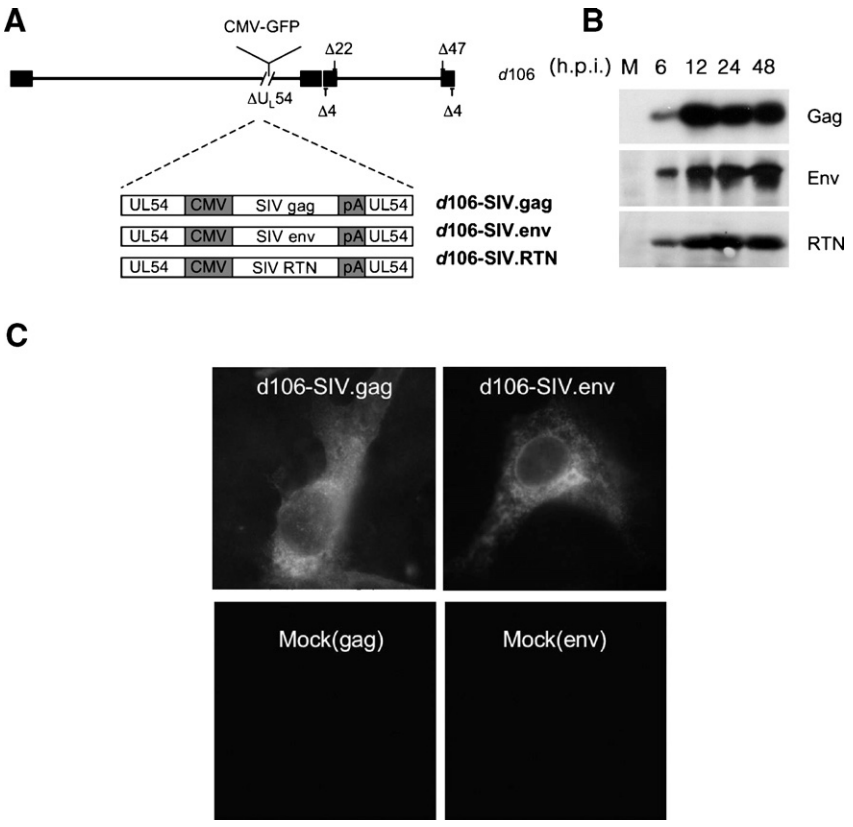

Fig. 8. Properties of d106 recombinants expressing SIV proteins. (A) Genomic maps of *d*106 recombinants. SIV coding sequences were inserted into the *UL54* gene in each recombinant by homologous recombination. CMV, promoter/enhancer sequences of the CMV IE gene; PA, polyadenylation signal. (B) Expression of SIV proteins was detected by western blotting. Vero cells were infected with the indicated viruses or mock infected. Cells were harvested at indicated times, and lysates were resolved by SDS–PAGE. SIV proteins were detected following incubation with specific monoclonal antibodies described in the text. (C) Localization of SIV gag and env protein in cells infected with the *d*106 vectors. Vero cells were infected with *d*106-SIV.gag or *d*106-SIV.env, fixed with 2% formaldehyde, permeabilized with cold acetone at 22 hpi, and stained with specific monoclonal antibodies against gag or env.

protein (results not shown). Second, we constructed a rev-tat-nefΔ fusion protein expression cassette as described in Materials and methods. The expression cassettes were inserted into the pPs27pd1 transfer plasmid (Rice et al., 1989) and then introduced into the U_L54 locus in the *d*106 genome by homologous recombination as described in Materials and methods. SIV protein expression by these three recombinants was analyzed by western blots (Fig. 8B). Vero cells were mock-infected or infected with *d*106-SIV.gag, *d*106-SIV.env, or *d*106-SIV.RTN. Proteins in the cell lysates were resolved by SDS–PAGE, and blots were probed with anti-gag, anti-gp120, or anti-Nef monoclonal antibody (Fig. 8B). SIV gag expression was detected by 6 hpi, peaked at 12 hpi, and was maintained in the cells through at least 48 hpi. SIV env expression was detected by 6 hpi and increased through 48 hpi. The rev-tat- nef fusion protein was expressed by 6 hpi, also peaked at 24hpi and was maintained through at least 48 hpi. Thus, *d*106 virus recombinants were capable of long-term expression of these SIV proteins.

Immunofluorescence studies were conducted to determine the intracellular location of env and gag in cells infected with these two *d*106 vectors. In *d*106-SIV.gag-infected cells, gag was distributed throughout the cytoplasm (Fig. 8C). In cells infected with *d*106-SIV.env, reticular staining characteristic of the ER and bright perinuclear fluorescent staining consistent with localization of env in the Golgi apparatus was observed (Fig. 8C).

Finally, the kinetics of SIV env gene expression was examined in cells infected with two HSV-1 replication-defective mutant vectors, *d*106-SIV.env and *d*81 (HSV-1 *ICP27* gene deletion mutant vector) (Murphy et al., 2000). Env protein was expressed at higher levels in *d*106-SIV.env-infected cells and increased through at least 48 hpi (Fig. 9). Env was expressed at lower levels in *d*81-infected cells and peaked at 24 hpi and then decreased by 48 hpi in these cells. Therefore, *d*106 gives high levels of transgene expression that are maintained through at least 48 hpi.

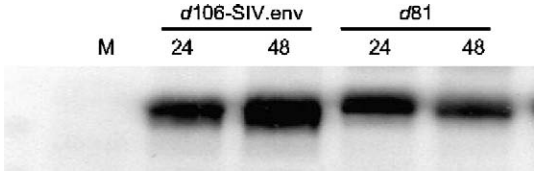

Fig. 9. Kinetics of SIV env protein expression in recombinant virus-infected cells. Vero cells were infected with *d*106-SIV.env or *d*81 (MOI=1) and harvested at the indicated times. Env was detected by western blotting.

Discussion

There continues to be a dire need for novel vaccine approaches, especially for use as AIDS vaccines. In this study, we have examined the vaccine vector properties of an HSV-1 viral strain that has four of its five immediate-early genes inactivated by mutations. We previously used a replication-defective strain that was deleted for the IE *ICP27* gene as part of a study that showed partial protection against mucosal challenge of rhesus macaques with the pathogenic SIVmac239 virus (Murphy et al., 2000). Here we studied an HSV-1 strain, *d*106 with additional IE gene mutations (Samaniego et al., 1998), as a second-generation vaccine vector. We observed that *d*106 shows many of the properties desirable in a vaccine vector: limited cytopathogenicity, high level expression of transgenes, ability to induce durable immune responses, and an ability to transduce dendritic cells and induce their maturation to lymph nodes.

Limited viral gene expression and cytopathogenicity

The enhanced immunogenicity of the transgene product expressed by *d*106 as compared with other HSV-1 replication-defective mutant strains is likely to be, at least in part, due to the fact that *d*106 expresses only two HSV proteins, the IE ICP0 and early (E) ICP6 protein, the large subunit of the viral ribonucleotide reductase. Mutational inactivation knocks out expression of four IE proteins, ICP4, ICP27, ICP22, and ICP47. ICP4, ICP27, and ICP22 are all required for viral gene expression (Roizman and Knipe, 2001). Thus, there is limited HSV gene expression and competition for antigenic presentation and immune responses. In addition, the limited gene expression means that there is little cytopathogenicity due to effects of newly expressed viral gene products. HSV-1 ICP4 and ICP27 are required for inhibition of cellular transcription (Spencer et al., 1997) and ICP27 is required for inhibition of RNA splicing (Sandri-Goldin, 1998). ICP27 is also needed, with vhs, to shut off host protein synthesis, likely because of its effects on host transcription and RNA splicing but potentially because of a direct effect on translation (Ellison et al., 2005; Fontaine-Rodriguez et al., 2004). The lack of host shutoff and limited cytopathogenicity allow the *d*106-infected cells to remain viable and express the transgene for at least a few days. This is also reflected in increased dendritic cell maturation and migration to lymph nodes following infection.

Reduced immune evasion

Inhibition of host gene expression downregulates the expression of MHC class I on HSV-infected cells (Salio et al., 1999; Walev et al., 1992). Thus, the inactivation of ICP4 and ICP27 will prevent this immune evasion effect induced by viral infection. In addition, HSV ICP47 blocks peptide transport into the endoplasmic reticulum (York et al., 1994). Therefore, mutational inactivation of the *ICP47* gene should enhance the presentation of MHC I epitopes in *d*106 virus-infected cells. Finally, ICP22 has been reported to inhibit MHC class II presentation (Barcy and Corey, 2001), and inactivation of the *ICP22* gene should prevent this immune evasion effect of HSV.

Efficacy in HSV-immune animals

As we observed for other replication-defective mutant viruses (Brockman and Knipe, 2002), and others observed for amplicon vectors (Hocknell et al., 2002), *d*106 is capable of inducing normal antibody responses in HSV-immune mice. Others have reported that HSV immunity significantly reduces immune responses to the transgene expressed by an HSV-1 replication-defective mutant virus (Lauterbach et al., 2005); thus, there are differences in experimental systems or viral vector strains and further studies of this property are warranted.

Role of ICP0 in stimulating transgene expression

Although ICP0 is needed to stimulate transgene expression from the HSV-1 genome (Samaniego et al., 1998), ICP0 has also been shown to block several aspects of the host immune response, including type I interferon induction (Lin et al., 2004; Melroe et al., 2004). Thus, an improved vector might encode an ICP0 molecule that can activate transgene expression without blocking the host immune response, if these activities of ICP0 can be separated.

Amplicon vectors

An additional approach that shares some of the advantages of the multiple IE gene deletion mutant is the amplicon vector, which contains a DNA replication origin, packaging signals, and the transgene of interest. In cells where helper functions are provided by a helper virus or plasmids, the amplicon plasmid is packaged as genome length (152 kbp) concatamers, allowing for high levels of transgene expression in transduced cells (Geller and Breakefield, 1988; Spaete and Frenkel, 1982). HSV-derived amplicon systems have been produced for use as vaccine vectors. In one system cells are cotransfected with a BAC containing a packaging signal-deleted HSV genome, needed to create the HSV delivery vehicle, and an HSV amplicon plasmid, containing the HSV packaging signal and the transgene of interest (Suter et al., 1999). The resulting amplicon virus is devoid of any HSV DNA with the exception of the packaging signal. HSV amplicons expressing HIV gp120 are highly immunogenic in mice (Hocknell et al., 2002) and show some protection of hu/SCID mice challenged with HIV (Gorantla et al., 2005). Thus far, titers of these amplicon vectors seem to be limiting for vaccine production.

In conclusion, this study indicates that a multiple IE gene-deleted, replication defective HSV-1 recombinant, *d*106, can effectively express foreign genes and induce strong cellular and humoral responses in mice, raising its potential for use as a vaccine vector in primate studies. Ongoing research involves the studies of these vectors in nonhuman primates (Kaur et al., 2007 (companion paper)) and studies to further optimize the *d*106 virus for transgene expression and immunogenicity.

Materials and methods

Cells and viruses

Vero, V8-27 cells containing the HSV-1 *ICP8* (U_L29) and *ICP27* (*U_L54*) genes (Da Costa et al., 2000), and E11 cells containing the HSV-1 *ICP4* and *ICP27* genes (Samaniego et al., 1998) were cultured as described. The HSV-1 *d*106 mutant virus (Samaniego et al., 1998) is derived from the wild-type (wt) strain KOS. The HSV-1 *d*81 replication-defective recombinant expressing SIV env was described previously (Murphy et al., 2000). Each recombinant was grown and assayed for infectivity [plaque forming units (PFU)/ml] in the appropriate complementing cell lines (V8-27 and E11) as described (Brockman and Knipe, 2002). Stocks of the viruses (stocks as infected-cell lysates or cell-free virus stocks) were generated as described previously (Da Costa et al., 2000).

Plasmids

The transfer plasmids used in this study are derived from plasmid pPs27pd1, which has a 6.1-kilobase pair (kbp) insert of the HSV-1 genome that contains the entire *ICP27* (*U_L54*) gene and flanking sequences (Rice et al., 1989). Plasmid p27-lacZ1 has an *ICP27* gene deletion and a lacZ expression cassette insertion (Rice and Knipe, 1990). The modified simian immunodeficiency virus (SIV) gag (pCMVSgag55) and env expression plasmids were described previously (Rosati et al., 2005). To express the rev, tat, and nef proteins, the rev and tat ORFs were fused to a mutated form of the nef gene that is unable to down-regulate MHC (Murphy et al., 2000), as described below. Expression cassettes containing the SIV ORFs downstream from the human cytomegalovirus immediate-early (CMV IE) promoter were constructed as described previously (Murphy et al., 2000), and inserted into pds27pd1.

Construction of the SIV rev-tat-nef fusion protein expression cassette (RTN)

The major immediate-early promoter/enhancer of human cytomegalovirus virus (HCMV) was PCR-amplified from the pCMV-revFLAG plasmid using primers 1A and NoflagCMV (Table 1), which contain *Eco*RI and *Bam*HI sites flanking the HCMV sequence. The open reading frame for SIV rev was PCR-amplified from a pCMV-revFLAG plasmid (rev sequence originally from pgREVS (Malim et al., 1989) and cloned into pCMVTag2B) using primers KozakRev and 1B, which include *Bam*HI and *Hin*dIII sites added to the 5′ and 3′ ends of rev sequence and ligated to human CMV to generate a 1060 bp fragment, CMV-rev. The tat open reading frame was PCR-amplified from the SIV tat clone (Malim et al., 1989) using primers 2A and 2B, which include *Hin*dIII and *Sal*I restriction sites added to the 5′ and 3′ ends of tat sequence, respectively, generating a 413-bp fragment. The nef delta 239–240 reading frame (deleted for codons 239 and 240 so that the protein does not down-regulate MHC class I) was PCR-amplified from plasmid pnef-delta 239–240 (Swigut et al., 2004) using primers 3A and RTN3C (Table 1), which include restriction sites for *Sal*I and *Avr*II, generating an 800-bp fragment. An SV40 polyadenylation signal sequence was PCR-amplified from plasmid prevFLAG, using primers 4A and 4B (Table 1), which include restriction sites for *Avr*II and *Eco*RI added to the ends of the SV40 polyA sequence, to generate a 370-bp fragment. The individual PCR fragments were ligated together to generate the rev-tat-nef (RTN) fusion cassette of size 2643 bp:

*Eco*RI-CMV-rev-*Hin*dIII-tat-*Sal*I-nef-*Avr*II-SV40polyA-*Eco*RI
1060 bp–413 bp–800 bp–370 bp

The RTN cassette with *Eco*RI ends was cloned into the unique *Eco*RI site of plasmid p6.3TKdeltaKpn (Murphy et al., 2000) to generate pTK-RTN, with the coding sequence for the fusion protein in the opposite orientation of the HSV-1 TK sequence. The plasmid was transfected into human 293 cells and lysates were analyzed by western blotting with anti-nef antibody.

Table 1
Primer pairs used in this study

1A:	5′ GGA CGA ATT CAT GCA TTA GTT ATT AAT AGT
NoflagCMV:	5′ CGC GGA TCC GCC ACC GCG GTG GAG CTC
Kozak Rev:	5′ GAC GGA TCC GCC ACC ATG AGC AAT CAC GAA AG
1B:	5′ GCC GAA GCT TGT CCT GAG GAC TTC TCG AAT
2A:	5′ GGA CAA GCT TAT GGA GAC ACC CTT GAG GGA
2B:	5′ CCG TGT CGA CTC TGC CAA GGC CAG GAG CTG
3A:	5′ GGA CGT CGA CAT GGG TGG AGC TAT TTC CAT
RTN3C:	5′ CCT GCC TAG GTC AGC GAG TTT CCT TCT TGT
4A:	5′ GGA CCC TAG GCA CTC GAT CGC CCT TCC CAA
4B:	5′ CCT GGA ATT CTG CAG TGA AAA AAA TGC TTT
UL48F1	5′ TATCCAGAGCGCCGTAGGGG 3′
UL48B1	5′ CTCCCACCCTGCATGGCAAC 3′

Transfection and recombinant virus isolation

HSV DNA was purified from infected cell lysates by sodium iodide gradient centrifugation (Walboomers and ter Schegget, 1976). Cotransfection of the infectious viral DNA and plasmid insert DNA was performed using Lipofectamine reagent (Invitrogen). Plaque purification of recombinant viruses with agarose overlay medium was performed as described previously (Rice and Knipe, 1990).

Construction of the d106-LacZ virus

A recombinant plasmid d27-LacZ was cotransfected with infectious *d*106 viral DNA into E11 cells. When the progeny viruses from this transfected culture were plated onto E11 cells in the presence of X-gal, approximately 1% of the plaques were blue. A blue plaque was picked, and after three rounds of purification, one of the virus clones was designated *d*106-LacZ. Recombination into the *UL54* locus was confirmed by the loss of fluorescence due to replacement of the *GFP* gene. The *d*106-LacZ virus was unable to form plaques on Vero cells but formed plaques efficiently on E11 cells (results not shown).

Construction of the d106-SIV.gag recombinant virus

Plasmid TKΔgag was constructed by insertion of the SIV p55 gag ORF from the RNA-optimized cassette pCMVSgag55 (Rosati et al., 2005) into the *Eco*RI site of p6.3TKΔKpn (Murphy et al., 2000). Plasmid TKΔgag was restricted with *Eco*RI and treated with Mung bean nuclease to drop out a 2.9 kbp CMV-SIV gag cassette. The transfer plasmid pSd27-1 plasmid DNA was cleaved with *Bam*HI, treated with mung bean nuclease to remove the single stranded extensions, treated with calf intestinal phosphatase, and ligated to the SIV gag cassette. The resulting plasmid was linearized and cotransfected with *d*106 viral DNA into E-11 cells. Viral recombinants were identified in a screen for non-fluorescent plaques because homologous recombination leading to the incorporation of the cassettes in the *ICP27/UL54* locus replaces the GFP expression cassette.

Construction of the d106-SIV.env recombinant virus

Plasmid TKΔenv was constructed by insertion of the SIV gp160 env ORF from the RNA-optimized construct pCMBkanR-SenvCTE (Von Gegerfelt and Felber, 1997) into the *Eco*RI site of p6.3TKΔKpn (Murphy et al., 2000). TKΔenv was restricted with *Eco*RI and treated with mung bean nuclease to drop out a 4.4-kbp CMV-SIV env cassette. The transfer plasmid pSd27-1 plasmid DNA was cleaved with *Bam*HI, treated with mung bean nuclease to remove the single stranded extensions, treated with calf intestinal phosphatase, and ligated to the SIV env cassette. The resulting plasmid was linearized and cotransfected with *d*106 viral DNA into E-11 cells. Viral recombinants were identified in a screen for non-fluorescent plaques because homologous recombination leading to the incorporation of the cassette in the *ICP27/UL54* locus replaces the GFP expression cassette.

Construction of the d106-SIV.RTN recombinant virus

Plasmid pTK-RTN, constructed as described above, was restricted with *Eco*RI and treated with mung bean nuclease to drop out a 4.4 kbp CMV-SIV rev-tat-nef (RTN) cassette. The transfer plasmid pSd27-1 plasmid DNA was restricted with *Bam*HI, treated with mung bean nuclease to remove the single stranded extensions, treated with calf intestinal phosphatase, and ligated to the SIV rev-tat-nef cassette. The resulting plasmid was linearized and cotransfected with *d*106 viral DNA into E-11 cells. Viral recombinants were identified in a screen for non-fluorescent plaques because homologous recombination leading to the incorporation of the cassette in the *ICP27/UL54* locus replaces the GFP expression cassette.

Analysis of viral proteins

Infected cell proteins were radiolabeled by incubating approximately 6×10^5 cells with 37.5 μCi of [^{35}S]-methionine per ml of methionine-free medium for 30 min at the indicated time points. The labeled viral proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as previously described (Knipe and Spang, 1982). For western blot analysis of β-galactosidase and SIV proteins, proteins were separated by SDS–PAGE, electroblotted to nitrocellulose, and analyzed using indicated primary antibodies and a chemiluminescence western blotting kit (Boehringer Mannheim).

Monoclonal antibodies

The monoclonal antibodies used in this study were as follows; anti-β-galactosidase mouse monoclonal antibody (Promega); anti-SIV mac251 gp120 antibody (KK65), Nef antibody (17.2) and gag antibody (KK64). The antibodies against SIV proteins were obtained from the NIH AIDS Research and Reference Reagent Program.

Immunofluorescence

Indirect immunofluorescence was performed as described previously (Quinlan et al., 1984). In brief, Vero cells were grown on coverslips and infected. At the indicated times, the cells were fixed in cold acetone and reacted with indicated primary antibody, washed with PBS and then reacted with FITC-conjugated goat anti-rabbit immunoglobulin. Fluorescence images were viewed with an Axioplan 2 (Zeiss) microscope equipped with an Orea cooled CCD camera (Hamamatsu Photonics). A Zeiss 63X plan Apochromat objective was used. Images were collected with the OpenLab software package (Improvision). GFP expression was visualized directly after fixation of infected cells.

β-Galactosidase enzyme assays

Vero cells were infected with d106-LacZ or HD-2 at MOI=1. At various times post infection, cells were lysed in reporter lysis buffer and incubated in assay buffer containing *o*-nitrophenyl1-β-D-galactopyranoside (ONPG). β-Galactosidase enzymatic activities in cell lysates were determined using an enzyme assay system (Promega, Madison, Wis.) according to the manufacturer's recommendations. Protein concentrations were calculated by comparison to a standard curve of freshly prepared β-galactosidase protein (Sigma) on the basis of absorbance at 410 nm.

Protein concentrations were calculated based on the basis of OD_{410}.

UV inactivation of viruses

Viruses were partially inactivated by exposure to 254-nm UV light for 10 min at a distance of 5 cm as described previously (Morrison and Knipe, 1994). Subsequent plaque titrations revealed that UV treatment reduced viral infectivity by approximately 2,000-fold.

Mice and inoculations

Animal studies were conducted in accordance with National Institutes of Health (NIH) and Harvard University guidelines.

Six-week-old female BALB/cJ or C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and acclimated for 1 week prior to use. The mice were inoculated s.c. in the left flank with 2×10^6 PFU of viruses, or mock-infected by s.c. inoculation with uninfected Vero cell lysate, followed by one or more booster inoculations at 2- or 3-week intervals. Inoculations consisted of virus stock diluted into a volume of 20 μl of sterile, endotoxin-free 0.9% sodium chloride solution (Sigma), per mouse. In some cases, mice were pre-immunized with 2×10^6 PFU of WT KOS1.1 virus once or twice by subcutaneous (s.c.) inoculation into the rear flank.

PCR detection of HSV DNA in lymph node tissue

After inoculation, both inguinal lymph nodes (LNs) of mice were removed at the indicated times. LNs were lysed into 150 mg/ml of proteinase K and DNA was extracted by phenol/chloroform extraction. A part of HSV-1 *UL*48 coding sequence (nucs. 103672-104116, 444 bp) was amplified by PCR from 50 ng of extracted DNA per reaction, using UL48F1 (Table 1) as the forward primer and UL48B1 (Table 1) as the reverse primer. The amplification consisted of an initial 5-min denaturation step at 94 °C followed by 35 cycles of denaturation (94 °C, 30 s)–annealing (60 °C, 30 s)–extension (72 °C, 30 s) and a final extension at 72 °C for 5 min.

Serum collection and ELISA

Blood samples were collected by retro-orbital plexus puncture, and sera were prepared using Becton Dickinson Microtainer serum separators (VWR) and then stored at −20 °C prior to analysis. Enzyme-linked immunosorbent assays (ELISAs) to determine antigen-specific IgG titers were conducted as described previously (Da Costa et al., 1999). Briefly, 96-well Nunc Maxisorp microtiter plates (VWR) were coated with HSV-1 virions (Advanced Biotechnologies Inc.) at 50 ng per well or with β-galactosidase (Sigma) at 250 ng per well in 50 μl of sodium bicarbonate buffer (pH 9.6) (Sigma) overnight at 4 °C. Plates were blocked with phosphate-buffered saline (PBS, pH 7.4) containing 5% (wt/vol) nonfat milk for 1 h at 37 °C and washed with PBS containing 0.05% Tween 20 (PBS/T) using a Skatron CellWasher 600 (Molecular Devices). Serial two-fold dilutions of mouse sera (from 1:100 to 1:12,800) in PBS/T were added and incubated for 2 h at 37 °C. Following serum antibody binding, plates were washed with PBS/T and then incubated with a rabbit anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (1:1000 dilution; Sigma) for 1 h at 37 °C. Plates were washed with PBS/T and developed by incubation with the alkaline phosphatase substrate *p*-nitrophenyl phosphate (Sigma) for 20 min at room temperature, and results were read at 405 nm on a VersaMax microplate reader (Molecular Devices).

The IgG antibody titer was calculated as the mean reciprocal $\log_2$ value of the last dilution resulting in an optical density (OD) reading 0.2 units above that of a control serum (background) (Brockman and Knipe, 2002). In each case, negative OD readings at the 1:100 dilutions were scored as positive at a 1:50 dilution (reciprocal dilution $50=\log_2 5.65$), and this value (5.65) was used as the limit of detection and subtracted from all results.

ELISPOT assay

To measure $CD8^+$ T cell responses specific for HSV glycoprotein B or β-galactosidase, an ELISPOT assay was performed using a BD ELISPOT Set (BD Biosiences Pharmingen). Purified anti-IFN-γ monoclonal antibody was used to coat 96-well ELISPOT plates at 5 μg/ml in PBS at 4 °C overnight. After washing with 0.05% Tween-20 in PBS (PBST), 200 μl of 10% FCS in DMEM was added to each well for 2 h at 37 °C to block non-specific binding. Freshly prepared splenocytes diluted to 1×10^5 or 2×10^4 cells in 100 μl of DMEM with MHC class I-restricted peptides at 10 μg/ml were placed in each well and incubated at 37 °C for 24 h. Two peptides were used in this assay; MHC class-I restricted β-gal peptide (DAPIYTNV) or MHC class I-restricted HSV-1 gB (SSIEFARL). Cells treated under the same condition but incubated without peptides were used as a negative control. PMA (50 ng/ml; Sigma) and Ionomycin (1 μg/ml; Sigma) were added to the positive control well. The plates were washed with PBST and incubated for 2 h at RT with 2 μg/ml of biotinylated anti-mouse IFN-γ in each well. The plates were then washed and treated with avidin-HRP at 1:100 dilution for 1 h at RT. After extensive washing, AEC Substrate Reagent (BD Biosciences) was added to develop spots in the plates for 20 min. The plates were rinsed and air-dried before spots were counted using a dissecting microscope.

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