HIV-1$_{89.6}$ Gag Expressed from a Replication Competent HSV-1 Vector Elicits Persistent Cellular Immune Responses in Mice

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Abstract

We have constructed a replication competent, $\gamma_1$34.5-deleted Herpes Simplex Virus type 1 (HSV-1) vector (J200) that expresses the *gag* gene from Human Immunodeficiency Virus type-1, primary isolate 89.6 (HIV-1$_{89.6}$), as a candidate vaccine for HIV-1. J200 replicates *in vitro*, resulting in abundant Gag protein production and accumulation in the extracellular media. Immunization of Balb/ c mice with a single intraperitoneal injection of J200 elicited strong Gag-specific CD8 responses, as measured by intracellular IFN-γ staining and flow cytometry analysis. Responses were highest between 6 weeks and 4 months, but persisted at 9 months post-immunization, the last time-point evaluated. These data highlight the potential utility of neuroattenuated, replication-competent HSV-1 vectors for delivery of HIV-1 immunogens.

Keywords

HSV-1 vaccine vector; Gag; HIV-1 vaccine

1. Introduction

During early attempts in the development of a vaccine for Human Immunodeficiency Virus type-1 (HIV-1) antibody responses were elicited by immunization with recombinant HIV-1 envelope glycoproteins. While this approach can prevent infections with viruses containing glycoproteins closely related to the original immunogen, it has failed to generate the broadly protective response required for a successful vaccine, given the diversity of circulating HIV clades [1–6]. The current strategy for development of a vaccine has shifted to presentation of a variety of HIV-1 recombinant proteins with novel delivery vectors and/or adjuvants to elicit T-cell mediated immunity (CMI) with an emphasis on cytotoxic CD8+ lymphocyte (CTL) responses. HIV-specific CTL responses can recognize and kill HIV infected cells and are effective in controlling HIV-1 replication during the course of natural infection [7–9]. Vaccines

which elicit these types of responses can also control the replication of pathogenic retroviruses and protect against the development of immunodeficiency and death in nonhuman primate (NHP) models [10–19]. The current mechanisms for induction of potent CTL mediated immunity include immunogen delivery by attenuated viral vectors, bacterial vectors, direct plasmid DNA injection, and co-delivery of vaccine adjuvants (reviewed in [20–22]).

Attenuated viral vectors have received the most attention among the current vaccine candidates that have been developed to elicit HIV-specific CTL responses. These vectors in development may not be fully effective in humans, and other approaches may be needed. Herpes simplex virus type-1 (HSV-1) with deletions in the $\Delta\gamma_1 34.5$ gene is replication-competent but is aneurovirulent [23]. Attenuated HSV-1 recombinants are attractive as HIV-1 vaccine vectors for multiple reasons: *i*) herpesvirus vectors can infect dendritic cells and stimulate potent, long-lasting CMI responses [24–28]; *ii*) attenuated HSV can establish latency and reactivate with the potential to prolong an immune response [29,30]; *iii*) several nonessential genes can be deleted and replaced with multiple foreign gene inserts (up to 30 kb) [31]; *vi*) HSV $\Delta\gamma_1 34.5$ viruses have been safe during human use in clinical trials, including intracranial injection at high doses [32–35]; and *v*) although prior immunity to HSV has a variable effect on vector efficacy in gene therapy applications, it does not impair either antibody or CMI responses elicited against immunogens expressed from HSV vectors [36–39].

Replication competent HSV-1 attenuated by a deletion in the thymidine kinase gene *tk-*) has been tested as an SIV vaccine vector in the NHP model. Immunized animals had measurable SIV specific CMI and reduced viral loads after infection with a pathogenic SIV [40]. However, recombinant HSV attenuated by the *tk-* phenotype alone remain neurovirulent and are not suitable as a vaccine vectors in humans [41].

More recently, a replication defective HSV-1 vector with multiple immediate-early gene deletions has been characterized as an SIV vaccine vector [39,42]. This virus boosts immune responses to DNA vectors in rhesus macaques and results in decreased plasma viral loads after SIV challenge [42]. HSV-1 amplicon vectors have induced potent Gag-specific T cell responses in BALB/c mice, giving further support for the use of HSV-1 as a vector for HIV-1 vaccine delivery [43]. However, difficulties in achieving high titers of amplicon vectors may prove to be a limiting factor for vaccine production. Moreover, replication competent HSV-1 vectors have the potential to elicit enhanced immune responses versus those elicited by immunization with replication defective vectors. This is due to their ability to replicate in the host, resulting in increased immunogen production. In order to develop a replication competent vector without neurovirulent properties, we have constructed a $\Delta\gamma_1 34.5$ HSV-1 that expresses the HIV-1$_{89.6}$ *gag* gene (J200) and tested the ability of this vector to elicit CMI responses in mice. These data demonstrate that HSV vectors continue to be promising candidates for HIV vaccine development and warrant further studies, including the introduction of additional HIV-1 antigens capable of eliciting a broad and potent cellular immune response.

2. Materials and Methods

2.1 Cells

Vero cells (American Type Culture Collection {ATCC}, Rockville, MD) were grown and maintained in Minimal Essential Medium (Mediatech Inc, Herndon, VA) containing 7% fetal bovine serum. The human 143 thymidine kinase minus cells (143tk-, ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech) supplemented with 10% fetal bovine serum. Rabbit skin cells (originally acquired from Dr. J. McClaren, University of New Mexico, Albuquerque, NM, USA) were maintained in DMEM supplemented with 5% fetal bovine serum.

2.2 Plasmids and Viruses

HSV-1 (F) strain is a low passage clinical isolate used as a prototype HSV-1 strain [44,45]. The HIV-1$_{89.6}$ proviral clone in pUC19 was obtained from the NIH AIDS Research and Reference Reagent Program. The entire HIV-1$_{89.6}$ *gag* gene and part of the *pol* gene containing the viral protease (referred to herein as *gag-pro* gene) was amplified by PCR using the following primers: 5' primer (89.6-763LKZ), 5' - GACTCGAGGAGGCTAGAAGGAGAACCATGG-3' and 3' primer (89.6-2576R) 5' - CCGTCTAGACTGTGACAGTTTCAATAGG-3' and subcloned into the TOPO TA cloning vector (Invitrogen, Corp.). The *gag-pro* gene was removed from this vector by digestion with EcoRI and XhoI, and subcloned into identical sites in plasmid pCIM to generate pCMGP89.6. pCIM, originally derived from pSI (Promega, Corp), was constructed as follows: the SV40 late polyadenylation signal was removed from pSI by digestion with EcoRI and ClaI, and replaced with the Mason-Pfizer monkey virus polyadenylation sequence (MPMVpA) and cytoplasmic transport element (CTE) [46]. The MPMVpA/CTE was PCR-amplified from pSR2 (E. Hunter, unpublished) with primers that contained unique restriction sites for EcoR1 (5' end) and ClaI (3' end). The SV40 promoter was removed by restriction digestion with BglII and PstI and replaced with the CMV immediate early promoter, from pCI (Promega, Corp). To construct the shuttle plasmid pJP200, the entire 3.6 kb *gag-pro* expression cassette, including the CMV promoter and intron just upstream of the *gag-pro* gene, was removed from pCMGP89.6 by digestion with BamHI and BglII, the ends blunted using DNA polymerase Klenow fragment, and subcloned into the HSV-1 shuttle plasmid pRB4878 [47] (kind gift of B. Roizman, University of Chicago). pRB4878 was digested with BstEII and StuI to remove the Egr-1 promoter and hepatitis B polyadenylation sites, and the overhanging sequences were filled in as above. This shuttle plasmid contains γ$_1$34.5 flanking sequences which facilitate recombination into this site when co-transfected with HSV DNA.

The parent recombinant HSV-1 virus R3659 has been described previously [48]. In R3659, the native thymidine kinase (*tk*) gene contains a 500 bp deletion, and both copies of the γ$_1$34.5 neurovirulence gene have been replaced with the *tk* gene expressed from the viral α27 promoter for use as a drug selection marker. The recombinant HSV-1 virus J200 was constructed using a previously described strategy [47,49]. Briefly, plasmid pJP200 was co-transfected with R3659 viral DNA, and recombinant, *tk* negative viruses were selected using the standard *tk* selection method [44]. Introduction of the HIV-1$_{89.6}$ *gag-pro* expression cassette into the γ$_1$34.5 locus was confirmed by Southern blot hybridization (data not shown). Clones J200.71 and J200.91 were selected for further characterization.

2.3 Metabolic labeling of proteins and immunoprecipitation

To demonstrate production of HIV-1 Gag polyproteins, Vero cells were seeded in T-25 tissue culture flasks one day prior to infection with J200 clones 71, 91, or R3659 (negative control without Gag expression) virus using a multiplicity of infection (MOI) of 10. Three hours post infection (hpi), cells were starved for 30 min in growth medium lacking methionine and cysteine (Met$^-$Cys$^-$), then 3 mls of Met$^-$Cys$^-$ medium containing 100 µCi of [^{35}S] Express Protein Labeling Mix (Perkin Elmer NEN, Boston, MA) was added to each flask. Flasks were returned to 37°C, 5% CO$_2$ incubator for an additional 14 h. At 17 hpi, cell culture media was removed and infected cells were treated on ice with lysis buffer A (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris, pH 7.5) and the lysate was centrifuged in a microfuge at 14,000 rpm for 5 minutes. The supernates were transferred to new tubes, PMSF and SDS were added to achieve final concentrations of 0.1 mg/ml and 0.1%, respectively. The samples were precleared by addition of 1 µl normal human serum, 60 µl of formalin-fixed *Staphylococcus aureus* Protein A, and incubating for 1 h at room temperature on a rocker. After centrifugation at 20,000 × g for 10 min, supernates were transferred to new tubes, and 1 µl of a 1:1000 dilution of HIV-1 infected patient serum was added for immunoprecipitation of

labeled Gag proteins. Immune complexes were precipitated using 30 µl of Protein A, followed by centrifugation as before. Complexes were washed three times in lysis buffer A containing 0.1% SDS, once in 20 mM Tris, pH 8.0, and pellets were resuspended in 30 µl protein loading buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel. The Prestained Benchmark Marker (Life Technologies) ladder was used as a visual marker. After electrophoresis, gels were stained in Coomassie Blue, destained in 10% (vol/vol) acetic acid and 15% (vol/vol) methanol, and soaked in Enhance (Dupont, NEN) for 30 minutes. Gels were dried and exposed to Kodak XAR film for 3 days ($-70°C$).

2.4 Western blot detection of Gag polyproteins

Vero cells were seeded in 6-well plates one day prior to infection with J200 or R3659 virus in MEM with 1% FBS for 2 hours using a multiplicity of infection (MOI) of 5. The cells were then fed with MEM with 7% FBS and harvested at 6, 12, 18, or 24 hpi and lysed with SDS-PAGE gel loading buffer (20% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, 6% (weight/vol) SDS, 62.5 mM Tris-HCl pH 6.8, 0.02% (weight/vol) bromophenol blue). The cell culture media was collected at each time point and centrifuged in a microfuge at 5,000 rpm for 5 min. Supernates were loaded onto a 20% weight/volume solution of sucrose in phosphate buffered saline in a SW41 rotor and centrifugation at 27,000 rpm for 16 h. The pellets were resuspended in SDS-PAGE gel loading buffer. Following SDS-PAGE, separated proteins were transferred to nitrocellulose filter paper (Protran 0.45 micron pore size, Schleicher and Schuell, Keene, NH) at 250 mA for 90 minutes in a transfer buffer of 50 mM Tris-HCl, 400 mM glycine, 0.1% SDS, 20% methanol using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Following blocking (10% dried milk in 10mM Tris-HCl pH 8.0, 150 mM NaCl, with 0.1% Tween 20, or TBST), the filter was then incubated in TBST containing HIV-Ig (NIH AIDS Research & Reference Reagent Program) at a dilution of 1:5000. The HRP-labeled secondary antibody (goat anti-human IgG, PerkinElmer) was then added at a dilution of 1:20,000 in TBST. The secondary antibody was detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and autoradiography.

2.5 In vitro and in vivo replication assays

Replication assays were conducted *in vitro* in Vero cells as described previously [47,49] at 12, 24, 48 and 72 hpi using an MOI of 1.0. For *in vivo* replication, 8 week old Balb/c mice were first anesthetized, then injected intramuscularly into the right hind gastrocnemius with 1×10^7 pfu of either R3659 or J200 (clone 71), in 50 µl total volume in the presence of dextran sulfate (100 µg/ml). At days 2, 4, and 7, two mice/group were sacrificed and the infected gastrocnemius removed, weighed and diluted to 20% wt/vol in a 50:50 mixture of sterile milk:DMEM/1% FBS. Samples were homogenized and subjected to three freeze/thaw ($-80°$C/37°C) cycles. Homogenates were sonicated prior to titering on Vero cells to determine viral recovery.

2.6 Mice and immunization

Female, 5–6 week old pathogen-free Balb/c mice were obtained from the Frederick Cancer Research and Development Center, National Cancer Institute. All animal studies were conducted in accordance with guidelines for animal use and care established by The University of Alabama at Birmingham Animal Resource Program and the Institutional Animal Care and Use Committee (protocol #010704285). Mice were immunized by intraperitoneal (i.p.) injection, using 200 µl of the desired virus dose (diluted in saline), or saline only. For assessment of Gag-specific CD8 responses, mice were challenged i.p. five days prior to sacrifice with 2.5×10^7 pfu of either vDK-1, a recombinant vaccinia virus that expresses HIV-1

Gag (vDK-1, NIH AIDS Research & Reference Reagent Program), or vSC11, a recombinant vaccinia virus constructed by homologous recombination with the shuttle plasmid pSC11 [50], which does not contain the *gag* gene.

2.7 Intracellular IFN-γstaining and flow cytometry analysis

Splenocytes freshly harvested at sacrifice were resuspended to a final concentration of 2×10^7 cells/ml in DMEM containing 10% FBS and 50 µM 2-mercaptoethanol. To assess intracellular gamma interferon production, splenocytes were cultured in 96 well flat-bottom tissue culture dishes with or without the addition of a major histocompatibility complex class I-restricted HIV-1 p24 peptide (AMQMLKETI) at 200ng/ml for 6 hours at 37°C in growth medium containing 10% T-STIM and GolgiPlug (BD Pharmingen, San Diego, CA), which blocks cytokine secretion from the Golgi apparatus. As a positive control, naïve splenocytes were stimulated with 50 ng/ml PMA and 1 µM ionomycin. After stimulation, cells were chilled on ice, transferred to round-bottom 96 well plates and pelleted by centrifugation at 600 rpm for 5 minutes at 4°C. Cells were first stained for CD8 expression by incubation with phycoerythrin-conjugated monoclonal rat anti-mouse CD8 antibody (0.06 µg/sample, BD Pharmingen), diluted in FACS buffer (PBS containing 2% bovine serum albumin, 0.2% sodium azide) for 30 minutes on ice in the dark. Cells were then washed 3 times in FACS buffer, resuspended in 100 µl of Cytofix/Cytoperm (BD Pharmingen), incubated as before for 20 minutes and washed three times with 1X Perm/Wash buffer (BD Pharmingen). Cells were resuspended in a 1:100 dilution of FITC-conjugated rat anti-mouse gamma interferon (IFN-γ) antibody (BD Pharmingen) in Perm/Wash buffer and incubated as before for 30 minutes. Cells were washed three times in 1X Perm/Wash buffer, twice in FACS buffer, and resuspended in 200 µls PBS containing 2% w/v paraformaldehyde. Stained cells were acquired using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using FCS Express (De Novo Software, Ontario, Canada).

3. RESULTS

3.1 Construction of a conditionally replication competent HSV-1 that expresses HIV-1$_{89.6}$ Gag

To maximize expression of HIV-1 antigens from our $\Delta\gamma_1 34.5$ HSV-1 vaccine vector, we first constructed a new vector (pCIM) for expression of HIV-1 genes that contained the CMV immediate early promoter and the Mason-Pfizer monkey virus (M-PMV) polyadenylation signal and cytoplasmic transport element (CTE). The M-PMV CTE has previously been shown to mediate transport of unspliced viral transcripts out of the nucleus for protein synthesis in the absence of Rev protein and its *cis*-acting response element (RRE) [46]. The *gag-pro* sequence from the primary isolate HIV-1$_{89.6}$ [51] was cloned into pCIM, and the entire expression cassette was removed and introduced into a $\gamma_1 34.5$ targeting vector to generate pJP200, as described in Materials and Methods. Two candidate viruses, J200.71 and J200.91, were selected following homologous recombination between pJP200 and R3659 viral DNA. Figure 1 illustrates the genomic organization of R3659 and J200. The presence of the HIV-1$_{89.6}$ *gag-pro* expression cassette within the $\gamma_1 34.5$ site was confirmed by restriction digest analysis of the viral DNA and Southern blot hybridization (data not shown).

3.2 HIV-1$_{89.6}$ gag-pro is efficiently expressed in J200-infected Vero cells

To demonstrate Gag protein production within J200-infected Vero cells, metabolic labeling studies were performed. At 3 hpi, cells were continuously labeled for 14 hours and HIV specific proteins were extracted from cell lysates by immunoprecipitation with serum from HIV-1 infected patients, and analyzed by SDS-PAGE. As shown in Figure 2A, the HIV-1 p55 Gag polyprotein, as well as the p41 Gag product, were detected in cell lysates from Vero cells infected with J200 clones 71 and 91, but not in cells infected with R3659 as a negative control. The p24 major capsid protein, a Gag protease cleavage product, was also visible in infected

cell lysates. The p41 band represents a truncated Gag product in which an internal downstream initiation codon was utilized. An additional band present in the J200-infected cell lysates, but not the R3659-infected cell lysates, at ~47kD is presumed to be a partial Gag cleavage product.

To demonstrate the release of Gag proteins over time, Vero cells were infected with J200 or R3659 (as a negative control) and cell lysates and culture media were collected at 6, 12, 18, and 24 hpi. HIV Pr55^{Gag} polyproteins were detected by Western blot starting at 12 hpi in the J200 cell lysate and 18 hpi in the cell culture media after centrifugation through 20% (weight/volume) sucrose (Figure 2B). The absence of both Pr55^{Gag} cleavage products in the culture media and mature or immature Gag virus-like particles after imaging with electron microscopy (data not shown) suggests that viral budding with proteolytic processing does not proceed with efficiency in this system. The mechanism for the release of Pr55^{Gag} into the cell culture media is not clear, but may be a consequence of HSV cytopathic effect and cell lysis.

3.3 In vitro *and* in vivo *replication of J200*

The efficiency of J200 replication in vitro was compared to its parent $\Delta\gamma_1 34.5$ HSV-1, R3659, and to wild type HSV-1 (F) strain, using Vero cells, which are permissive to HSV-1 infection. At 12, 24, 48, and 72 hours after infection (MOI=1) with wild type, R3659, or J200 clones 71 or 91, cells were harvested and viral recovery determined. No significant differences were seen in replication over time of either J200 clone as compared to HSV-1 (F) and R3659 (data not shown). Clone 71 was selected for all subsequent studies and is herein referred to as J200.

To assess *in vivo* replication efficiency over time, 1×10^7 pfu of either J200 or R3659 was injected intramuscularly into the hind leg gastrocnemius of Balb/c mice. At days 2, 4, or 7, mice (2/virus/timepoint) were sacrificed and the injected gastrocnemius was dissected and homogenized, and the homogenate titered on Vero cells. At day 2, the average titer from R3659 was 7.1×10^3 pfu/ml, whereas the average titer of J200 was 5.5×10^5 pfu/ml. No virus was recovered from R3659-infected muscle at either day 4 or day 7 following injection. For J200, the average titer recovered at day 4 was 6.3×10^5 pfu/ml from mice infected with this virus. However, by day 7, no virus was recovered from either of the J200-infected samples.

3.4 Gag-specific CD8$^+$ T cell responses in mice following immunization with J200

The ability of J200 to elicit Gag-specific CD8+ T cell responses was evaluated in mice by intracellular IFN-γ analysis. Balb/c mice were primed by a single i.p. administration of 1×10^7 or 5×10^7 pfu of J200 or R3659 virus, whereas control animals received saline only. At 11 weeks post-immunization, responses were boosted by i.p. injection with either a recombinant vaccinia virus (vv) that expresses HIV-1 Gag (vDK-1, referred to here as vv-Gag) or a vaccinia virus control (SC11, referred to as vv-only). Vaccinia virus replicates well in mice, and has been previously used to boost CTL responses after priming with HIV-1 Gag expressed from vaccine vectors in mice [52,53]. Splenocytes were harvested and Gag-specific CD8$^+$ T cell responses were analyzed five days following vaccinia boost by stimulation with the MHC Class I-restricted Gag peptide AMQMLKETI and assessing IFN-γ production by intracellular cytokine staining. As shown in Figure 3 (right panels), 3.1% and 2.9% of CD8$^+$ T cells isolated from mice immunized with 5×10^7 and 1×10^7 pfu of J200, respectively, were producing intracellular IFN-γ following vv-Gag challenge and peptide stimulation. The interferon CTL response is Gag-specific: only 0.3–0.4% of splenocytes demonstrated intracellular IFN-γ production in the absence of Gag peptide (Figure 3, left panels). In R3659-immunized, vv-Gag challenged mice, 0.3–0.4% of CD8$^+$ T cells were positive for intracellular IFN-γ with or without peptide stimulation (data not shown).

To determine the timing of onset for Gag-specific CD8$^+$ T cell responses elicited in response to J200 immunization, as well as the persistence of these responses, mice were immunized as

above with J200 or R3659, or received saline injection only. At 3, 9, 26 and 39 weeks post-immunization, mice were sacrificed and their spleens isolated as described above. Five days prior to sacrifice at each time point, mice were boosted as above with either vv-Gag or vv-only. For all but the last time point, four J200-immunized mice received vv-Gag and two received vv-only as a control for effects of vaccinia challenge on CD8+ T cells. For the last time point, three J200-immunized mice received vv-Gag and one received vv-only as control for effects of vaccinia challenge. Splenocytes were harvested and analyzed individually for each mouse and the proportion of IFN-γ positive CD8+ cells was averaged for each treatment group. At 3 weeks following J200 immunization, the earliest time point assessed, more than 5.0% of CD8+ T cells (as determined by FACS analysis) on average were producing intracellular IFN-γ in response to vv-Gag boost *in vivo* and stimulation with the Gag-specific peptide in vitro. In the absence of peptide stimulation, the mean percent of CD8+ cells stained positive for IFN-γ was 0.5% in 3 out of 4 samples. Administration of vv-Gag boost after a control primary immunization with R3659 did not elicit a comparable response. J200 primary immunization without a vv-Gag boost was also insufficient to elicit a substantial response. The average vv-Gag boosted and Gag peptide-specific responses dropped to 2.5% by 9 weeks following J200 immunization (Figure 4), consistent with the first experiment (Figure 3), and remained elevated as compared to responses in the R3659 immunized controls. Even at 39 weeks, the last time point assessed, the average Gag-specific CD8+ T cells producing IFN-γ from J200-immunized mice was 2.2%, versus 0.4% for R3659 controls. In contrast, none of the other immunization groups showed increased levels of Gag-specific CD8+ T cells in response to Gag-peptide stimulation, as compared to control samples which were not peptide-stimulated. Plots included in Figure 4A are from one of the samples for each time point and are representative for results of the samples in the given treatment group. The values indicated within each plot in Figure 4A represent the means of all the samples included for that timepoint. The mean Gag-specific CD8+ T cell responses detected for each treatment group over time as compared to the control groups are shown in Figure 4B.

4. DISCUSSION

An ideal HIV vaccine would elicit a broadly neutralizing antibody response which would protect the vast majority of individuals from infection. Immunization strategies designed to elicit neutralizing antibody responses are effective in protecting against an infection with a viral strain closely related to the immunogen. However, these antibody responses have failed to protect against challenge with heterologous strains, and thus have not yet reached the threshold that will be required for a universally effective vaccine. In contrast, while vaccines that elicit CMI responses do not prevent infection in animal models, they are effective against a variety of viral strains by reducing viral loads and preventing disease associated with viral infection. Predictive correlates of protection have not yet been established for CMI elicited by an HIV vaccine. However, HIV and/or SIV-specific CD8+ responses are associated with viral suppression in the course of natural infection and protection of immunized NHPs from advanced disease, following pathogenic retrovirus challenge [12–17,54]. As long as vaccines with neutralizing immunity remain elusive, the best hope for the field lies with the development of vaccines effective at reducing viral loads in the infected host. Vaccines that elicit broadly effective CMI responses are anticipated to significantly prolong survival, and inhibit transmission. The latter is crucial to curtail the rate of new infections in developing nations that lack access to antiretroviral therapy.

A diverse set of recombinant, attenuated viral vectors have been selected for their ability to stimulate CMI to heterologous antigens, and explored as vectors for HIV vaccines (reviewed in [11,21]. Some of these candidates have not yet been tested in humans but are non-toxic and elicit significant CMI responses in animal models. These include alphaviruses [55–57], polioviruses [58], and rhabdoviruses [52]. Candidate vaccine vectors in more advanced stages

of development are under evaluation in human clinical trials. Canarypox vectors are well tolerated, but their immunogenicity has been disappointing: HIV-specific CD8+ CTL responses measured at any one timepoint after vaccination are present in only 20–30% of human subjects [59,60]. While these phase I/II trials were not designed to test vaccine efficacy, breakthrough HIV infections have occurred with equal frequency in the vaccinated and unvaccinated control subjects [61]. Nevertheless, since the significance of the CTL responses as a correlate of protection is not entirely clear, a large Phase III trial is underway to measure the efficacy of a canarypox vector as part of an HIV vaccine regimen. While awaiting the canarypox Phase III results, modified Vaccinia Ankara (MVA) [62–64] and adenovirus vaccine vectors[24,65] are being evaluated in both preclinical and Phase I and II patient trials. Both vectors stimulate potent CMI responses in primates and a majority of human subjects. However, prior immunity to each vector may potentially interfere with initial vaccine responses and/or booster doses required to maintain host immunity. Given the potential limitations for leading vector candidates, and the lack of adequate assays to identify correlates of immunity in humans, there is a clear need for the development of novel vaccines.

Recombinant attenuated $\Delta\gamma_1 34.5$ HSV-1 has been studied as an oncolytic agent that is safe for use in humans and also has several characteristics of an attractive vaccine vector candidate. These unique properties include low-level replication in animal tissue which may elicit long lasting CMI responses after single dose administration and expression of foreign genes even in the presence of pre-existing immunity to the vector [38] combined with the complete absence of neurovirulence [23].

In this pilot study, the J200 virus replicates in animal tissue following intramuscular injection and produces significant levels of Gag protein. A single dose of J200 elicits CD8$^+$ CMI following challenge with a heterologous viral vector (vv-Gag) up to 9 months later. The magnitude of the cellular immune response to J200 prime/vv-Gag challenge is similar in magnitude to prior observations with the most immunogenic attenuated viral vectors [52,66] and thus may also be effective at preventing advanced disease following pathogenic challenge. Further development of these $\Delta\gamma_1 34.5$ HSV vaccine vectors will include the incorporation of HIV-1 *env* with accessory genes and measuring the breadth of the immune response against a spectrum of CTL epitopes across the entire range of vaccine immunogens, rather than the single Gag epitope used in this study.

A persistent state of immune memory and subsequent response to boost immunizations or pathogenic challenge are fundamentally essential for a successful vaccine. Although CTL activity is not measurable after the J200 primary injection, the vigorous response following vv-Gag boost clearly demonstrates the induction of a durable immune memory state which persists through an interval of at least 39 weeks. Local replication of the J200 vector likely contributes to the persistent effects observed after a single injection. Despite the inability to demonstrate prolonged replication of J200 following i.m. administration, persistent CD8 responses were elicited. This supports the hypothesis that the level of protective immune responses elicited by an antigen can be influenced by the delivery vector; in this case, an attenuated HSV. Persistent effect after a single dose has also been observed with other attenuated, replication competent vaccine vectors and this property may translate into enhanced vaccine efficacy [52,67,68].

Pre-existing host immunity has interfered with immune responses elicited by both of the viral-based vectors of the advanced HIV-1 vaccine candidates in humans, with adenoviral vectors affected more heavily than the poxvirus vectors as a group. The issue of pre-existing immunity towards HSV-derived vectors has also been investigated in murine models, with conflicting results. Neither antibody nor cellular proliferative responses elicited in mice immunized with a replication-defective HSV vector were affected by prior exposure to HSV [38]. Additionally,

treatment of tumor-bearing mice with oncolytic HSV was not adversely effected even in mice with prior HSV-1 immunity [36]. Other studies in mice seropositive for HSV-1 have indicated that humoral and cell-mediated immune responses are significantly reduced following immunization with replication-defective HSV vectors as compared to immunization of HSV-1 naïve mice[69]. In humans, the response to HSV oncolytic therapy for glioma in clinical trials has not been correlative with pre-existing HSV-1 seropositivity [35].

In summary, we have provided new evidence supporting the use of HSV-based vectors for delivery of HIV-1 antigens as vaccines. We report for the first time an attenuated but replication competent HSV vector (J200) engineered to express HIV-1 Gag protein. This virus elicits potent Gag-specific CD8$^+$ T cell responses following a single intraperitoneal immunization. The CD8$^+$ responses elicited from this immunization persist at least 9 months. Further, prior studies with the parent virus of J200 have demonstrated its safety in mice, nonhuman primates, and in Phase 1 trials in brain tumor patients with compromised immune systems. Future studies utilizing this vector and this murine model will test other HIV-1 immunogens and vectors expressing cytokine adjuvants. Finally, studies will also be performed to assess the role of CD4$^+$ or other responses that may mediate a state of immune memory.

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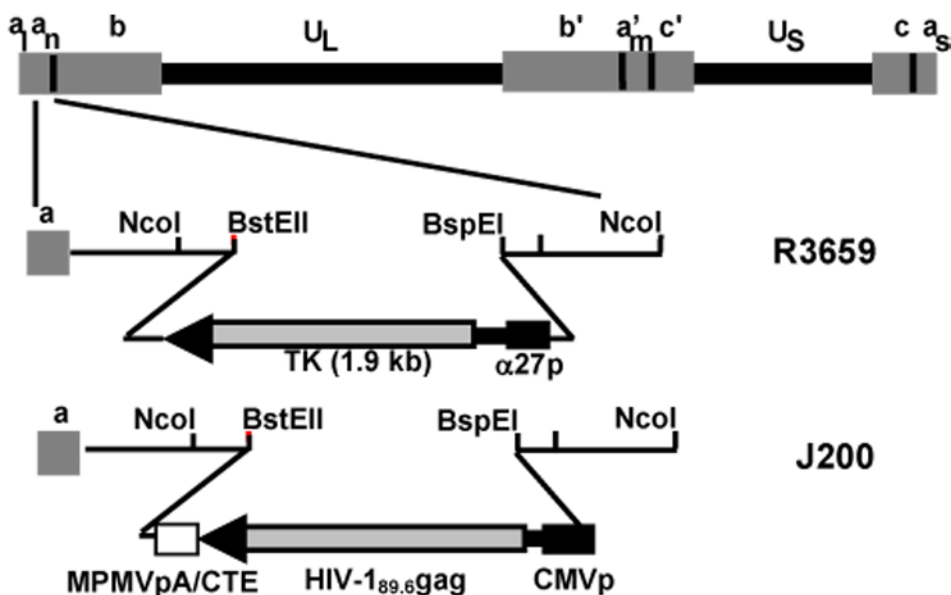
Figure 1.
Schematic diagram of $\gamma_1 34.5$-deleted HSV-1. Both copies of the HSV-1 $\gamma_1 34.5$ gene were replaced with the CMV-driven HIV-$1_{89.6}$ Gag expression cassette (only terminal repeat location is indicated on diagram). U_L, unique long; U_S, unique short; a,a', b,b', c,c', inverted repeat sequences; MPMVpA/CTE, Mason-Pfizer monkey virus polyadenylation signal/ cytoplasmic transport element; TK, thymidine kinase; $\alpha 27p$, HSV alpha 27 gene promoter; CMVp, cytomegalovirus immediate early promoter; R3659, parent virus of J200. Not shown schematically: the native tk gene in R3659 has been deleted.
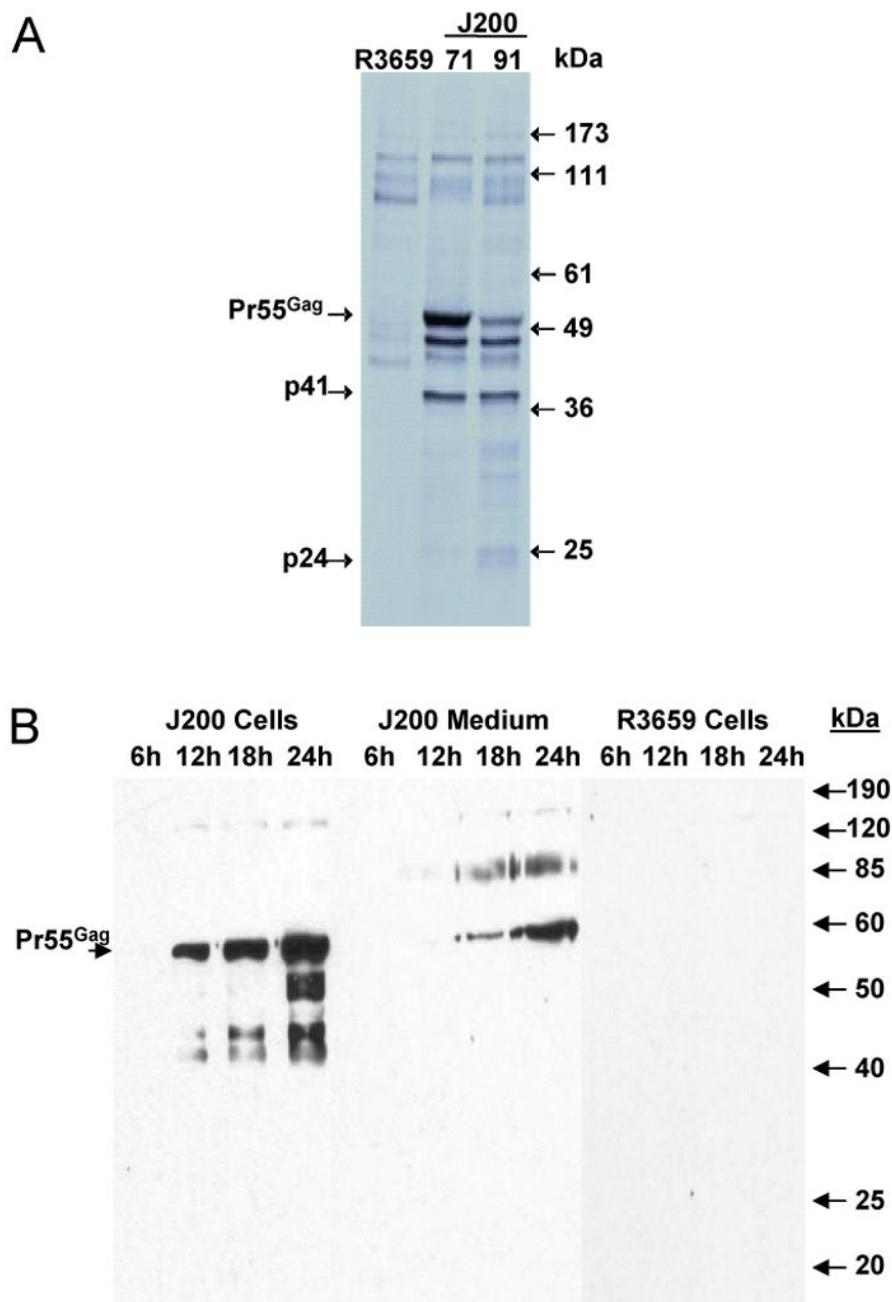
Figure 2.
Expression of HIV-1$_{89.6}$ Gag from J200-infected Vero cells. Vero cells were infected with either J200 or R3659, and protein expression was assessed by metabolic labeling and immunoprecipitation (panel A) or Western blot (panel B). Pr55^{Gag}, the p55 precursor Gag polyprotein; p41, protein product as a result of transcriptional initiation at a downstream ATG; p24, Gag cleavage product, the major HIV-1 capsid protein; kDa, protein standard size markers. 71 and 91 are individual clones of J200.
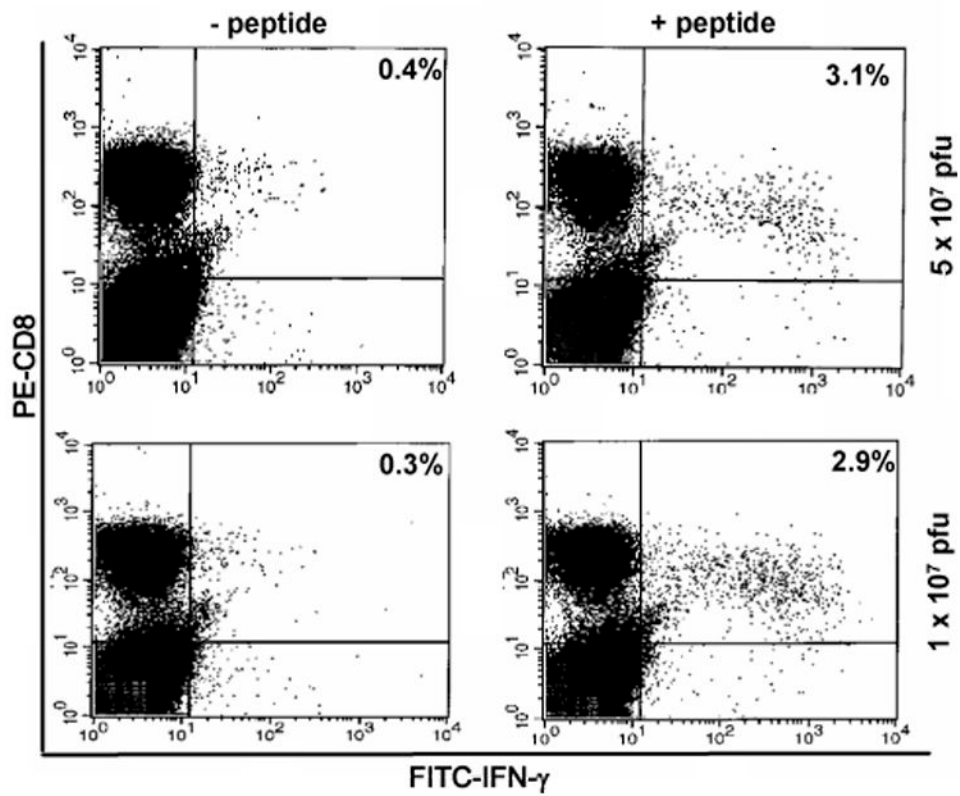
Figure 3.
Intracellular cytokine staining and flow cytometry analysis of splenocytes stained for CD8 (PE-labeled) and intracellular IFN-γ (FITC-labeled) production at 11 weeks post-immunization. Top panels: immunization dose was 5×10^7 pfu J200. Bottom panels: immunization dose was 1×10^7 pfu J200. For details, see text.

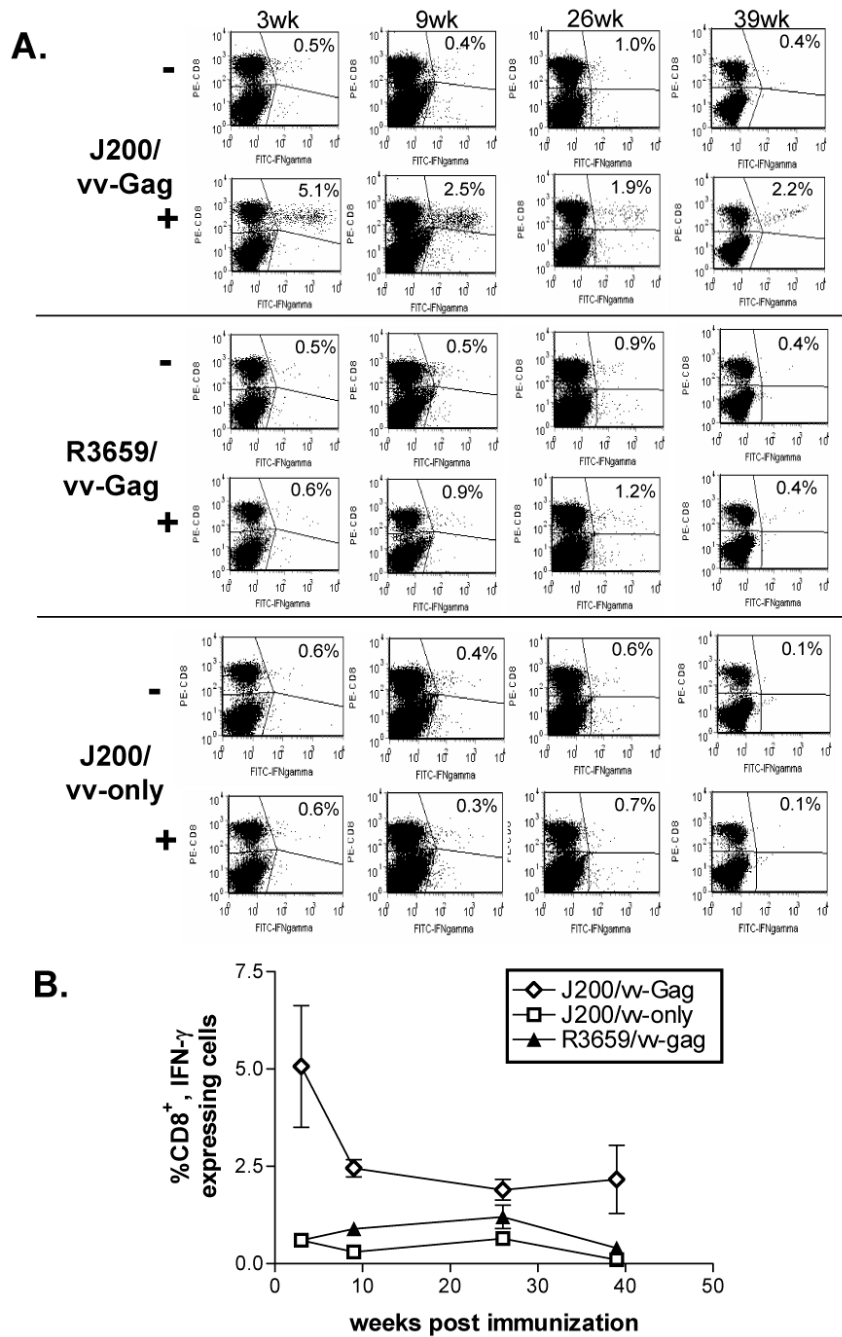**Figure 4.**
Intracellular cytokine staining and flow cytometry analysis of Gag-specific CD8+ responses over time in Balb/c mice immunized with 1×10^7 J200 i.p. Splenocytes were isolated from mice challenged 5 days prior i.p. with either vvGAG (vDK1) or vvonly (SC11), as indicated in Materials and Methods. Splenocytes were cultured in the presence (+) or absence (−) of the MHC class I-restricted HIV-1 p24 peptide (AMQMLKETI) for 6 hours prior to staining for both surface CD8+ (PE-CD8, vertical axis) and intracellular IFN-γ (FITC-IFNgamma, horizontal axis). A. Representative plots from one of the samples for each time point is shown. The average percent of positive Gag-specific IFN- γ-secreting CD8+ T cells is indicated in the upper right hand corner of each plot. B. Line graph indicating the average responses for all of

the mice within a given immunization group over time. Only the samples stimulated with p24 peptide are shown.