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HSV-1 amplicon vectors elicit polyfunctional T cell responses to HIV-1 Env, and strongly boost responses to an adenovirus prime

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Abstract

HSV-1 amplicon vectors elicit strong T-cell responses to encoded antigens but the qualitative nature of these responses is poorly understood. Antigen-specific CD4⁺ and CD8⁺ T-cell responses to amplicon and adenovirus (rAd5) vectors encoding HIV-1 gp120 were assessed following immunization of mice, by performing intracellular cytokine staining for IFN γ , IL-2 and TNF α , following stimulation of splenocytes with a HIV-1 Env peptide pool. The quality of the primary T-cell response to amplicon and rAd5 vectors was strikingly similar, but there were qualitative differences in responses to amplicon vectors that incorporated different promoters upstream of gp120 - suggesting that promoters can significantly influence immune response quality. When prime-boost combinations were studied, a rAd5 prime and amplicon boost elicited the highest T-cell response. Furthermore, protocols that incorporated a rAd5 prime consistently elicited a greater proportion of polyfunctional CD4⁺ T-cells - regardless of boost. This suggests that initial priming can shape immune response quality after a boost. Overall, these findings provide insight into effective vector combinations for HIV-1 vaccine development.

Keywords

HIV-1/AIDS vaccine; adenovirus vector; HSV-1 amplicon vector

1. INTRODUCTION

Efforts to develop a vaccine against HIV-1 are a major global health priority and a range of immunization strategies are being actively explored. One focus is the development and optimization of viral vectors to deliver HIV-1 antigens. Virus-based vaccine vectors under

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investigation include adenoassociated virus (AAV) [1,2], adenovirus [3-8], alphaviruses [9-13], poxviruses [14,15] and herpes simplex virus type 1 (HSV-1) [16-18].

Helper-free HSV-1 amplicon vectors are replication-defective, “gutless” vectors that possess compelling advantages as potential vaccine delivery platforms (reviewed in [16]). These advantages include (i) their ability to elicit strong cellular immune responses to encoded antigens [19-21], (ii) their broad host cell tropism and ability to directly transduce antigen presenting cells, including dendritic cells (DC), both *in vivo* and *in vitro* [21,22]; (iii) their favorable safety profile and lack of expression of virally-encoded immunomodulatory genes; and (iv) their very large transgene capacity (up to 150 kb) [23,24].

Our laboratory has previously shown that a single injection of a helper-free HSV-1 amplicon vector can elicit potent [19,20] and durable [19] immune responses to an encoded HIV antigen even in the face of preexisting immunity to HSV [19]. While the magnitude of the cellular immune response elicited by amplicon vectors has been extensively studied, little is known about the quality of that response, or the effectiveness of heterologous prime-boost immunization regimens that combine amplicon vectors with another viral vaccine modality. The present study was designed to address these questions.

For the prime-boost analysis, we focused on replication-defective recombinant adenovirus-5 (rAd5) vectors. These have been shown to elicit durable and strong CD8⁺ T-cell responses to encoded antigens in naïve hosts [3], but their effectiveness is limited by pre-existing antibodies in many human subjects and by the rapid induction of virus-neutralizing antibodies following the delivery of an initial vaccine dose [25,26]. The use of heterologous prime boost regimens that include diverse virus vectors in combination with adenovirus may help to overcome these limitations. We therefore performed experiments to examine the magnitude and quality of immune responses elicited by a heterologous prime boost regimen combining HSV-1 amplicon and rAd5 vectors that encoded the same HIV-1 antigen (gp120).

We also performed a careful comparison of the magnitude and quality of the T cell response that was elicited by HSV-1 amplicon and rAd5 vectors encoding the same antigen, at both acute and late time points following an initial priming immunization. Antigen-specific CD4⁺ and CD8⁺ T cell responses were assessed by intracellular cytokine staining for IFN γ , IL-2 and TNF α , following stimulation of splenocytes with a HIV-1 Env peptide pool. We focused on IL-2, IFN- γ and TNF- α production because T cell responses to vaccination or infection are characterized by populations of cells that produce various combinations of IFN- γ , TNF- α or IL-2 [27-30]. These differences have profound implications for effector function and development into memory cells. For example, CD4⁺ T cells that secrete only IFN- γ have minimal intrinsic proliferation capacity and a limited capacity to develop into memory cells, when compared to cells that produce IL-2 or IL-2 plus IFN- γ [31-33]. In addition, multifunctional cells that secrete all three cytokines have been shown to produce higher levels of cytokine per cell [34,35], and the frequency of such multifunctional cells has been shown to be a correlate of vaccine-mediated protection against infection with *Leishmania major* [34], and of non-progressor status in HIV-1 infection [36].

The results of our studies show that gp120-encoding HSV-1 amplicon vectors and rAd5 vectors elicit primary T cell responses that are similar both in magnitude and quality. In contrast, HSV-1 amplicon vectors that incorporated different promoter elements upstream of gp120 elicited qualitatively and quantitatively distinct antigen-specific T cell response profiles.

When prime-boost immunization regimens were studied, the combination of a rAd5 prime followed by HSV-1 amplicon boost elicited the highest overall CD4⁺ and CD8⁺ T cell response. In addition, protocols that incorporated a rAd5 prime consistently elicited the greatest proportion of polyfunctional CD4⁺ T cells (i.e., cells that secreted all three cytokines that were

assayed). Thus, a rAd5 prime, HSV-1 boost regimen elicited the best overall combination of T cell response magnitude and quality.

2. MATERIALS AND METHODS

2.1 Viral vectors

All vectors contained a human codon-optimized gene encoding HIV-1 gp120 (*syngp120*); this gene was derived from the clade B MN strain of HIV-1, and included a V3 loop substitution from HIV-1 LAI, that introduced an immunodominant H2D^d restricted V3 epitope (RGPGRFVFI), recognized in BALB/c mice [37,38]. Three different HSVgp120 amplicon vectors and an E1-deleted, replication defective type 5 adenovirus (rAd5) vector were constructed. The three HSVgp120 amplicon vectors all contained the same HIV-1 gp120 insert, placed under the transcriptional control of three different promoters: the major human cytomegalovirus immediate early promoter (*cmv*), a modified *cmv* promoter containing regulatory sequences from the human T-cell leukemia virus type 1 LTR (*cmvr*) [39], and the herpes simplex virus immediate early 4/5 promoter (*hsv*). HIV-1 gp120 expression in the rAd5 vector was transcriptionally controlled by the *cmv* promoter.

For all experiments, an amplicon vector encoding *E. coli* β -galactosidase (HSV β GalZ) was used as a negative control.

2.2 Construction of HSV-1 amplicon vectors

The pVAX1 plasmid, which contains a basic *cmv* promoter/enhancer, was purchased from Invitrogen (Carlsbad, CA) and modified by inserting the HSV-1 origin of replication and packaging signals into the *Pml*I site, to create pVAX.HSV(*cmv*).

pVAX.HSV(*cmv*) was further modified by (1) excising the *cmv* promoter/enhancer and the multiple cloning site (MCS) by digesting with *Mlu*I and *Pme*I and (2) ligating a new MCS linker created from the following oligonucleotide (and its complement): 5'-*Mlu*I-AGCGCTCCGCGGACCGGTCCTAGGATCGATACTAGTCAATTGAGTACTGCTAGCGTTTAAACCTTAAGAAGCTTCTGCAGGATATCGCGGCCGCTCGAGTCTAGAGGGCCC-PmeI-3'. This resulted in the creation of the promoterless vector, pVAX.HSV.

The *cmvr* promoter was PCR amplified from plasmid VRC 8400 (provided by the NIH VRC) using forward (5'-*Afe*I-TACTGAGAGTGCACCATATGCG-3') and reverse (5'-*Age*I-TGTCGACGACGGTGAAGTGCAGA-3') primers and the resulting 1.2kb DNA fragment cloned into promoterless pVAX.HSV using *Afe*I and *Age*I, to generate pVAX.HSV(*cmvr*).

pVAX.HSV(*cmv*)gp120 was created by PCR amplifying *syngp120* from the plasmid pHSVgp120 [19] using forward (5'-*Avr*II-CTCGAGATCCATTGTGCTC-3') and reverse (5'-*Sal*I-TTACCGCTTCTCGCGCTGCACCAC-3') primers and then cloning the resulting 1.6kb DNA fragment into *Nhe*I and *Xho*I digested pVAX.HSV(*cmv*).

pVAX.HSV(*cmvr*)gp120 was created by PCR amplifying *syngp120* from the plasmid pHSVgp120 using forward (5'-*Mfe*I-CTCGAGATCCATTGTGCTCTAAA-3') and reverse (5'-*Not*I-TTACCGCTTCTCGCGCTGCACCACGCG-3') primers and cloning the resulting 1.6kb DNA fragment into pVAX.HSV(*cmvr*) using *Mfe*I and *Not*I.

The construction of pHSV(*hsv*)gp120 has been described [19], as has the process of vector packaging [19,20]

2.3 Construction of rAd5 vector

The adenovirus type 5 vector (rAd5) used in this study was prepared using the AdEasy™ vector system (Qbiogene, Carlsbad, CA) [40]. The syngp120 insert was PCR-amplified and cloned into the adenovirus shuttle vector pShuttle; this vector contains a CMV promoter upstream of the inserted gp120 gene insert [41]. Viral titers were determined in genome units, by real-time PCR using Taqman probes and primers directed to the adenovirus hexon gene, as described [41].

2.4 Mice

Female BALB/c mice, aged 5 to 6 weeks, were obtained from Taconic Laboratories (Germantown, NY) and maintained according to University of Rochester and National Institutes of Health guidelines. 28-gauge 0.5-inch, ½ cc insulin syringes (Becton Dickinson, Franklin Lakes, NJ) were used for injections.

2.5 Immunization of mice

Groups of 4 mice were injected intramuscularly (i.m) in the left hind quadriceps with virus inocula that were diluted into a final volume of 100µL phosphate buffered saline (PBS). Two series of experiments were conducted.

These experiments were performed using a dose of 10^6 genome units of the HSV-1 amplicon vectors, and 10^7 genome units of adenovirus vectors. Our intention was to compare the quality of the immune response elicited by these two vectors, and not to perform a direct head-to-head comparison of the relative potency of the two platforms. Thus, we deliberately avoided using maximum tolerated vector dose levels. Rather, we selected vector dose levels that, based on prior experience, were expected to yield antigen-specific cellular immune responses of roughly equivalent magnitude.

In the first series of experiments, mice were inoculated with 1×10^6 genome units (g.u.) of HSVgp120, 1×10^7 g.u. of rAd5gp120, 1×10^6 g.u. of HSVLacZ or a combination of 5×10^5 g.u. of HSVgp120 and 5×10^6 g.u. of rAd5gp120 as summarized in Table 1A. Mice were euthanized 12 days post prime.

In the second series of experiments, mice were primed with 1×10^6 genome units of HSVgp120, 1×10^7 g.u. of rAd5gp120 or 1×10^6 g.u. of HSVLacZ. They were then boosted, 72 days post prime, with 1×10^6 g.u. of HSVgp120 or 1×10^7 g.u. of rAd5gp120 as outlined in Table 1B. Mice were euthanized 12 days post boost.

2.6 Sample collection and splenocyte preparation

Serum samples were collected by the retro-orbital route in 1.7 ml tubes containing 20µL of heparin (Sigma-Aldrich), separated by centrifugation and stored at -80°C until further manipulation.

Spleens were homogenized by gentle grinding between the frosted ends of autoclaved glass slides and a single cell suspension of splenocytes prepared by passing the homogenized cells through a 70µm strainer (Becton Dickinson, Franklin Lakes, NJ) into cold RPMI (Gibco-BRL) supplemented with 10% inactivated FBS (Hyclone), 50µM β-mercaptoethanol and PSG (Gibco-BRL). Cells were stained with trypan blue, counted and used immediately.

2.7 Intracellular cytokine staining (ICS)

5×10^6 splenocytes were stimulated with a 15-mer HIV-1 MN Env peptide pool (2 µg/ml each peptide; NIH AIDS Research & Reference Reagent Program) that was supplemented with the

immunodominant V3 peptide (RGPGRFVFI; Sigma-Genosys). Cells were exposed to peptide in the presence of 5 µg of purified anti-mouse CD28 (BD Pharmingen) and 5 µg of purified anti-mouse CD49D (BD Pharmingen) for 6 hours at 37°C/5%CO₂. Golgi plug (BD Pharmingen) was added during the final 4 hours of incubation. Cells were then stained with anti-mouse CD16/32 (Fc block) antibody followed by surface staining.

Cells in experimental Series 1, were surface stained with a cocktail of anti-CD3 PE-Cy7, anti-CD4 PerCP, and anti-CD8 PE-Cy5. Cells were then fixed, permeabilized with cytofix cytoperm (BD Pharmingen) and stained with a cocktail of anti-CD3 PE-Cy7, anti-CD4 PerCP, anti-CD8 PE-Cy5, anti-IFNγ PE, anti-IL2 FITC and anti-TNFα APC.

Cells in experimental Series 2, were surface stained with a cocktail of anti-CD3 PE-Cy7, anti-CD4 PerCP, anti-CD8 Alexa 430 and anti-CD44 Alexa 405. Cells were then fixed and permeabilized with cytofix cytoperm (BD Pharmingen) and stained with a cocktail of anti-CD3 PE-Cy7, anti-CD4 PerCP, anti-CD8 AlexaFluor 430, anti-CD44 AlexaFluor 405, anti-IFNγ PE, anti-IL2 FITC and anti-TNFα APC.

1×10⁶ cells per sample were acquired on a BD LSRII flow cytometer (BD Biosciences) and FACS data were analyzed using FlowJo software (TreeStar, Ashland, OR).

2.8 Statistical analysis

Statistical analyses were performed using GraphPad PRISM software version 4.0 (GraphPad Software, Inc). Comparison of cellular immune responses, between groups, was performed using one-way nonparametric Analysis of Variance (ANOVA) followed by Tukey-Kramer post test. P values were considered to be significant if less than 0.05.

3. RESULTS

3.1 Analysis of CD8⁺ and CD4⁺ T-cell immune responses, following primary immunization with different viral vector

HSV-1 amplicon vectors elicit potent immune responses to encoded HIV-1 antigens [19,20]. However, there is little available information on the overall quality of the amplicon-induced cellular immune response, and on the ability of amplicon vectors to effectively synergize with another viral immunization modality. In the present study, we conducted an in depth, head-to-head comparison of the quality of the immune response elicited by a helper-free HSV-1 amplicon vector and a replication-defective recombinant human adenovirus-5 (rAd5) vector encoding the same HIV-1 antigen.

To assess the overall quality of the acute immune response to an amplicon-vectored HIV-1 antigen, 6 groups of BALB/c mice (4/group) were immunized using the schedule outlined in Table 1A. Animals were inoculated with one of three HSV-1 amplicon vectors which encoded HIV-1 gp120 under the transcriptional control of three different promoters: (1) the major human cytomegalovirus (CMV) immediate early (*cmv*) promoter, (2) a modified *cmv* promoter containing regulatory sequences from the human T-cell leukemia virus type 1 LTR (*cmvr*) [39], and (3) the herpes simplex virus immediate early 4/5 promoter (*hsv*). Additional groups of animals received a recombinant adenovirus type-5 encoding the same HIV-1 gp120 gene (rAd5gp120 (*cmv*)), under the control of the CMV promoter (*cmv*) or a mixed inoculum containing the rAd5gp120 (*cmv*) vector and the HSVgp120 (*hsv*) vector. Mice were sacrificed 12 days post immunization, splenocytes collected and HIV-1 envelope (Env)-specific T-cells quantitated by performing intracellular cytokine staining for interferon gamma (IFNγ), interleukin 2 (IL2) and tumor necrosis factor alpha (TNFα). We gated on CD3⁺CD8⁺ and CD3⁺CD4⁺ T-cells and assessed the production of IFNγ in these cell populations. We then analyzed IL2 and TNFα production by both the IFNγ-positive and IFNγ-negative cells, and

the frequency of cells producing a single cytokine, or a combination of two or more cytokines was calculated.

As shown in Figure 1, both the HSV amplicon and rAd5 vectors elicited a strong CD8⁺ and CD4⁺ Env-specific T cell response, when compared to mice immunized with an irrelevant antigen (HSV_{LacZ}). There was no significant difference in the frequency of cytokine producing CD8⁺ or CD4⁺ T-cells in the rAd5gp120 immunized mice compared to the HSV(*cmv*)gp120 or HSV(*hsv*)gp120 immunized mice. Mice immunized with the HSV(*cmv*)gp120 amplicon produced a significantly higher frequency of cytokine producing CD8⁺ and CD4⁺ T-cells when compared to all of the other vectors, while co-delivery of the HSV(*hsv*)gp120 amplicon vector and the rAd5gp120 vector yielded a response that was statistically indistinguishable from that elicited by either rAd5gp120 or HSV(*hsv*)gp120 alone.

Qualitative dissection of the Env-specific CD8⁺ (Figure 2A) and CD4⁺ (Figure 2B) T-cell response provided additional insight into the nature of the cellular immune response elicited by the various vectors. The CD8⁺ T-cell response (Figure 2A) was very similar between the rAd5 and amplicon vector platforms, and was comprised of two distinct cell populations: the majority of cells produced IFN γ alone (CD3⁺CD8⁺IFN γ ⁺IL2⁻TNF α ⁻), while a smaller subset of cells produced both IL2 and IFN γ (CD3⁺CD8⁺IFN γ ⁺IL2⁺TNF α ⁻).

In contrast, the CD4⁺ T-cell response (Figures 2B and 2C) showed a broader spectrum of cytokine producing cells, with many more cells that were capable of simultaneously producing two or more cytokines. For both vector platforms, the largest single population of cytokine-producing CD4⁺ T cells were cells that produced IFN γ alone. The relative proportion of these cells was roughly similar in animals that received the HSV-1 amplicon or rAd5 vectors alone, and was greater in mice that received the combination of both vectors (as a single combined inoculum).

The HSV-1 amplicon and rAd5 platforms both elicited significant numbers of dual cytokine-producing cells (especially IFN γ and IL2 positive cells) and multifunctional cells (i.e., IFN γ ⁺IL2⁺TNF α ⁺ cells). The CD4⁺ T cell cytokine profiles was especially similar in the case of the vectors that contained the same basic CMV promoter element upstream of gp120 [i.e., HSV-1 (*cmv*) and rAd5 (*cmv*)]. In contrast, comparison of the response elicited by the three different HSV-1 amplicon vectors revealed subtle differences in the qualitative nature of the CD4⁺ T cell response. This is most apparent in the frequency of CD4⁺ T cells that produced IL2 alone (*hsv* > *cmv* > *cmvr*; Figure 2B), as well as in the frequency of cells producing IFN γ alone (*cmvr* > *cmv* = *hsv*) and IFN γ plus TNF α (*cmvr* > *cmv* = *hsv*) (Figure 2C).

3.2 Heterologous vector prime-boost incorporating HSV-1 amplicon enhances the frequency of cytokine producing CD8⁺ and CD4⁺ T-cells

To determine whether amplicon vectors may be effectively combined with another viral immunization modality, BALB/c mice (4/group) were primed with rAd5 gp120 or HSV(*hsv*)gp120 amplicon at day 0 and boosted 72 days post prime with HSV(*hsv*)gp120 or rAd5gp120 (Table 1B). Mice were sacrificed 12 days post boost, splenocytes collected and Env-specific T-cells quantitated by performing intracellular cytokine staining for IFN γ , IL2 and TNF α . We gated on CD3⁺CD8⁺ and CD3⁺CD4⁺ T-cells and assessed the production of IFN γ in these cell populations. We then analyzed IL2 and TNF α production by both the IFN γ -positive and IFN γ -negative cells, and the frequency of cells producing a single cytokine, or a combination of two or more cytokines was calculated.

As shown in Figure 3, the magnitude of the Env-specific CD4⁺ and CD8⁺ T cell response was significantly increased when mice which were primed with HSV(*hsv*)gp120 or rAd5gp120 received either a homologous or heterologous boost (compared to mice that did not receive a

booster). The magnitude of the CD8⁺ T-cell response in the heterologous prime-boost combinations is impressive, but similar to that elicited by other efficient heterologous prime-boost regimens that incorporate virus vectors. For example, priming of BALB/c mice with a single-cycle vesicular stomatitis virus (VSV)-Env vector and subsequent boosting with a heterologous vaccinia-Env construct generated approximately 25% tetramer-positive CD8⁺ T cells post-boost [42].

The data in Fig. 3 further show that a homologous amplicon prime-boost combination elicited a larger frequency of antigen-specific, cytokine producing CD8⁺ and CD4⁺ T-cells than a homologous rAd5 (rAd5gp120) prime-boost. However, the greatest CD8⁺ and CD4⁺ responses were detected in animals that received a priming immunization with rAd5gp120 and a subsequent boost with HSV(*hsv*)gp120. Somewhat unexpectedly, the reverse combination (amplicon prime, followed by adenovirus boost) was considerably less effective. These findings show that the HSV-1 amplicon vector is a highly effective boost to a heterologous rAd5 prime, and that this heterologous prime-boost combination leads to considerably stronger responses than can be achieved with either vector alone.

3.3 Viral vector influences the spectrum of functional T-cells in a prime boost regimen

The cellular immune response in mice that received the prime-boost immunization regimens was also analyzed at a qualitative level, and the data are summarized in Figure 4. The immune response profiles in each figure have been divided into three groups: (1) Mice primed with HSV(*hsv*)gp120 or rAd5gp120 and receiving no boost, (2) mice primed with HSV(*hsv*)gp120 or rAd5gp120 and receiving a rAd5gp120 boost and (3) mice primed with HSV(*hsv*)gp120 or rAd5gp120 and receiving an HSV(*hsv*)gp120 boost.

Analysis of the CD8⁺ T-cell cytokine profile (Figure 4) shows a narrow spectrum of cytokine producing cells, regardless of the immunization regimen or vector employed. The response was dominated by cells producing IFN γ alone; multifunctional cells were much less frequent and were principally limited to cells that produced the combination of IFN γ and TNF α . In contrast, the CD4⁺ T-cell response (Figure 4) revealed a broad spectrum of cytokine producing cells in all three groups.

Immunization with a combination of rAd5gp120 (prime) and HSV(*hsv*)gp120 (boost) led to an increase in the magnitude of each of the seven possible antigen-specific, cytokine-producing CD4⁺ T cell subpopulations, relative to all of the other immunization regimens (Figure 4B). These differences achieved statistical significance in four cases (marked by asterisks in Figure 4B). In addition, a homologous prime-boost immunization regimen using the HSV(*hsv*)gp120 amplicon vector led to a significant elevation in the frequency of antigen-specific CD4⁺ T cells producing IFN γ only (Figure 4C).

Immunization with HSV(*hsv*)gp120 or rAd5gp120 alone (in the absence of a boost) resulted in very similar patterns of antigen-specific CD4⁺ T cell cytokine production when assessed 84 days after the original inoculation. This memory T cell response was principally composed of cells producing two or more cytokines, with a large fraction of multifunctional cells (ie. cells that produced IFN γ , IL2, and TNF α). This is consistent with the maturation of the acute CD4⁺ T-cell response (Figure 2), and the acquisition of greater functionality in the antigen-specific CD4⁺ memory T cell population.

Boosting immunization at day 72 resulted in both a quantitative increase in the CD4⁺ response (Figure 3) and a qualitative shift in the nature of that response (Figure 4). Regardless of the boost that was given, animals that received a priming inoculation with the HSV-1 amplicon vector exhibited a distinct CD4⁺ response profile, compared to animals that received priming inoculation with the rAd5 vector. This was characterized by a relative increase in the proportion

of cells that produced IFN γ alone and a relative decrease in the proportion of multifunctional cells (i.e., cells that produced IFN γ , IL2, and TNF α) (Figure 4). Overall, the quality of the CD4 $^{+}$ T-cell response was superior in rAd5-primed animals, as compared to HSV-1 amplicon-primed animals, while the rAd5 prime/HSV-1 amplicon boost elicited the best combination of immune response magnitude and quality (Figures 3 and 4).

4. DISCUSSION

The quality of the cellular immune response, and the elicitation of a polyfunctional signature in the cytokine profile of antigen-specific CD4 $^{+}$ and CD8 $^{+}$ T-cells has become recognized as an important predictor of the effectiveness of naturally-acquired and vaccine-induced immune responses to HIV-1 and its simian counterpart, SIV [33,43-53]. In this study, we therefore sought to characterize the quality of the CD4 $^{+}$ and CD8 $^{+}$ T-cell response that is induced following immunization with HSV-1 amplicon vectors. Related objectives were to compare the quality of this CD4 $^{+}$ and CD8 $^{+}$ T-cell response to the response elicited by immunization with rAd5, and to determine whether HSV-1 amplicon vectors could be effectively used in a prime boost regimen with another viral vector modality.

Qualitative analyses of the primary T-cell response to an amplicon-vectored HIV-1 antigen revealed a potent CD8 $^{+}$ and CD4 $^{+}$ T-cell response that was characterized by high numbers of CD4 $^{+}$ cells that produced only a single cytokine (IFN γ). The CD4 $^{+}$ T-cell cytokine profiles elicited by the two vectors were strikingly similar, particularly when the encoded HIV-1 antigen was expressed from the same promoter. In contrast, there were distinct differences in the CD4 $^{+}$ T-cell cytokine profiles elicited by HSV-1 amplicon vectors that contained alternative promoter elements upstream of the HIV-1 gp120 insert. This was unexpected, since all of the promoters used are considered to be broadly and constitutively active. Amplicon vectors containing these promoters exhibit a relative hierarchy of antigen expression, as follows: *cmvr* > *cmv* > *hsv* [20]. However, the differences in antigen expression levels between the three promoters are generally modest in most cell types, except for primary dendritic cells [20]; thus, the relationship between antigen expression levels and immune response quality remains to be determined.

A major difference in the CD4 $^{+}$ T-cell response elicited by Env-encoding HSV-1 amplicon vectors with alternative promoters was reflected in the frequency of single-positive IL-2 producing cells. These cells likely represent primed, but uncommitted (precursor) CD4 $^{+}$ T cells (Thpp) [54,55]. This uncommitted CD4 $^{+}$ T-cell population produces IL-2 and several chemokines, but few of the cytokines that characterize Th1 or Th2 cells [56] - as a result, they may have the ability to recruit other immune cells and to provide flexibility in shaping future responses.

These results also underscore the importance of transcriptional promoter elements in influencing not only the magnitude of the cellular immune response to amplicon-encoded HIV-1 antigens [20], but also the quality of that response. It will be of interest to conduct future studies to determine whether promoter-dependent differences in the levels of antigen expression, either globally or in specific cell types, result in differences in the quality of the resulting CD4 $^{+}$ T-cell response to virus or plasmid vectors. It also suggests that it may be important to consider promoter effects when making comparisons of immune response quality between different vaccine vector platforms.

Studies in preclinical animal models and in HIV-1 seronegative human volunteers have shown that antigen-specific cellular immune responses to HIV-1 antigens can be enhanced when heterologous vectors are combined in a prime-boost strategy. The most frequently used combination is a DNA prime followed by a viral vector boost; this often results in an increase

in the magnitude and durability of the antigen-specific cellular immune response (reviewed in [57]). Consistent with this, a DNA prime, followed by an amplicon boost, results in an increase in the magnitude of the CD8⁺ T-cell responses to an encoded HIV antigen in mice [58].

We therefore analyzed immune responses to prime-boost regimens that included both homologous and heterologous combinations of HSV-1 amplicon and rAd5 vectors encoding the same HIV-1 antigen. For these experiments, we chose to allow primed mice to ‘rest’ for 72 days before boosting. Since HSV-1 amplicon vectors induce high and transient levels of transgene (antigen) expression [22], we expected that the overwhelming majority of antigen specific T cells present at the time of the boost would therefore be memory cells. This prediction was consistent with the strikingly polyfunctional nature of the CD4⁺ T-cell population in mice that received only a priming immunization with HSV-1 amplicon or rAd5 vectors (and no boost). In both cases, the antigen-specific CD4⁺ T-cell population underwent a dramatic shift between an early time point post-immunization (day 12; Figure 2) and the late time point (day 84; Figure 4).

The functional diversity of the gp120-specific CD4⁺ T cell population was much greater at 84 days following vector immunization than at the effector phase, 12 days following immunization. This is consistent with the changes in the functional signatures of antigen-specific CD4⁺ T cells that occur over time, in response to cell differentiation, establishment of immune memory, and changes in antigen load [33,46]. HSV-1 amplicon vectors are known to elicit a sharp, acute burst of antigen exposure that is extinguished within a week following vector delivery [22], while adenovirus vectors may continue to produce low levels of antigen over a protracted period [59]. Thus, the broad similarity in the functional CD4⁺ T cell profiles elicited by these vectors at both the effector (day 12; Figure 2) and memory phases (day 84; “prime only” groups, Figure 4) is of interest. Whether the functional T cell signatures elicited by the two vectors may diverge progressively over time, due to persistent low level antigen production by adenovirus vectors, is uncertain and will require further study.

HSV-1 amplicon and rAd5 vectors both proved effective in boosting the magnitude of the Env-specific CD4⁺ and CD8⁺ immune response that was elicited by an initial priming immunization. HSV-1 amplicon vectors were uniformly more effective as a boost than rAd5 vectors, when used either in homologous or heterologous prime-boost combinations, perhaps because HSV-1 amplicon vectored vaccines are relatively resistant to pre-existing antiviral immunity [19], unlike rAd5 vectored vaccines [26,60-63]. The beneficial effect of HSV-1 amplicon vector boosting was especially evident in the magnitude of the CD8 responses, which was greatest in the heterologous prime-boost regimen that incorporated a rAd5 vector prime and a HSV amplicon boost. The magnitude of the CD8⁺ T cell response to this heterologous prime-boost regimen is similar to that reported in BALB/c mice immunized with a single cycle VSV-Env construct and then boosted with a vaccinia-Env vector [42]. This suggests that the rAd5 vector prime and HSV amplicon boost combination elicits CD8⁺ T cell responses that compare favorably in magnitude with those elicited by some of the most effective prime-boost combinations presently available.

In comparing the various prime-boost combinations, it is striking that a rAd5 vector prime plus HSV amplicon boost considerably outperformed the reciprocal regimen (HSV amplicon prime, followed by rAd5 boost). The underlying reasons for this will require further investigation, but it is possible that the ability of adenovirus type-5 vectors to elicit potent innate immune responses and local inflammatory reactions, which are essential in initiating primary immune responses, plays a role in this process [64-66].

We also examined the quality of the antigen-specific T cell response that was elicited by the various immunization regimens. Regimens that incorporated the rAd5 vector prime

consistently generated a CD4⁺ T cell response profile that was characterized by high levels of multifunctional cells, regardless of which vector was used as a boost. In contrast, regimens that incorporated the HSV-1 amplicon prime yielded low levels of multifunctional cells, regardless of the boosting immunization. Thus, the initial priming immunization exerted a durable effect on the qualitative nature of the boosted response. If this finding is generalizable to other vector platforms and other antigens, it may have important implications for HIV vaccine development.

Overall, experiments show that the heterologous combination of a rAd5 vector prime and a HSV amplicon vector boost yielded the best combination of cellular immune response magnitude and quality. This may provide a useful way to extend the utility and effectiveness of rAd5 vectors, and an alternative or adjunct to the use of nonhuman or rare adenovirus serotype vectors [26,60,67]. These findings point the way forward to novel and more effective vector combinations for HIV-1 vaccine development.

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Figure 1. Quantitative analysis of the Env-specific T-cell response following single dose injection of rAd5 and HSV-1 amplicon vectors into mice

rAd5 and HSV-1 amplicon vectors encoding HIV-1 gp120 under the transcriptional control of different promoters were used in this experiment. Promoter elements including the standard CMV major immediate early promoter-enhancer (*cmv*), a hybrid CMV promoter and HTLV-1 LTR enhancer (*cmvr*) or the HSV-1 4/5 immediate early promoter (*hsv*); see text for details). These vectors were inoculated either alone or in combination (HSV & rAd5 group) into the left hind quadriceps of BALB/c mice (4/group; doses are indicated in Table 1A). Animals were sacrificed 12 days later and splenocytes were harvested. Env-specific responses were assayed by intracellular cytokine staining for CD8⁺T-cell (**A**) and CD4⁺T-cell (**B**) production of IFN γ , TNF α and IL2. The data represent the total percentage of T cells that produced any of the measured cytokines in response to stimulation with the Env peptide pool. Data shown are for individual mice; horizontal bars represent the mean cytokine production for each group. Statistical analysis was performed by a non-parametric one-way ANOVA. All values from immunized animals showed statistically significant induction of Env-specific T cell responses, versus mice immunized with amplicon particles encoding an irrelevant protein (HSV:LacZ; $p < 0.05$).

Figure 2. Cytokine profile of the Env-specific T-cell response following single dose injection of rAd5 and HSV-1 amplicon vectors into mice

rAd5 and HSV-1 amplicon vectors encoding HIV-1 gp120 from the indicated promoter elements (*cmv*, *cmvr*, *hsv*; see Fig. 1 legend), were inoculated either alone or in combination (HSV & rAd5 group) into the left hind quadriceps of BALB/c mice (4/group) at the doses denoted in Table 1A. Animals were sacrificed 12 days later and splenocytes were harvested. Env-specific responses were assayed by intracellular cytokine staining for CD8⁺T-cell and CD4⁺T-cell production of IFN γ , TNF α and IL2. Analysis was performed by gating on CD8⁺ and CD4⁺ T-cells for the production of IFN γ . IFN γ -positive and IFN γ -negative cells were then separately gated, to assess antigen-specific production of IL2 and TNF α . The frequency of cells producing a single cytokine, or two or more cytokines was calculated using FlowJo

software. The CD8⁺ (**A**) and CD4⁺ T-cell (**B**) data are presented here as pie charts; each pie chart represents the mean cytokine profile for splenocytes from each group of mice. Each slice (different color) of a pie denotes a distinct subset of cytokine producing cells for that group of immunized mice, after subtraction of non-specific background staining (determined from mice immunized with HSV_{LacZ}). (**C**) Quantitative data for the CD4⁺ T-cell response. The percentage of CD4⁺ T-cells that produced each of the seven possible combinations of IFN γ , TNF α and IL2 in response to stimulation with the Env peptide pool is shown. Data represent mean values for each group of four mice; horizontal bars represent the standard error of the mean. Statistical analysis was performed by two-way ANOVA with Bonferroni's post-test. * Different from all groups ($p < 0.01$); ** different from all groups ($p < 0.001$). Note that panel C includes background staining data (determined from mice immunized with HSV_{LacZ}); no background subtraction was applied to the data in this panel.

Figure 3. Quantitative analysis of the Env-specific T-cell response following heterologous vector prime boost injections of rAd5 and HSV-1 amplicon vectors into mice

BALB/c mice (4/group) were primed with rAd5 (1×10^7 genome units) and HSV-1 amplicon (1×10^6 genome units), encoding HIV-1 gp120, by inoculation into the left hind quadriceps at day 0. Mice were boosted 72 days post prime, with HSVgp120 (1×10^6 g.u.) or rAd5gp120 (1×10^7 g.u.) as summarized in Table 1B. Animals were sacrificed 12 days post boost and splenocytes were harvested. Env-specific responses were assayed by intracellular cytokine staining for: CD8⁺T-cell production of IFN γ , TNF α and IL2 in splenocytes from HSV-1 amplicon (A) and rAd5 (B) primed mice; CD4⁺T-cell production of IFN γ , TNF α and IL2 in splenocytes from HSV-1 amplicon (C) and rAd5 (D) primed mice. The data represent the total percentage of T cells that produced any of the measured cytokines in response to stimulation with the Env peptide pool. Data shown are for individual mice; horizontal bars represent the mean cytokine production for each group. Statistical analysis was performed by a non-parametric one-way ANOVA followed by a Tukey-Kramer post test, using GraphPad Prism. Statistically significant enhancement in T-cell responses, versus mice that were not boosted (None) is denoted by “+” signs as follows: +, $p < 0.01$; ++, $p < 0.001$. Statistically significant

enhancement in T cell responses, versus mice that were boosted with rAd5, is denoted by “ Δ ” signs as follows: Δ : $p < 0.01$; $\Delta\Delta$: $p < 0.001$.

Figure 4. Cytokine profile of the Env-specific T-cell response following heterologous vector prime boost injections of rAd5 and HSV-1 amplicon vectors into mice

BALB/c mice (4/group) were primed with rAd5 (1×10^7 genome units) and HSV-1 amplicon (1×10^6 genome units), encoding HIV-1 gp120, by inoculation into the left hind quadriceps at day 0. Mice were boosted 72 days post prime, with HSVgp120 (1×10^6 g.u.) or rAd5gp120 (1×10^7 g.u.) as summarized in Table 1B. Animals were sacrificed 12 days post boost and splenocytes were harvested. Env-specific responses were assayed by intracellular cytokine staining for CD8⁺T-cell and CD4⁺T-cell production of IFN γ , TNF α and IL2. Analysis was performed by gating on CD8⁺ and CD4⁺ T-cells for the production of IFN γ . IFN γ -positive and IFN γ -negative cells were then separately gated, to assess antigen-specific production of IL2 and TNF α . The frequency of cells producing a single cytokine, or two or more cytokines was calculated using FlowJo software. (A) The CD8⁺ and CD4⁺ T-cell data are presented here as pie charts; each pie chart represents the mean cytokine profile for splenocytes from each group

of mice. Each slice (different color) of the pies denotes a distinct subset of cytokine producing cells for that group of immunized mice, after subtraction of non-specific background staining (determined from mice immunized with HSVlacZ). **(B)** Quantitative data for the CD4⁺ T-cell response. The inset Figure legend denotes the various prime-boost combinations used (Ad prime only = rAd5gp120 prime only; Ad-Ad = rAd5gp120 prime and boost; Ad-HSV = rAd5gp120 prime plus HSVgp120 boost; HSV prime only = HSV(*hsv*)gp120 prime only; HSV-Ad = HSVgp120 prime plus rAd5gp120 boost; HSV-HSV = HSVgp120 prime and boost; lacZ = HSVlacZ only). The percentage of CD4⁺ T-cells that produced each of the seven possible combinations of IFN γ , TNF α and IL2 in response to stimulation with the Env peptide pool is shown. Data represent mean values for each group of four mice; horizontal bars represent the standard error of the mean. Statistical analysis was performed by two-way ANOVA with Bonferroni's post-test. * Different from all groups ($p < 0.01$); ** different from all groups ($p < 0.001$). Note that panel B includes background staining data (determined from mice immunized with HSVlacZ); no background subtraction was applied to the data in this panel.

TABLE 1A

Immunization scheme for experimental series 1

Group #	Group	Promoter	Dose
1	HSV gp120	hsv	1×10^6
2	HSV gp120	cmv	1×10^6
3	HSV gp120	cmvr	1×10^6
4	rAd5 gp120	cmv	1×10^7
5	HSV gp120	hsv	5×10^5
	rAd5 gp120	cmv	5×10^6
6	HSV Lac Z	hsv	1×10^6

TABLE 1B

Immunization scheme for experimental series 2

Prime Immunogen (Day 0)	Boost Immunogen (Day 72)
HSV gp120	HSV gp120 rAd5 gp120 No Boost
rAd5 gp120	HSV gp120 rAd5 gp120 No Boost
HSV Lac Z	No Boost