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Strong cellular and humoral anti-HIV Env immune responses induced by a heterologous rhabdoviral prime–boost approach

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Abstract

Recombinant rhabdovirus vectors expressing human immunodeficiency virus (HIV) and/or simian immunodeficiency virus (SIV) proteins have been shown to induce strong immune responses in mice and rhesus macaques. However, the finding that such responses protect rhesus macaques from AIDS-like disease but not from infection indicates that further improvements for these vectors are needed. Here, we designed a prime–boost schedule consisting of a rabies virus (RV) vaccine strain and a recombinant vesicular stomatitis virus (VSV) both expressing HIV Envelope (Env). Mice were primed and boosted with the two vaccine vehicles by different routes and in different combinations. Mucosal and systemic humoral responses were assessed using enzyme linked immunosorbent assay (ELISA) while the cellular immune response was determined by an IFN-γ ELISPOT assay. We found that an immunization combination of RV and VSV elicited the highest titers of anti-Env antibodies and the greatest amount of Env-specific IFN-γ secreting cells pre- and post-challenge with a recombinant vaccinia virus expressing $HIV_{89.6}$ Env. Furthermore, intramuscular immunization did not induce antigen-specific mucosal antibodies while intranasal inoculation stimulated vector-specific IgA antibodies in vaginal washings and serum. Our results show that it is feasible to elicit robust cellular and humoral anti-HIV responses using two different live attenuated Rhabdovirus vectors to sequentially prime and boost.

Keywords: Immune response; Prime; Boost

Introduction

Although the Acquired Immunodeficiency Syndrome (AIDS) and its causative agent, HIV-1, was discovered over 20 years ago, it remains a global health dilemma. In 2003, the HIV/AIDS pandemic killed approximately 3 million people and infected approximately 5 million more, raising the number of people currently living with HIV/AIDS to more than 40 million (AIDS epidemic update, 2003; Joint United Nations Programme on HIV/AIDS [http://www.unaids.org]). Numerous candidate vaccine designs and regimens investigated in rhesus monkeys and humans have had limited successes in combating HIV, however, insights from these trials may pave the way for a safe and efficacious vaccine in the future (McMichael and Hanke, 2003).

HIV poses challenging obstacles in the development of an efficacious prophylactic or therapeutic vaccine. First, HIV-1 conceals its conserved receptor and co-receptor binding sites in heavily glycosylated crevices, which limits the induction of antibodies against these domains (Leonard et al., 1990; Robinson, 2002). Second, the weak

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non-covalent interactions between the envelope subunits allow the glycoprotein to be shed in various forms during natural infection. This "viral debris" exposes mainly non-relevant antibody epitopes (McKeating et al., 1991; Moore et al., 1990). As a result, antibodies are generated to these envelope subunits that fail to recognize neutralizing epitopes present on the mature envelope oligomers on intact virions (Parren et al., 1999). Third, the ability of HIV to integrate into the host genome and remain as an inactive provirus permits it to escape immune surveillance and thus establish reservoirs in T-cell, macrophage, and monocyte compartments (Blankson et al., 2002). Lastly, the error prone HIV reverse transcriptase gives rise to mutant viruses that can escape both B-cell- (Kwong et al., 2002) and T-cell-mediated immune responses (McMichael, 1998).

Nonetheless, previous studies have revealed that both humoral and cellular immune responses play crucial roles in the immune response against HIV-1 (Ferrantelli et al., 2002). The humoral immune system is composed of two distinct effectors: mucosal and systemic antibodies. HIV predominantly infects via mucosal openings and antibodies lining these cavities might have an important role for virus neutralization at the onset of infection. Conversely, once infection is established, systemic antibodies are important in the control of viral spread.

Passive transfer of neutralizing monoclonal antibodies (nmAb) has been shown to protect chimpanzees (Emini et al., 1992), monkeys (Hofmann-Lehmann et al., 2001; Mascola et al., 1999, 2000; Parren et al., 2001), and severe combined immunodeficiency mice populated with human peripheral blood lymphocytes (hu-PBL-SCID mice) (Parren et al., 1995; Safrit et al., 1993) from infection with simian-human immunodeficiency virus (SHIV) or laboratory-adapted strains of HIV-1. Moreover, human monoclonal antibodies (mAb) have also been demonstrated to neutralize laboratory-adapted HIV strains as well as primary HIV isolates in vitro (Ferrantelli and Ruprecht, 2002).

Additional studies have demonstrated that cellular immune responses induced by $CD8^+$ cytotoxic T-lymphocytes (CTLs) and $CD4^+$ helper T-lymphocytes (HTLs) play crucial roles in suppressing HIV replication in humans (Borrow et al., 1994; Greenough et al., 1997; Koup et al., 1994; Rinaldo et al., 1995; Rowland-Jones et al., 1995) and SIV infection in macaques (Cranage et al., 1997; Gallimore et al., 1995; Jin et al., 1999). Despite this, even in the presence of a robust cellular response, the emergence of CTL escape mutants in macaques has been observed (Barouch et al., 2002, 2003) suggesting that activation of the humoral and cellular immune response is necessary for a potent HIV vaccine (McMichael and Hanke, 2003; McMichael et al., 2002).

To date, heterologous prime–boost protocols using a variety of vectors have been tested for their ability to generate anti-HIV immune responses. These include: naked DNA (Barouch et al., 2000; Subbramanian et al., 2003; Woodberry et al., 2003), recombinant proteins (Letvin et al., 1997; Mantis et al., 2001), modified vaccinia Ankara (MVA) (Amara et al., 2002; Gherardi et al., 2004; Hanke et al., 2002; McConkey et al., 2003), fowlpox virus (Clements-Mann et al., 1998; Kent et al., 1998), adenovirus (AdV) (Buge et al., 1999), influenza virus (Flu) (Gherardi et al., 2003; Gonzalo et al., 1999; Li et al., 1993), and vesicular stomatitis virus (VSV) (Haglund et al., 2002; Rose et al., 2000, 2001). In this study, we assessed the ability of an RV/VSV protocol to activate mucosal and systemic immune responses in mice when administered via an intranasal or intramuscular route.

Results

Rhabdoviral boost enhances both humoral and cell-mediated immunity

We previously demonstrated that RV vectors expressing HIV-1 Env are able to induce potent and long-lasting anti-HIV-1 Env cellular responses in mice (McGettigan et al., 2001). However, current vaccine research in non-human primate models strongly indicates that even potent cellular responses are not sufficient to protect from an AIDS-like disease due to CTL escape mutants (Barouch et al., 2002, 2003). For this reason, strong humoral responses against HIV-1 gp160 have been suggested as keys to a successful HIV-1 vaccine. Our previous results demonstrated that anti-HIV-1 Env humoral responses were detected after priming with an RV-based vaccine expressing HIV-1 Env, followed by a boost with recombinant HIV-1 gp120 protein (Schnell et al., 2000). Here, we analyzed an alternate vaccine strategy using two different Rhabdoviral vectors expressing HIV-1 Env to prime and boost. For this approach, three groups of BALB/c mice were immunized intramuscularly with a RV vector expressing SHIV-$1_{89.6P}$ Env ecto and transmembrane domain fused to the cytoplasmic domain of the RV G (RV-89.6P). Five weeks after the first immunization, one group was boosted with the same RV vector while another was boosted with a heterologous vector, vesicular stomatitis virus (VSV), expressing HIV-$1_{89.6}$ Env (VSV-89.6). A third group was not boosted and served as a control (Fig. 1A). Nine weeks post-prime immunization, all mice were challenged intraperitoneally with a recombinant vaccinia virus expressing HIV-$1_{89.6}$ Env (rVV-89.6). Sera from each mouse were collected as described in Materials and methods to measure anti-HIV-1 Env antibody response. In addition, 5 days post-challenge spleens were removed to evaluate the cellular immune response to HIV-1 Env.

Seroconversion to HIV-1 Env and RV G in serum was analyzed by ELISA and the results are shown in Fig. 2. Mice that received a single inoculation with RV-89.6P did not seroconvert to HIV-1 Env before or after rVV-89.6 challenge (Fig. 2A, Mice A1–A3 and Table 1), although vector-specific seroconversion was detected

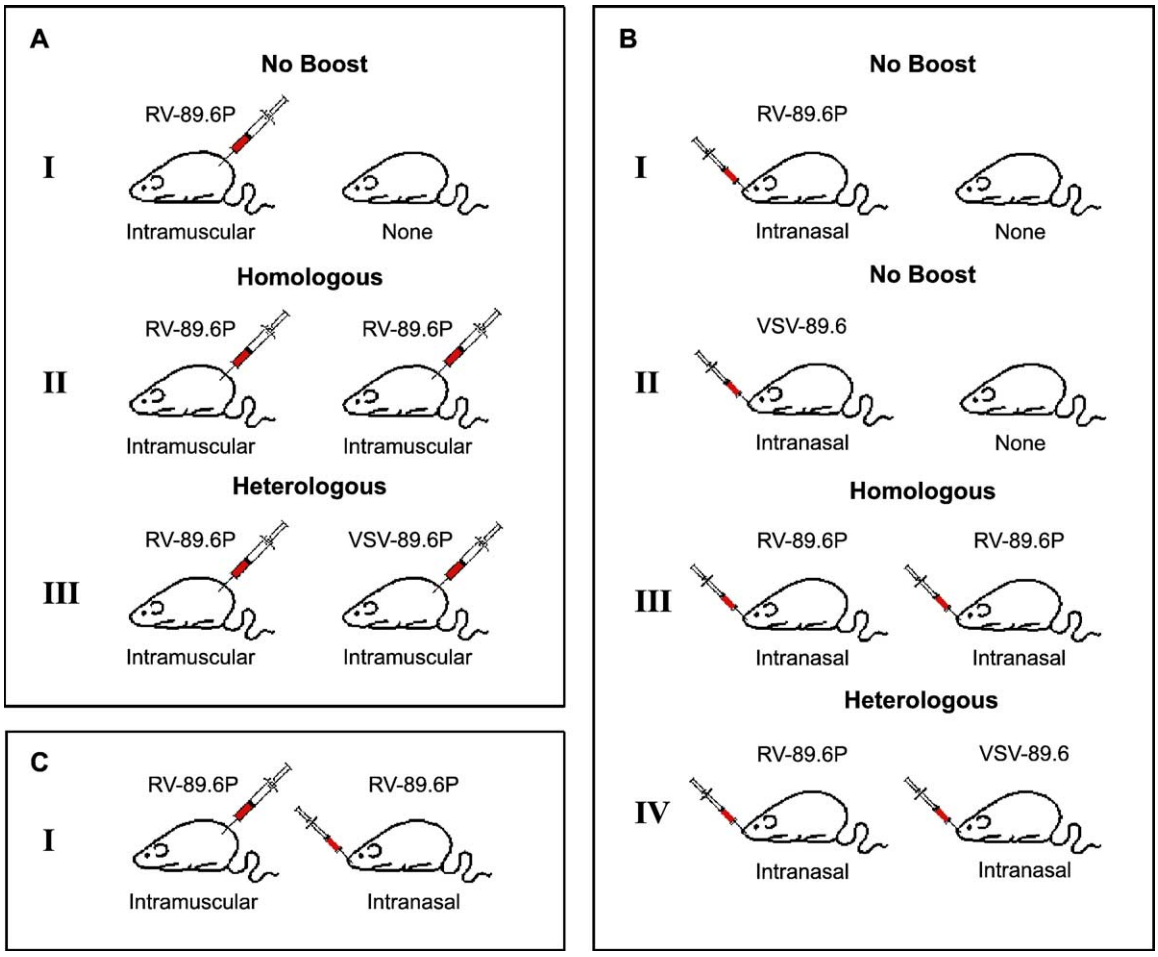

Fig. 1. Prime–boost immunization groups. All mice were primed immunized intramuscularly (A and C) or intranasally (B) and boosted (or not boosted) intramuscularly (A) or intranasally (B and C) 5 weeks post prime.

against RV G (Fig. 2A, white bars). This is consistent with our previous findings that a boost with recombinant gp120 was necessary to induce seroconversion to HIV-1 Env (Schnell et al., 2000). However, mice that received two inoculations with RV-89.6P seroconverted to HIV-1 Env after challenge with rVV-89.6 (Fig. 2B, Mice B1-B3 and Table 1) showing that two inoculations with RV is adequate to prime for seroconversion following rVV-89.6 challenge but did not lead to seroconversion on its own. Of note, those mice that received a single inoculation with RV-89.6P, followed by a boost with VSV-89.6 seroconverted to Env at week 8 (3 weeks post-boost) and elicited consistently high titers of HIV-1 Env-specific antibodies 5 days post-challenge (Fig. 2C, Mice C1–C3 and Table 1). These results indicate that homologous boosting increases the HIV-1 Env specific antibody responses, whereas a heterologous viral vector induces a more potent humoral response to HIV-1 Env.

After determining that priming and boosting with two different vectors expressing HIV-1 Env induced the highest anti-Env antibody titers, we next wanted to measure the effects of this vaccine regimen on the cellular response. Although CTL escape mutants were detected in rhesus macaque studies (Barouch et al., 2002, 2003), others indicate that strong cellular responses will also be necessary for an effective HIV-1 vaccine (Kaech et al., 2002; Seder and Hill, 2000; Yang, 2003). This is based on the finding that exposed but uninfected individuals have HIV-1-specific CTLs without detectable antibodies (Rowland-Jones et al., 1995) and that long-term nonprogressors are asymptomatic probably due to strong memory CTL activity (Rinaldo et al., 1995). In addition, there is a correlation between a strong CTL activity and slow disease progression (Rinaldo et al., 1995). More importantly, we wanted to ensure that the strong humoral response detected above did not interfere with the generation of a robust cellular response induced by our vaccine vehicle. Five days post-challenge with rVV-89.6 HIV-1 Env, mice were sacrificed, spleens removed and the cellular response determined by IFN-γ specific ELISPOT. As shown in Fig. 2D, approximately 2000 splenocytes per million cells were secreting IFN-γ after a single inoculation with RV-89.6P following a challenge with rVV-89.6. Boosting with the same vector, RV-89.6P, elicited approximately a 2-fold increase in spot-forming cells when compared with those mice that received a single inoculation (Fig. 2E). The

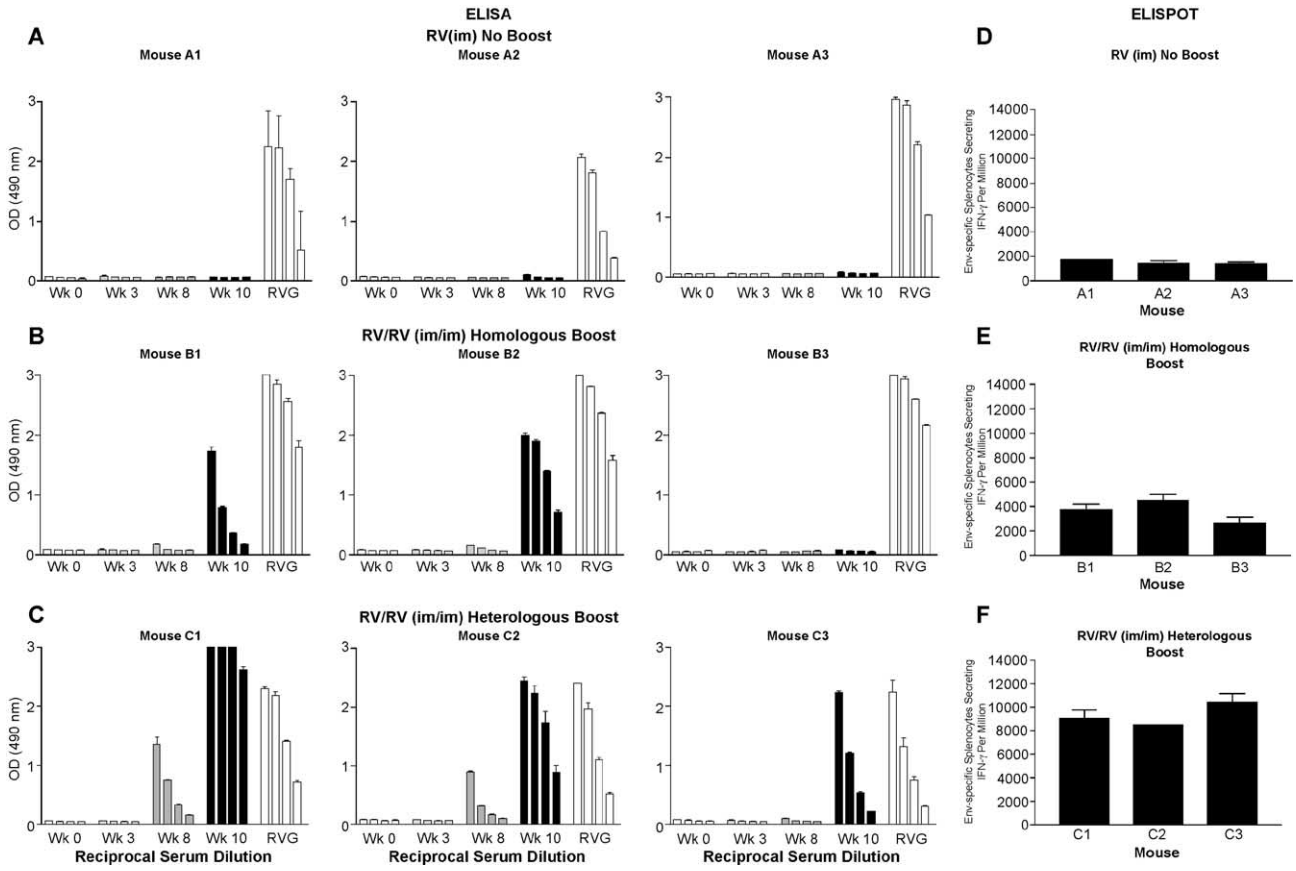

Fig. 2. Three groups of 5 BALB/c mice each were immunized intramuscularly with 3.0×10^5 ffu of RV-89.6P. Five weeks post-prime inoculation; mice were not boost (A, D), boosted with 3.0×10^5 ffu of RV-89.6P (B, E) or boosted 3.0×10^5 ffu of VSV-89.6 (C, F). ELISA was used to detect antigen-specific IgG antibodies in sera on weeks 0, (pre-bleed), 3 (post-prime), 8 (post-boost), and 10 (5 days after challenge with rVV-89.6). Seroconversion to RV-based vaccine is indicated by RV G antibodies (white bars) at week 8. The four bars of each time period represent the optical density at each reciprocal dilution (100, 300, 900, and 2700). Four weeks after boost, mice were challenged with rVV-89.6 and 5 days later, the mice were sacrificed and their spleens removed. Single-cell suspensions were prepared and the ELISPOT assay was used to detect splenocytes that secreted IFN-γ after in the presence of the HIV-1 89.6R10 peptide (a CTL epitope conserved between 89.6 and 89.6P). Data above show three of five mice from each immunization group.

heterologous boost, however, induced more than a 4- and 2.5-fold increase in spot-forming cells when compared with a single inoculation or homologous boost, respectively (Fig. 2F). Thus, these data demonstrate that the heterologous prime boost combination of RV and VSV enhances both the humoral response (Fig. 2C), and significantly increases the cellular response to HIV-1 Env (Fig. 2F).

Mucosal inoculation induces a systemic response

The above experiment demonstrated that a heterologous prime–boost regimen augmented both the systemic humoral and cellular immune response to HIV-1 Env when the vaccine was administered via an intramuscular route. Often, the initial contact with HIV-1 occurs in the mucosa due to sexual contact. Therefore, a potential HIV-1 vaccine might need to induce a mucosal immune response in addition to a systemic immune response to provide sterilizing immunity. While an intramuscular administration of a vaccine is not likely to induce a mucosal immune response, several studies have demonstrated immunization through a mucosal route can elicit both mucosal and systemic immunity (Gherardi et al., 2003, 2004; Staats et al., 1996).

Previously, a recombinant VSV expressing influenza virus hemagglutinin administered intranasally was shown to induce a systemic humoral response that protected mice from a lethal influenza virus challenge (Roberts et al., 1998). Others have demonstrated that a mucosal route of immunization is sufficient to elicit a systemic cellular immune response (Gherardi et al., 2003). The combination of a robust mucosal and systemic immune response could potentially neutralize HIV-1 upon encounter and/or limit virus propagation in vivo. To evaluate the viability and effectiveness of a mucosal prime–boost strategy to elicit both systemic and mucosal immune responses, BALB/c mice were immunized intranasally with RV-89.6P. One group was boosted intranasally with RV-89.6P (homologous) and the second group boosted intranasally VSV-89.6 (heterologous) 5 weeks after the first immunization. The last group was not boosted and served as a control. An additional group was inoculated intranasally with VSV-89.6 and also served as a control (Fig. 1B). A

Table 1
ELISA was used to detect antigen (oligomeric gp140)- and vector (RV G)-specific IgG and IgA antibodies both in sera and/or vaginal washings post-challenge with rVV-89.6 (week 10)

	Humoral response		Mucosal response			
	RVG	HIV-1 89.6	RVG		HIV-1 89.6	
			S	VW	S	VW
Intramuscular						
RV/None	≥1:900	ND	ND	ND	ND	ND
RV/RV	>1:2700	1:2700	ND	ND	ND	ND
RV/VSV	≥1:900	>1:2700	ND	ND	ND	ND
Intranasal						
RV/None	>1:2700	ND	1:2	1:18	ND	ND
RV/RV	>1:2700	1:300	1:2	1:54	ND	ND
RV/VSV	1:900	>1:2700	1:2	1:6	ND	ND
VSV/None	ND	ND	ND	ND	ND	ND
IM/IN						
RV/RV	1:2700	ND	ND	ND	ND	ND

(S = serum, VW = vaginal washing, and ND = none detected).

lower dose was used to infect the VSV group due to residual disease caused by the vector (two out of five mice died after immunization with 10^5 pfu of VSV-89.6). All groups were challenged intraperitoneally 9 weeks post prime immunization with rVV-89.6. Serum was collected via peri-orbital bleeding and vaginal washings were collected in parallel to measure the induction of a mucosal humoral response. Spleen were harvested days after challenge with rVV-89.6 to assess the anti-HIV-1 Env cellular memory response.

Pooled serum antibodies directed towards oligomeric HIV-1 Env and RV G were analyzed by ELISA as described above and the results are shown in Fig. 3. Not surprisingly, mice that received a single immunization of RV-89.6P did not seroconvert to Env during the immunization schedule or post-challenge (Fig. 3A and Table 1), although we detected

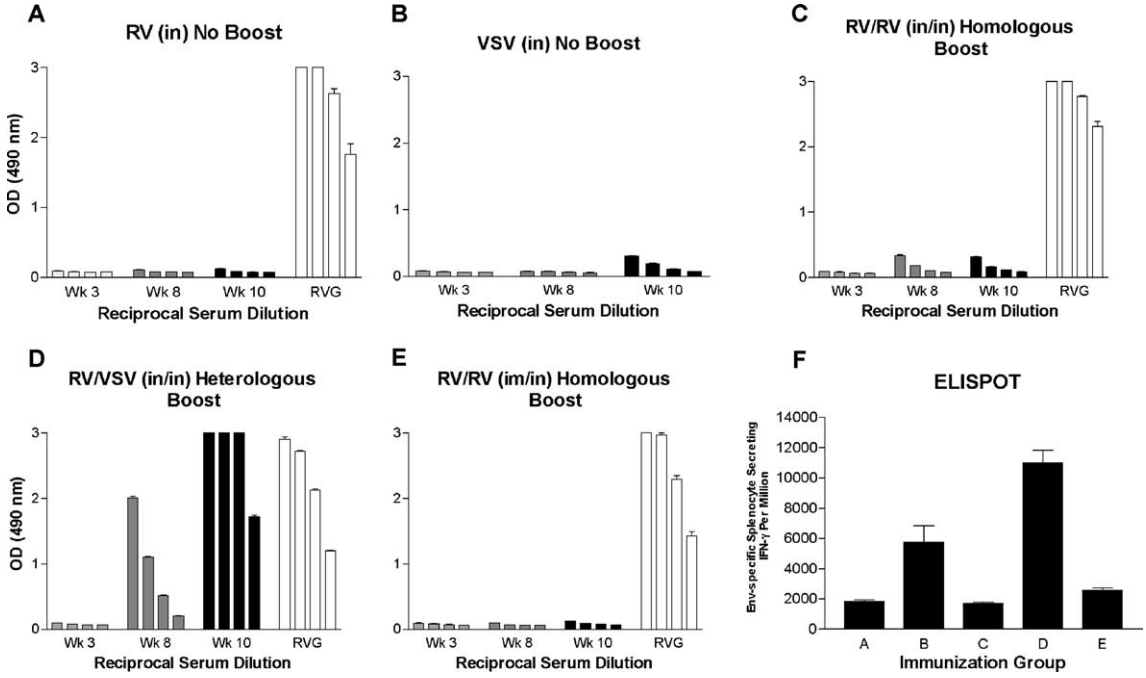

Fig. 3. Four groups of 5 BALB/c mice each were immunized intranasally with 4.0×10^5 ffu of RV-89.6P (A, C and D) or VSV-89.6 (B). Five weeks post-prime inoculation; mice were not boost (A and B), boosted with 4.0×10^5 ffu of RV-89.6P (C) or boosted 4.0×10^5 pfu of VSV-89.6 (D). Another group was primed intramuscularly with 3.0×10^5 ffu of RV-89.6P and 5 weeks later, boosted intranasally with 4.0×10^5 ffu of RV-89.6P (E). ELISA was used to detect antigen-specific IgG antibodies in sera pooled from five mice per group on weeks 0, (pre-bleed), 3 (post-prime), 8 (post-boost), and 10 (5 days after challenge with rVV-89.6). Seroconversion to RV-based vaccine is indicated by RV G antibodies (white bars) at week 8. The four bars of each time period represent the optical density at each reciprocal dilution (100, 300, 900, and 2700). Another group was prime intramuscularly with 3.0×10^5 ffu of RV-89.6P and 5 weeks later, boost intranasally with 4.0×10^5 ffu of RV-89.6P (E). Four weeks after boost, mice were challenged with rVV-89.6 and 5 days later the mice were sacrificed and their spleens removed. Single-cell suspensions pooled from two mice per group were prepared and the ELISPOT assay was used to detect splenocytes that secreted IFN-γ after in the presence of the HIV-1 89.6R10 peptide (F). ELISA and ELISPOT data represent pooled samples.

vector-specific antibodies on week 8 (Fig. 3A, RVG, white bars). These data are in agreement with our previous results that seroconversion to HIV-1 Env following immunization with RV-89.6P requires a boost with either a recombinant gp120 protein (Schnell et al., 2000) or a viral vector expressing HIV-1 Env (Figs. 2B and C). On the other hand, we detected a very low response to HIV-1 Env 5 days post-challenge in mice that received a single immunization with VSV-89.6 (Fig. 3B). Mice that received two inoculations of RV-89.6P did not generate the high amount of HIV-1 Env antibodies (Fig. 3C) as was observed in intramuscularly immunized mice after challenge (Fig. 2B and Table 1). Mice that were immunized intranasally with RV-89.6P followed by a heterologous intranasal boost with VSV-89.6, notably, seroconverted to Env on week 8 (3 weeks post-boost) and induced high titers of HIV-1 Env antibodies 5 days after challenge (Fig. 3D and Table 1).

A benefit of inoculation via the nasal cavity is the activation of the mucosal immune system, specifically the production of IgA antibodies. Since the mucosa is the predominant route of natural HIV-1 infection, it is equally important to measure the IgA response mounted by potential vaccines. The above experiments demonstrate that immunization with two different Rhabdovirus-based vectors administered intranasally can efficiently induce systemic humoral immune responses. Next, we assessed if the intranasal prime–boost regimen, as described above, could elicit HIV-1 Env specific IgA antibodies in the vaginal mucosa.

A summary of the RV G- and Env-specific IgA ELISA data are shown in Table 1. Overall, no anti-HIV-1 Env IgA antibodies were detected in vaginal washings of intranasally immunized mice. However, there were vector-specific RV G-IgA antibodies in both vaginal washings and serum in intranasally inoculated mice that were not observed in intramuscularly immunized mice (Table 1). These data show that although intranasal administration of an RV-based vaccine did not elicit detectable mucosal antibodies directed against HIV-1 Env, they did induce the production of vector-specific mucosal antibodies.

Previous results from this lab indicate that BALB/c mice that received a single immunization of an RV vector expressing HIV-1 Gag via the intramuscular or intranasal route produced approximately equivalent cellular responses as measured by the amount of IFN-γ secreting splenocytes (McGettigan, Tan and Schnell, unpublished data). Presently, we wanted to evaluate if a mucosal immunization with an RV vector expressing HIV-1 Env is comparable to an intramuscular inoculation in inducing systemic cellular response. Additionally, we also sought to examine whether an intranasal heterologous immunization scheme using RV and VSV expressing Env could increase the number of antigen-specific IFN-γ secreting splenocytes.

The systemic cellular memory response to HIV-1 Env was determined 5 days after challenge with rVV-89.6. Two mice from each group were sacrificed, and their spleens removed and pooled. The ELISPOT assay, as described above, was utilized to assess the amount of Env-specific splenocytes secreting IFN-γ. As shown in Fig. 3F (group A), about 2000 splenocytes secreted IFN-γ after a single intranasal inoculation with RV-89.6P while about 6000 splenocytes were induced after a single intranasal inoculation with VSV-89.6 (Fig. 3F, group B). An intranasal boost using the same RV vector, RV-89.6P, did not augment the cellular response (Fig. 3F, group C); in contrast to what was observed after an intramuscular boost using the same RV vector (Fig. 2E). This is consistent with the lower antibody titer we observed in RV/RV intranasal (i.n./i.n.) compared to the RV/RV intramuscular (i.m./i.m.) immunized mice. On the other hand, a heterologous intranasal boost with VSV-89.6 induced about a 5.5-fold increase of IFN-γ secreting cells over mice immunized once or twice with the RV vector (Fig. 3F, group D). ELISA and ELISPOT data from intranasally immunized mice demonstrate that a homologous boost with the same RV vector, RV-89.6P, did not significantly increase systemic humoral or cellular response, unlike what was observed in intramuscularly immunized mice. Nonetheless, these data indicate that a heterologous prime–boost regimen with RV and VSV is able to enhance both systemic humoral and cellular responses to HIV-1 Env when administered intranasally.

Heterologous route of vaccine immunization

Our current data demonstrate that a vaccine regimen consisting of two different Rhabdovirus vectors to prime and boost elicited the strongest systemic humoral and cellular immune responses to Env. We next wanted to evaluate whether a combination of different routes of administration to prime and boost could increase the efficiency of the immune response to Env. We hypothesized that since intramuscular immunization does not induce a mucosal vector-specific response, the intranasal inoculation of RV-89.6P could induce the production of antibodies lining the mucosal cavity. Therefore, we immunized BALB/c mice intramuscularly with RV-89.6P followed by an intranasal boost with the same viral vector 5 weeks after prime immunization (Fig. 1C). Nine weeks post-prime immunization; the mice were challenged intraperitoneally with rVV-89.6. Sera and vaginal washings were collected throughout the immunization protocol.

Mice that received an intramuscular prime and intranasal boost did not seroconvert to Env as shown in Fig. 3E via an ELISA assay. However, they did produce vector-specific RVG antibodies on week 8 confirming successful immunization with the Rhabdovirus-based vaccine. Anti-Env and anti-RVG IgA antibodies were not detected in the vaginal washings throughout the experiment (Table 1). These results reveal that the heterologous route of vaccine administration using RV-based vectors expressing HIV-1 Env does not elicit the production of systemic or mucosal anti-Env antibodies.

To determine the effects of a heterologous route of vaccine administration on the cellular immune response, we harvested the spleens 5 days after challenge with rVV-89.6 and processed them for an IFN-γ ELISPOT. As shown in Fig. 3F,

Table 2
Virus neutralizing activity against HIV-1 (strain MN) of sera from mice immunized as outlined in Fig. 1 were determined five days after rVV-89.6 challenge

Immunization	i.m.			i.n.		Control
VNA	RV only	RV/RV	RV/VSV	RV/VSV	VSV only	WRB pool
50%	<20	<20*	109	24	<20	691
80%	<20	<20	47	<20	<20	232
90%	<20	<20	28	<20	<20	121

The WRB pool indicates a pool of HIV-1-infected patients.

intramuscular/intranasal vaccination regimen using the same RV vector produced approximately 3000 IFN-γ secreting cells. This cellular immune response, however, is not significantly different from mice that received two immunizations via the same route (Figs. 2E and 3F). Hence, this experiment indicates that administration of RV-based vaccines through theses two different routes does not enhance systemic humoral or cellular immune responses.

Heterologous prime–boost immunization induce HIV-1 neutralizing antibodies

Lastly, we analyzed if the observed Env-specific antibodies identified in these experiments are associated with neutralizing activity against HIV-1. For this preliminary study, the laboratory-adapted HIV-1 strain MN was utilized. As shown in Table 2, the best neutralizing activity was observed for pooled sera from mice immunized intramuscularly with RV and VSV expressing HIV-1 Env, whereas the same approach resulted in reduced titers via the intranasal route. Of note, these results indicate that the ELISA data for seroconversion correlate well with neutralization activity against a laboratory-adapted HIV-1 strain. It is interesting to note that a single inoculation with RV or VSV via any route did not induce any neutralizing activity.

Discussion

Results presented here indicate that the use of Rhabdovirus-based vaccines are able to elicit potent immune responses to HIV-1 Env. We have previously shown that RV vectors expressing HIV-$1_{89.6}$ Env are capable of eliciting anti-HIV Env neutralizing antibodies after a recombinant gp120 protein boost (Schnell et al., 2000) and long-lasting cellular immune responses after a single immunization (McGettigan et al., 2001). In the present study, we analyzed if it is possible to enhance these responses by using a live attenuated RV vector expressing $SHIV_{89.6P}$ Env to prime in conjunction with another Rhabdovirus, VSV, expressing HIV-$1_{89.6}$ Env. As described, cellular immune responses to HIV-1 Env were analyzed by the ELISPOT assay while systemic and mucosal humoral responses against HIV-1 Env and vector specific proteins were assessed using ELISA. Expansion of the cellular response using two distinct live vectors to prime and boost was initially shown against malaria using Flu and VV (Li et al., 1993). Succeeding studies have shown that heterologous prime–boost immunization is capable of expanding the cellular $CD8^+$ T cell response by as much as 30% (Estcourt et al., 2002). Gherardi et al. (2003) and Gonzalo et al. (1999) have shown that a prime–boost schedule consisting of Flu and VV or MVA elicited approximately a 16-fold increase in Env-specific and a 4.5-fold increase in Gag-specific (Nakaya et al., 2003) IFN-γ secreting splenocytes compared to homologous boosted mice. Studies using VSV to prime and vaccinia virus (VV) to boost increased the number of intracellular IFN-γ stained $CD8^+$ T cells by 12-fold when compared to mock boosted mice. Our results show that a boost with a heterologous vector (VSV) increases the amount of IFN-γ secreting cells by approximately 2.5- to 5-fold and 4.5- to 5.5-fold higher than homologously boosted and singly immunized mice, respectively. This is comparable with previous data, which demonstrates that a second dose of a heterologous vector is more efficient in expanding the cellular response.

In contrast to the cellular immune response, neutralizing antibodies against HIV-1 are more difficult to induce (Parren et al., 1999). Previous studies using VSV expressing glycoproteins from different serotypes to sequentially prime and boost was able to augment the anti-Env humoral responses by 2-fold in comparison to singly immunized mice (Rose et al., 2000). A similar approach using a combination of Flu/MVA showed a 4-fold increase in anti-Env antibody production, in comparison to a Flu/Flu control group (Gherardi et al., 2003). Both studies show that a heterologous prime boost approach either through the use of non-cross-reactive glycoproteins or different viral vectors induced a more robust immune response to HIV Env. Here, we found that heterologous (RV/VSV) boosted mice also produced high titers of HIV-1 specific antibodies as seen for Flu/MVA and VSV. Of note, to compare different prime–boost approaches, the experiments have to be performed in parallel. Homologous boosted (RV/RV) mice immunized intramuscularly also elicited significant amounts of anti-HIV-1 Env 5 days post-challenge, while singly inoculated (RV/None) mice did not seroconvert to HIV-1 Env during the course of the experiment. Previous studies have found that SHIV-89.6 and SHIV-89.6P have highly divergent neutralization epitopes (Crawford et al., 1999; Etemad-Moghadam et al., 1998; Montefiori et al., 1998). The absence of a high titer of anti-Env antibodies at 3 weeks post-boost in homologous boosted (RV/RV) mice and in single immunized (RV/None) mice

could be due to the difference of the antigens used during immunization (89.6P) and in the ELISA (89.6). Nonetheless, ELISA data from our heterologous regimen indicate that a combination of RV and VSV may be a better alternative than using glycoprotein exchange VSV vectors to elicit high titers of anti-Env antibodies in a Rhabdoviral vaccine regimen. This is also supported by the finding that only this combination induced neutralizing antibodies to HIV-1_{MN}.

The mucosal route of vaccine administration is an important consideration in the development of a prophylactic HIV-1 vaccine regimen. Prior studies have shown that mucosal vaccine administration can elicit both systemic humoral and cellular immune responses (Horner et al., 2001; Lemiale et al., 2003; Mantis et al., 2001; Staats et al., 1996). Likewise, our data show that intranasal immunizations can elicit production of both systemic anti-Env IgG antibodies (humoral) and IFN-γ secreting splenocytes (cellular). Generation of IgA antibodies is a chief criterion of a mucosal immune response and is ultimately important at the onset of natural HIV-1 infection. Several groups have observed production of HIV-1 specific IgA antibodies at mucosal surfaces after intranasal immunizations with naked DNA (Horner et al., 2001; Lemiale et al., 2003), Adenovirus-type 5 (Ad5) (Lemiale et al., 2003) recombinant gp41 (Mantis et al., 2001) and whole-killed HIV-1 (Dumais et al., 2002). While we did not observe a detectable level of anti-HIV-1 Env IgA in vaginal washings or in serum, we did detect IgA antibodies directed against RV G from intranasally immunized mice. It must be noted, however, that DNA-based and recombinant protein vaccine protocols usually consist of multiple boosts, while our regimen involves a single boost with RV or VSV.

Pre-existing immunity to vaccine vectors is a major concern when designing candidate vaccines whether it is generated naturally or artificially. Interestingly, a recent study found that an intranasal vaccination with an Ad5 expressing HIV-1 antigens did not overcome prior Ad5 immunity. Moreover, two intranasal immunizations with Ad5 had lower anti-HIV cellular and humoral immune response compared to its intramuscular counterparts (Lemiale et al., 2003). Consistent with this finding, we also observed that two intranasal immunizations with RV-89.6P had a slightly lower anti-Env cellular response and no humoral response compared to intramuscular immunized mice. However, in the presence of an anti-RV immune response, an intranasal boost with a different vector, VSV, expressing HIV-1 Env resulted in enhanced anti-Env cellular and humoral immune response, comparable to intramuscular (RV/VSV) immunized mice.

In conclusion, prime–boost approaches in HIV vaccine development consist mainly of DNA-prime and DNA virus boost regimens. Both have been shown to increase cellular and humoral responses compared to a single immunization, however, no approach has yet been able to protect a monkey model system from SIV/HIV infection (Barouch et al., 2002, 2003). Ultimately, mass vaccination against small pox and a high seroprevalence to adenovirus in the population may limit the use of DNA virus-based vaccine vectors. There are several advantages in using Rhabdoviral vectors in a prime–boost regimen. On one hand, there is little seroprevalence to either RV or VSV in the human population, which makes them excellent candidate vaccine vectors. Furthermore, pathogenic markers for RV have been well characterized such as the point mutation at a site in its glycoprotein that renders recombinant viruses apathogenic even when introduced intracranially in mice (McGettigan et al., 2003). Oral administration of RV vaccine strains have already been shown to be apathogenic in chimpanzees (World Health Organization, unpublished document W. H. O./Rab. Res./93.42) while rVSV expressing HIV-1 Env and SIV Gag protected monkeys from AIDS for up to 14 months post-challenge with SHIV-89.6P (Rose et al., 2001).

This study indicates that immunization using two rhabodviral vectors is more potent in inducing the adaptive immune response to HIV-1 Env when compared to homologous and non-boosted regimens. Furthermore, our current protocol highlights the feasibility of using Rhabdovirus-based vaccines in a prime–boost regimen.

Materials and methods

Viruses

The plasmid encoding the recombinant RV vector pSBN-333 was previously described (McGettigan et al., 2003). To construct a recombinant RV expressing the $SHIV_{89.6P}$ Env ecto-(ED) and transmembrane (TM) domains fused to the RV glycoprotein (G) cytoplasmic domain (CD), the $SHIV_{89.6P}$ Env ED and TM were amplified by PCR from pKB9SHIV(89.6P), (National Institutes of Health AIDS Research and Reference Reagent Program [ARRRP]) using Vent polymerase (New England Biolabs, Inc.) and the primers RP27 5′-GGG CTG CAG CTC GAG CGT ACG AAA ATG AGA GTG AAG GAG ATC AGG-3′ and RP32 5′-GCC CCG TTA ACT ATA GAA AGT ACA GCA AAA-3′. The PCR product was digested with *Bsi*WI–*Hpa*I (New England Biolabs, Inc.) and was cloned to pBS2H-NL4-3-G which contains the RV G CD (McKenna et al., 2003). The resulting plasmid was named pBS289.6P-RVG. To introduce the gene encoding the chimeric $SHIV_{89.6P}$ Env into pSBN-333, pBS289.6P-RVG was digested with *Bsi*WI–*Xba*I and the 2.2-kb fragment cloned into the previously *Bsi*WI–*Nhe*I-digested pSBN-333. The resulting plasmid was designated pRV-89.6P and the virus RV-89.6P recovered by standard methods (Schnell et al., 1994).

Recombinant VSVs (rVSV) expressing a chimeric HIV-$1_{89.6}$ Env fused to the VSV-tail (VSV-89.6) was a kind gift from John K. Rose (Johnson et al., 1997) (Yale University).

The relative level of HIV-1 Env expression in infected cells with similar recombinant Rhabdoviral vectors were

previously demonstrated for RV (Schnell et al., 2000) and VSV (Haglund et al., 2000; Johnson et al., 1998).

Immunization

Intramuscular: three groups of five 6- to 8-week-old female BALB/c mice (Harlan Sprague) were primed intramuscularly with 3.0×10^5 foci forming units (ffu) of RV-89.6P. Five weeks post-prime, one group was boosted intramuscularly with 3.0×10^5 ffu of RV-89.6P (homologous) and another group, with 3.0×10^5 plaque forming units (pfu) of VSV-89.6 (heterologous). The third group was not boosted and served as a control. Intranasal: Four groups of five 6- to 8-week-old female BALB/c mice were primed intranasally with either 4.0×10^5 ffu of RV-89.6P (3 groups) or 4.0×10^5 pfu VSV-89.6 (1 group). Five weeks after the first inoculation, one group (RV-89.6P primed) was boosted intranasally with 4.0×10^5 ffu of RV-89.6P (homologous) while another (RV-89.6P primed) with 4.0×10^5 pfu of VSV-89.6 (heterologous). The third group (RV-89.6P primed) and the VSV-89.6 primed mice were not boosted and served as controls. Intramuscular/Intranasal: five 6- to 8-week old female BALB/c mice were primed intramuscularly with 3.0×10^5 ffu of RV-89.6P followed by an intranasal boost with 4.0×10^5 ffu of RV-89.6P 5 weeks post prime. All mice were challenged intraperitoneally 9 weeks post priming with 1.0×10^7 pfu of VBD3-89.6 obtained from ARRRP, a recombinant vaccinia virus expressing HIV-$1_{89.6}$ Env (rVV-89.6).

Sample collection

Blood was collected retro-orbitally on weeks 0 (pre-bleed), 1, 3 (post-prime), 6, 8 (post-boost), and 10 (5 days after rVV-89.6 challenge). Blood samples were spun at 12,000 rpm for 5 min and serum was collected and stored at 4 °C. Vaginal washings were collected in parallel with blood samples. A micropipettor was used to flush 65 μl of phosphate buffered saline (PBS) four to six times into the vaginal cavity. Vaginal washings were then spun at 10,000 rpm for 15 min to remove cellular debris and supernatants were collected and stored at 4 °C.

RV G ELISA

RV G protein was purified from sucrose-purified RV virions as described previously (McGettigan et al., 2003). RV G was diluted in coating buffer (50 mM Na_2CO_3, pH 9.6) at a concentration of 200 ng/ml and plated in a 96-well ELISA Maxisorp plate (Nunc) with 100 μl per well. Plates were incubated overnight at 4 °C, washed three times with phosphate buffered saline [PBS]/Tween-20 (PBS [pH 7.4], 0.1% Tween-20) and blocked with blocking buffer (PBS [pH 7.4], 5% dry milk powder) for 30 min at room temperature (RT). Serial dilutions of sera or vaginal washings were added and plates incubated for 1 h at RT. Plates were washed three times, followed by the addition of horseradish peroxidase-conjugated goat anti-mouse IgG (γ chain specific, 1:5000) or rat anti-mouse IgA (α chain specific, 1:2000) (Southern Biotechnology Associates, Inc). Plates were incubated at 37 °C for 30 min, washed three times and 200 μl of OPD (*o*-phenylenediamine dihydrochloride; Sigma) substrate was added to each well. The reaction was stopped with 50 μl of 2M H_2SO_4 per well and plates were read at 490 nm.

Oligomeric HIV-$1_{89.6}$ gp140 ELISA

Supernatants from B-SC-1 cells infected with a recombinant vaccinia virus expressing oligomeric HIV-$1_{89.6}$ gp140 (a generous gift from Robert W. Doms, University of Pennsylvania) (Richardson et al., 1996), were used to coat a 96-well ELISA Maxisorp plate (Nunc). The following steps of the ELISA were identical to the RV G ELISA protocol described above.

IFN-γ ELISPOT

Five days after challenge with rVV-89.6, mice were sacrificed, spleens were removed, and single-cell suspensions were prepared. Red blood cells were removed with ACK lysing buffer (BioSource), and cells were washed twice in RPMI-10 supplemented with 10% fetal bovine serum (FBS). A 96-well multiscreen filtration plate (MAIPS4510, Millipore) was coated overnight at 4 °C with 10 μg/ml of rat anti-mouse IFN-γ monoclonal antibody (mAb, R4-6A2, PharMingen) in sterile PBS. The plates were blocked for 1 h with 5% bovine serum albumin (BSA) in PBS at 37 °C. The plates were prepared for the splenocytes by the addition of 100 μl of RPMI 1640 supplemented with 10% FBS and incubated for an additional 2 h. Two-fold dilutions of harvested splenocytes were plated in triplicate and incubated with or without the major histocompatibility complex class I-restricted peptide, 89.6R10, (IGPGRAFYAR), which contains a CTL epitope (for BALB/c mice) from the V3 loop shared between 89.6 and 89.6P Env for 16 h at 37 °C. Plates were washed 10 times with PBS containing 0.25% Tween-20 and then once with sterile distilled water. Wells were incubated with 5 μg/ml of biotinylated rat anti-mouse IFN-γ mAb (XMG1.2, PharMingen) for 2 h and then washed five times with 0.25% Tween 20 in PBS. The wells were treated with 1 mg/ml of horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch) in PBS containing 1% BSA, incubated for 2 h at room temperature, and washed four times. IFN-γ-secreting spot-forming cells were detected by the addition of 3, 3′-diaminobenzidine and 4-chloro-1-naphthol in cold methanol (McGettigan et al., 2003).

Virus neutralization

Mouse serum samples were aliquoted into 1.8 ml cryovials and heat-inactivated for 30 min at 56 °C in a

circulating water bath. The samples were then spun at 8000 RPM for 2 min to pellet cellular debris. Serum was initially diluted 1:5 in 15% FCS (Gemini) RPMI 1640 media (Quality Biological) supplemented with 1% L-glutamine (Quality Biological) and 1% Penicillin/Streptomycin (Quality Biological) (cRPMI). The initial dilution was followed by serial 4-fold dilutions, ending with 1:5120. As a "+" control for neutralization, a well-characterized pool of sera from HIV-1 infected individuals was diluted in a similar manner. Both HIV-1 negative human sera, diluted 1:5, and cRPMI were used as negative controls. The dilutions were done in a 96-well microtiter plate. Twenty-five microliters of each serum dilution were added to a 500-μl deep-well box. Sample sera were distributed in replicates of four, whereas control sera were done in replicates of eight. The T cell line adapted HIV-1 strain MN was grown and tittered in the human-transformed T-cell line H9 (NIH AIDS Reagent Program). Twenty-five microliters of MN, diluted to 100–200 $TCID_{50}$, was added to each well. The serum sample/virus mixtures were allowed to incubate for 30 min in a 37 °C/5% CO_2 incubator. During this time, H9 cells were prepared for infection, counted and resuspended at 2×10^6/ml in fresh cRPMI. After 30 min, 50 μl of cells were added to each well. The cultures were incubated overnight in a 37 °C/5% CO_2 incubator. On day 1, the cultures were washed three times (final volume of 500 μl/wash) with cRPMI. The plates were placed in a centrifuge for 10 min at 1000 RPM (Sorval Legend RT). After the final wash, 200 μl of cRPMI was added to each well and mixed with the cell pellets four to five times. The cells were then transferred to a 96-well round bottom plate and incubated at 37 °C/5% CO_2 for 4 days. On day 4, 100 μl of supernatant was removed from each well and lysis solution was added (100 μl of dPBS+ 20 μl of Lysis Buffer-Coulter Corp.). The plates were stored at −30 °C until p24 antigen ELISA analysis could be performed. After thawing the samples and bringing all reagents to room temperature, the ELISA procedure was performed according to the manufacturer's instructions (Coulter Corp.). Replicates of each sample were pooled for use in the assay. Control wells were also pooled. P24 production for each sample was measured against baseline viral growth in cRPMI. Fifty percent, 80%, and 90% neutralization titers were calculated for each sample (Kim et al., 2003).

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