

Enhanced cellular immunity to SIV Gag following co-administration of adenoviruses encoding wild-type or mutant HIV Tat and SIV Gag

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Abstract

Among candidate antigens for human immunodeficiency virus (HIV) prophylactic vaccines, the regulatory protein Tat is a critical early target, but has a potential for immune suppression. Adenovirus (Ad) recombinants encoding wild-type HIV Tat (Tat-wt) and a transdominant negative mutant HIV Tat (Tat22) were constructed and administered to mice separately or together with Ad-SIV_{gag}. Immunogenicity and effects on immune responses to the co-administered Gag immunogen were evaluated. Wild-type and mutant Tat recombinants elicited similar Tat-specific cellular and humoral immune responses. Co-administration of either Tat immunogen with Ad-SIV_{gag} induced modest but significant enhancement of Gag-specific interferon-gamma secreting T cells and lymphoproliferative responses. Neither the Ad-recombinant encoding Tat-wt nor Tat22 suppressed induction of anti-Tat or anti-Gag antibodies. Based on the immune responses observed in mice, both recombinants appear to be suitable vaccine candidates. Their contribution to protective efficacy remains to be determined in a non-human primate model.

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Introduction

The difficulties in developing a vaccine against HIV/AIDS have led to the realization that multi-component immunogens eliciting both humoral and cellular immune responses will be essential for achieving a successful vaccine (Ho and Huang, 2002; Letvin et al., 2002; Malkevitch and Robert-Guroff, 2004; McMichael and Rowland-Jones, 2001). Accumulating evidence from developmental HIV and simian immunodeficiency virus (SIV) vaccine studies indicates that vaccines encoding multiple viral antigens can

induce broader immune responses and/or greater protective efficacy against viral infection (Hel et al., 2002; Kong et al., 2003; Mossman et al., 2004; Negri et al., 2004; Ourmanov et al., 2000; Patterson et al., 2004; Zhao et al., 2003b). In addition to the viral structural and enzymatic proteins, Env, Gag, and Pol, viral regulatory, and accessory proteins are important potential vaccine components (Robert-Guroff, 2002). Tat, Rev, and Nef, in particular, have been targeted by several laboratories for vaccine development (Cafaro et al., 1999; Makitalo et al., 2004; Mossman et al., 2004; Muthumani et al., 2002; Negri et al., 2004; Osterhaus et al., 1999; Patterson et al., 2002; Richardson et al., 2002; Tikhonov et al., 2003; Verrier et al., 2002).

Because of its critical role in viral pathogenesis and infectivity (Chang et al., 1995), the transactivator protein,

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Tat, has received much attention (Ensoli and Cafaro, 2000–01; Gallo, 1999; Goldstein, 1996). HIV-1 Tat is a small nuclear protein, but it has a variety of activities. In addition to functioning as a transcriptional transactivator of HIV gene expression, Tat is released from productively infected cells and can be taken up and imported into the nucleus of many different cell types. There it promotes HIV replication or modulates the expression of cellular genes such as transcription factors, cytokines, and genes that regulate the cell cycle and are important for HIV replication (Barillari and Ensoli, 2002; Caputo et al., 2004).

Extracellular Tat binds specific cell receptors and phosphorylates tyrosine kinases, thus activating specific signal transduction pathways, and upregulates the expression of the HIV co-receptors CCR5 and CXCR4 (Barillari and Ensoli, 2002; Caputo et al., 2004). Extracellular Tat also has important effects on immunoregulatory functions (Caputo et al., 2004). In particular, bioactive soluble Tat selectively binds to and enters immature and mature dendritic cells (iDC and mDC, respectively), drives iDC maturation and activation toward a T helper 1 (Th-1) inducing phenotype (Fanales-Belasio et al., 2002), facilitates major histocompatibility complex (MHC) class I antigen presentation (Kim et al., 1997; Moy et al., 1996), and modulates proteasome catalytic subunit composition, modifying the hierarchy of epitopes presented in favor of subdominant and cryptic ones (Gavioli et al., 2004).

Extracellular Tat can also be immunosuppressive, and its immune suppressive effects are thought to contribute to AIDS pathogenesis (Gallo, 1999). Tat can induce apoptosis in T cells by a process involving activation of cellular cyclin dependent kinases (Li et al., 1995). Tat has been shown to upregulate Fas L on macrophages (Dockrell et al., 1998; Wu and Schlossman, 1997) and T cells (Kolesnitchenko et al., 1997), resulting in apoptosis of Fas expressing T cells. A critical report demonstrated that in mice native Tat immunization in complete Freund's adjuvant suppressed the induced antibody response to a co-administered antigen, and that this was abrogated by oxidation of the Tat molecule (Cohen et al., 1999).

Because it is produced early in the HIV replication cycle, vaccine-induced immune responses to Tat might inhibit Tat functions, abrogating both HIV transactivation and the deleterious effects of Tat on uninfected, bystander cells, as well as target early HIV infected cells. However, the potential use of Tat as a vaccine candidate is controversial. On the positive side, studies of HIV-infected patients and SIV-infected non-human primates suggest that an immune response to Tat has a protective role and may control the progression of the disease. Anti-Tat antibody responses and CTLs have been associated with non-progression to AIDS in infected individuals (Re et al., 2001; Reiss et al., 1990; van Baalen et al., 1997; Zagury et al., 1998; Rezza et al., 2005). Furthermore, a recent study

in macaques infected with SIV demonstrated that anti-Tat CTLs are key to control of early virus replication (Allen et al., 2000). As a vaccine candidate, however, mixed results have been obtained using different approaches. Immunization with active Tat protein has led to strong protection of cynomolgus macaques in pre-clinical vaccine studies against intravenous challenge with SHIV89.6P (Cafaro et al., 1999; Maggiorella et al., 2004). In contrast, immunization with either inactivated or native Tat has shown minimal or no protection of rhesus macaques against intrarectal (Pauza et al., 2000) or intravenous (Silvera et al., 2002) SHIV89.6P challenge, intrarectal SHIVBX08 challenge (Verrier et al., 2002), or intravenous SHIV33 or SHIV33A challenge (Goldstein et al., 2000). Whether these mixed outcomes reflect species differences, route or strain of challenge, or differences in the Tat immunogens or vaccine regimens is not clear. Taken together, these observations suggest that additional investigation of HIV-1 Tat vaccines is warranted.

The present study was undertaken to investigate the vaccine potential of a replicating Ad recombinant encoding HIV-1 Tat, a vaccine design aimed at inducing a more potent cellular immune response than would be expected for protein alone or DNA-based vaccines. We compared Ad-recombinants encoding both native Tat and a Tat mutant (tat22; Cys 22 to Gly) lacking transactivation ability (Caputo et al., 1996). Previously, intramuscular immunization of mice with plasmids encoding transdominant negative mutants of HIV-1 Tat, including Tat22, was shown to elicit a humoral response to wild-type Tat comparable to that induced by inoculation of wild-type *tat* DNA (Caselli et al., 1999). Here, we compared the immunogenicity of the two candidate vaccines and examined their effects on the immune response to a co-administered Ad-recombinant vaccine encoding SIV Gag.

Results

Construction of Ad-recombinants encoding wild-type and mutant tat genes

Ad type 5 host range mutant (Ad5hr)-recombinants containing the full-length cDNA of the HIV-1 wild-type *tat* gene (Ad5hr-HIV*tat-wt*) or the Tat transdominant negative mutant (Ad5hr-HIV*tat22*) under the transcriptional control of a hybrid gene regulation unit, consisting of a human CMV promoter followed by the Ad5 tripartite leader sequence, were constructed as described in Materials and methods. The Ad5hr vector was used to facilitate future efficacy studies in the rhesus macaque model by allowing replication of the Ad-recombinant. Western blot analysis showed the levels of *in vitro* expression of the wild-type and mutant *tat* genes were comparable following infection of human 293 cells with the Ad5hr-recombinants (data not shown).

Immunization of mice

Ad5hr-recombinants replicate in monkeys, whereas in mice, the inserted genes are expressed, but recombinant replication does not occur. Here, we assessed the immunogenicity of the recombinants in Balb/C mice prior to future testing in the SIV rhesus macaque challenge model. The protocol is outlined in Table 1 and detailed in Materials and methods. We immunized 10 mice each in groups I and II with an Ad5hr-recombinant encoding wild-type or mutant tat, respectively. Because of previous reports concerning suppression of antibody responses to other antigens co-administered with native Tat protein, we included immunization groups III–V, in which 10 mice each received Ad5hr-SIVgag alone or together with Ad5hr-HIVtat-wt or Ad5hr-HIVtat22. A sixth control group received empty Ad5hrΔE3 vector only. Two additional groups (VII and VIII) were included to examine a possible immune modulating effect of prior immunity to wild-type or mutant Tat by administering first Ad5hr-HIVtat-wt or Ad5hr-HIVtat22 alone and subsequently 4 weeks later, Ad5hr-SIVgag together with Ad5hr-HIVtat-wt or Ad5hr-HIVtat22. Immune responses were evaluated in these mice at week 6.

Antigen-specific cellular immune responses

An IFN- γ ELISPOT assay was used to assess Tat- and Gag-specific cellular immune responses elicited by the Ad5hr-recombinant vaccines. Ad5hr-recombinants encoding Tat-wt and Tat22 were equally immunogenic, with no differences observed in the number of Tat-specific IFN- γ -secreting spot-forming cells (SFC) between groups I and II following either the first or second immunizations (Fig. 1A). Similarly, the Tat-specific ELISPOT responses seen at both time points in Groups IV and V in which Ad5hr-SIVgag was co-administered were also equivalent. In all cases, except for Group II mice immunized with Ad5hr-HIVtat22, a second immunization with the same Ad5hr-recombinant significantly boosted the Tat-specific cellular

response ($P = 0.028, 0.071, 0.0031, \text{ and } 0.023$ for Groups I, II, IV, and V, respectively; Fig. 1A). A small but significant decrease in the number of IFN- γ -secreting cells in response to Tat peptides was seen in mice co-immunized with the Ad5hr-SIVgag recombinant and either Ad5hr-HIVtat-wt or -HIVtat22 following the second immunization (Group I vs. IV, $P = 0.0096$; Group II vs. V, $P = 0.0072$; Fig. 1B). Whether these minimal decreases of 20 mean SFC (10% decrease) for Ad5hr-HIVtat-wt and 41 mean SFC (19% decrease) for Ad5hr-HIVtat22 in the overall induced cellular response to Tat would be reproduced in primate models and affect protective efficacy will need to be evaluated in future studies.

As previously seen in the macaque model (Zhao et al., 2003a), the Ad5hr-SIVgag recombinant was also immunogenic in mice. A boosting effect of two sequential immunizations was seen in the Gag cellular immune response in all three groups that received this Ad5hr-recombinant ($P < 0.0001, P = 0.0094, P < 0.0001$ for Groups III, IV, and V, respectively; Fig. 1A). In contrast to the small decrease in cellular immune response to Tat seen upon co-administration of the Ad5hr-SIVgag recombinant, both the Ad5hr-HIVtat-wt and -HIVtat22 co-administrations had an enhancing effect on cellular responses to Gag. The number of Gag-specific IFN- γ -secreting cells seen following both the first and second immunizations was significantly increased (Group III vs. IV, $P = 0.0001$ and $P = 0.015$ post-first and -second immunization; Group III vs. V, $P = 0.0012$ and $P < 0.0001$ post-first and -second immunization; Fig. 1B). The increases in the Gag-specific responses observed 2 weeks after the first immunizations were 116 mean SFC (46%) and 78 mean SFC (31%) for mice co-immunized with the Ad5hr-HIVtat-wt and Ad5hr-HIVtat22 recombinant immunogens, respectively, and 240 mean SFC (59%) and 172 mean SFC (42%) respectively at week 6 following the second immunization. These results indicate that rather than suppressing cellular immunity, both wild-type and mutant Tat immunogens administered as Ad-recombinants can enhance the cellular immune

Table 1
Mouse immunization schedule

| Group | Immunogens | # Mice immunized week 0 | # Mice sacrificed week 2 | # Mice immunized week 4 | # Mice sacrificed week 6 |
|-------------------|--------------------------------------|-------------------------|--------------------------|-------------------------|--------------------------|
| I | Ad5hrΔE3-HIVtat-wt | 10 | 5 | 5 | 5 |
| II | Ad5hrΔE3-HIVtat22 | 10 | 5 | 5 | 5 |
| III | Ad5hrΔE3-SIVgag | 10 | 5 | 5 | 5 |
| IV | Ad5hrΔE3-HIVtat-wt + Ad5hrΔE3-SIVgag | 10 | 5 | 5 | 5 |
| V | Ad5hrΔE3-HIVtat22 + Ad5hrΔE3-SIVgag | 10 | 5 | 5 | 5 |
| VI | Ad5hrΔE3 vector | 10 | 5 | 5 | 5 |
| VII ^a | Ad5hrΔE3-HIVtat-wt | 5 | 0 | | |
| | Ad5hrΔE3-HIVtat-wt + Ad5hrΔE3-SIVgag | 0 | | 5 | 5 |
| VIII ^a | Ad5hrΔE3-HIVtat22 | 5 | 0 | | |
| | Ad5hrΔE3-HIVtat22 + Ad5hrΔE3-SIVgag | 0 | | 5 | 5 |

^a In groups VII and VIII, 5 mice each were first immunized at week 0 with Ad5hrΔE3-HIVtat-wt or Ad5hrΔE3-HIVtat22. The same mice were subsequently boosted at week 4 with the same recombinants together with Ad5hrΔE3-SIVgag, and sacrificed at week 6.

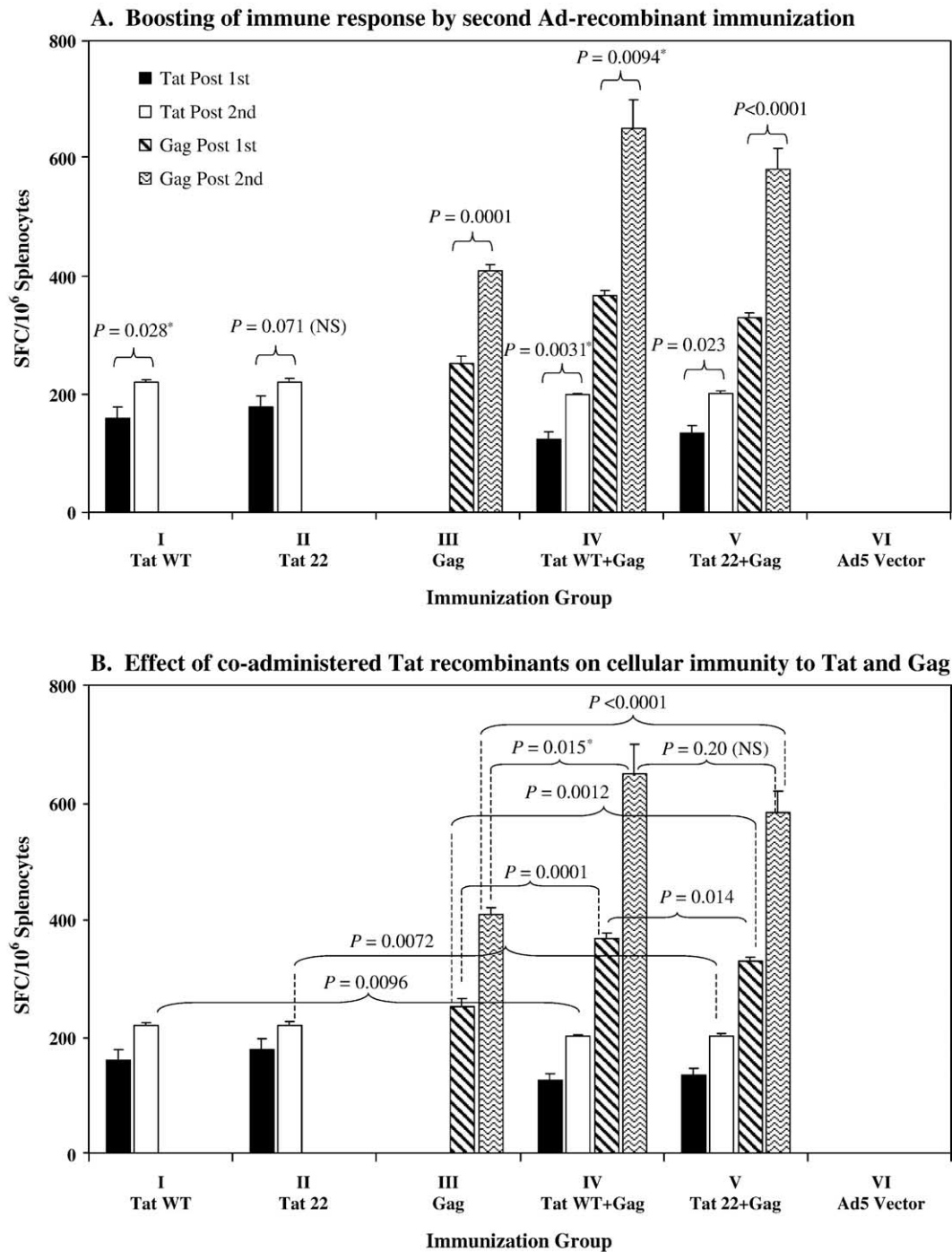


Fig. 1. IFN- γ ELISPOT responses to HIV-1 Tat and SIV Gag peptides following each Ad-recombinant immunization. Freshly isolated splenocytes were assessed for IFN- γ ELISPOT responses after in vitro exposure to peptide pools spanning the HIV-1_{IIIIB} Tat and SIV_{mac239} Gag proteins. Spot forming cells (SFC) are reported following subtraction of background spots observed in the presence of medium alone. Data are presented as mean SFC \pm standard errors of the means. Panels A and B present identical data in order to illustrate statistically significant differences between immunization groups. Statistical analyses were performed by first testing for homogeneity of variance in the responses for the two groups being compared. For groups with homogeneous variances, *t* tests were used. For groups with non-homogeneous variances, a Welch modified *t* test was employed, indicated by an asterisk. NS = not significant.

response to a co-administered antigen. Although the Gag-specific cellular responses were slightly higher following one co-administration with Ad5hr-HIV_{tat-wt} compared to Ad5hr-HIV_{tat22} (Group IV vs. V, post-first, $P = 0.014$), this difference disappeared following the second co-

administrations (Group IV vs. V, post-second, $P = 0.20$) as shown in Fig. 1B. Thus, overall, both the wild-type and mutant Tat immunogens exerted a similar enhancing effect on the immune response of the co-administered SIV Gag immunogen.

In view of the clear enhancement of Gag-specific cellular immunity observed in the co-immunization groups, we looked for immune modulation of cellular immune responses to the Ad5hr-vector itself, by evaluating IFN- γ secretion in response to the Ad5 fiber protein. No differences in ELISPOT responses to Ad5 fiber peptides were seen among any of the immunization groups. Mean Ad5 fiber-specific responses in Groups I through VI at week 2 ranged from 167 to 186 SFC/10⁶ splenocytes and at week 6 from 175 to 239 SFC/10⁶ splenocytes.

T cell proliferation assays were conducted to determine if the vaccine-induced cellular immune responses detected by ELISPOT assay were mirrored by responses to intact Tat protein and SIV p27. Proliferative responses to native and oxidized Tat protein were equivalent (data not shown). Therefore, only results with the oxidized protein are presented. Similar to results obtained for IFN- γ secretion, no difference was observed in the ability of Ad5hr-HIVtat-wt or -HIVtat22 to elicit potent Tat-specific T cell proliferative responses. The mean SI in response to Tat protein (Fig. 2) was similar following each immunization regardless of whether mice received a single Ad-recombinant encoding native or mutant Tat (Groups I and II), or were co-immunized with Ad5hr-SIVgag (Groups IV and V). A boosting of the Tat-proliferative response by a second recombinant administration was detected in immunization groups I and II ($P = 0.0001$ and 0.0003 , respectively), and in groups IV and V that received the co-administered Ad5hr-SIVgag ($P = 0.0030$ and 0.0002 , respectively). No proliferative responses to Tat above background levels were observed in Group VI mice that received only the Ad5hr-vector, indicating not only the specificity of the Tat-response in the immunized mice, but also the absence of non-specific

stimulation in vitro by the Tat protein preparation. A single administration of Ad5hr-SIVgag recombinant was sufficient to elicit a potent Gag-specific proliferative response that was not significantly boosted by the second administration. We speculate that the immunization regimen was too compressed to allow optimal boosting of this induced immune response.

As with cellular responses evaluated for SIV-specific IFN- γ -secreting cells, the SI in response to p27 Gag was also enhanced by co-administration of Ad5hr-recombinants encoding either Tat-wt or Tat22 (Fig. 2). Significant enhancement in the Gag proliferative response of 37% resulted from co-administration of the Ad5hr-HIVtat-wt recombinant after the week 0 immunizations (Group III vs. IV; $P = 0.037$; Fig. 2). Following the week 4 booster immunizations, the Gag proliferative responses in mice co-administered the Tat-wt recombinant also increased (36%) compared to mice that received the Gag recombinant alone, but in this case, statistical significance was not reached ($P = 0.079$). Co-administration of the Tat22 and Gag recombinants resulted in significant increases in the T cell proliferative response to p27 of 49% after both the first and second administrations (Group III vs. V; $P = 0.010$ and 0.029 , respectively; Fig. 2). Thus, the overall evaluations of immunity induced by the vaccination regimen indicated enhanced cellular immune responses to SIV Gag resulted from co-administration of the Ad-tat recombinant immunogens.

Humoral immune responses

Having shown that both the Ad5hr-HIVtat-wt and -HIVtat22 vaccines were immunogenic and able to elicit

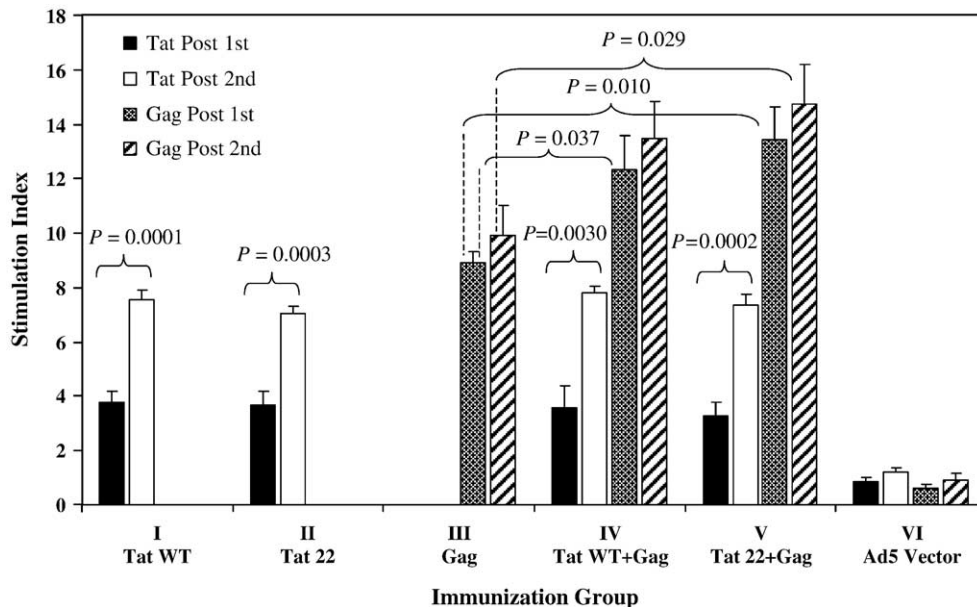


Fig. 2. Lymphoproliferative responses to oxidized HIV-1 Tat and SIV Gag antigens following each Ad-recombinant immunization. Freshly isolated splenocytes were assessed for T cell proliferative activity to Tat and Gag p27 proteins as described in Materials and methods. Data are presented as mean stimulation indices \pm standard errors of the means. All statistical analyses were performed by *t* test, as groups being compared had homogeneous variances.

Table 2
Antibody responses to Tat and Gag post-second immunization

| Group | Immunogen | Antibody titer (Geometric mean (range)) | |
|-------|---|--|-------------------|
| | | Tat | Gag (p27) |
| I | Ad5hrΔE3-HIVtat-wt | 528 (50~3,200) | |
| II | Ad5hrΔE3-HIVtat22 | 44 (10~1,600) | |
| III | Ad5hrΔE3-SIVgag | | 2462 (10~18,500) |
| IV | Ad5hrΔE3-HIVtat-wt + Ad5hrΔE3-SIVgag | 106 (10~3,200) | 3724 (220~20,000) |
| V | Ad5hrΔE3-HIVtat22 + Ad5hrΔE3-SIVgag | 69 (10~400) | 88 (10~800) |

Statistical analyses were performed by *t* test, as variances between groups were homogeneous. NS = not significant. The single statistically significant difference is indicated in bold.

Statistical analyses were performed by *t* test, as variances between groups were homogeneous. NS = not significant. The single statistically significant difference is indicated in bold.

potent cellular immune responses, we next evaluated humoral immunity elicited by the vaccines. While enhanced cellular immunity to a co-administered antigen was demonstrated above, previous reports have shown suppression of antibody responses to a co-administered antigen in the presence of active Tat protein (Cohen et al., 1999). Antibody titers induced following immunization with the Ad5hr-recombinants were relatively low overall.

However, 2 weeks after the second Ad-recombinant immunizations, antibody responses to Tat and SIV p27 were detected in the appropriate immunization groups (Table 2). Anti-Tat titers elicited by the Ad5hr-HIVtat-wt and Ad5hr-HIVtat22 immunizations were similar regardless of whether they were administered singly or together with the Ad5hr-SIVgag recombinant. No significant differences in Tat antibody titers were seen between groups I and II and IV and V. Further, unlike the immune modulation seen in the cellular immune responses, no significant increase or decrease was observed in either anti-Tat or anti-Gag antibody titers upon co-administration of Ad5hr-HIVtat-wt or -HIVtat22 with Ad5hr-SIVgag (compare groups I vs. IV and II vs. V for Tat antibodies; groups III vs. IV and III vs. V for Gag antibodies; Table 2). Overall, however, mice co-administered Ad5hr-HIVtat-wt and Ad5hr-SIVgag exhibited somewhat greater anti-Gag titers compared to mice co-administered Ad5hr-HIVtat22 and Ad5hr-SIVgag ($P = 0.030$). Mice in this same immunization group had also exhibited greater IFN- γ secretion in response to Gag peptides compared to mice co-immunized with Ad5hr-HIVtat22 and Ad5hr-SIVgag, significantly increased after the first immunization but not after the second (Fig. 1B), suggesting a possible advantage of the Tat-wt immunogen. This observation should be further evaluated in a primate model.

Ad5 neutralizing antibody titers were determined on mouse sera from all groups, and similar to results of ELISPOT assays for Ad5 fiber peptides, no significant differences were observed among the immunization groups.

Effect of prior Tat immunization on elicitation of immune responses to Gag

To evaluate the effect of prior Tat immunization on elicitation of immune responses to Gag, two immunization groups were studied in which mice were first immunized with Ad5hr-HIVtat-wt (Group VII) or Ad5hr-HIVtat22 (group VIII) alone, and then were co-administered the HIVtat-wt or HIVtat22 recombinant together with Ad5hr-SIVgag at week 4. Following the co-administration, cellular and humoral immune responses to Gag were compared at

Table 3
Effect of prior immunization with Ad-recombinants encoding Tat-WT or Tat22 on cellular response to co-administered Ad-recombinant encoding SIV Gag

| Prior immunity to Tat | Gag-specific IFN- γ -secreting cells (SFC/10 ⁶ splenocytes) | T cell proliferative response to p27 (Stimulation index) |
|-----------------------|---|--|
| To Tat-WT | | |
| No | 367 ± 9 | 12.32 ± 1.25 |
| Yes | 378 ± 7 | 9.82 ± 0.5 |
| To Tat22 | | |
| No | 329 ± 7 | 13.4 ± 1.23 |
| Yes | 405 ± 37 | 10.23 ± 0.52 |

Mean values ± standard error of the mean reported. *Statistical analysis performed by Welch's modified *t* test. All other analyses were by *t* test. NS = not significant.

week 6 to Gag responses following a single co-administration (week 2) in groups IV (Ad5hr-HIVtat-wt plus Ad5hr-SIVgag) and V (Ad5hr-HIVtat22 plus Ad5hr-SIVgag). The results are shown in Table 3. There were no significant differences in Gag-specific IFN- γ secretion between groups with regard to prior Tat exposure. In addition, proliferative responses to p27 were not significantly decreased by prior immunization with Ad5hr-HIVtat-wt. However, there was a marginally significant decrease in the proliferative response to Gag following prior immunization with Ad5hr-HIVtat22 ($P = 0.045$), again suggesting a possible benefit of using the Tat-wt immunogen. In general, two immunizations with the Ad5hr-SIVgag recombinant were needed for induction of a Gag-specific antibody response, so no prior effect of anti-Tat immunity on Gag antibody responses was observed in groups VII and VIII in which mice received only a single Ad5hr-SIVgag immunization.

Discussion

In the present study, we have demonstrated that both Tat-wt and the mutant Tat22, administered as Ad5hr-recombinants, are highly immunogenic in Balb/C mice. Cellular immune responses, including secretion of IFN- γ in response to Tat peptides and T cell proliferative responses to both native and oxidized Tat protein, were readily detected after a single immunization and were efficiently boosted by a second immunization with the same recombinant. Our immunization strategy using Ad vectored vaccines is aimed at eliciting cellular immunity and priming humoral immune responses. In keeping with this, as the experimental protocol did not include booster immunizations with Tat protein, antibody responses to Tat required two Ad-recombinant administrations for induction and overall were modest.

Not only were the Ad5hr-HIVtat-wt and -HIVtat22 recombinants immunogenic in mice, stimulating strong cellular responses to Tat, they also enhanced cellular immune responses to SIV Gag, co-administered as an Ad5hr-SIVgag recombinant. Modest enhancement of both Gag-specific IFN- γ secretion and T cell proliferation was observed. This is in apparent contrast to previous reports showing immune suppressive effects of Tat (Cohen et al., 1999; Dockrell et al., 1998; Kolesnitchenko et al., 1997; Li et al., 1995; Wu and Schlossman, 1997). However, there is an increasing body of evidence suggesting that the immunomodulatory activities of Tat are immunostimulating rather than immunosuppressive. In fact, recent data indicate that Tat in its native form selectively binds to and is taken up by DC in which it induces activation and maturation into mDC (Fanales-Belasio et al., 2002). In particular, DC exposed to Tat upregulate key co-stimulatory molecules such as CD40, CD80, CD86, LFA-1, MHC Class I and II antigens, and produce inflammatory (TNF- α) and Th-1-

polarizing (IL-12) cytokines and β -chemokines (RANTES, MIP-1 α , and MIP-1 β). Of importance, in vitro responses to allo and recall antigens are also increased (Fanales-Belasio et al., 2002).

Tat contains a cationic region, amino acids 48–60, identical in both the Tat-wt and Tat22 immunogens tested here, that increases expression of epitope/MHC class I complexes on the cell surface (Leifert et al., 2003; Lindgren et al., 2000). However, this mechanism would not have influenced immunity to SIV Gag, administered as a separate Ad5hr-recombinant. Therefore, the most likely explanation for the observed enhanced cellular immunity to Gag is an upregulation of inflammatory and immunostimulating cytokines and chemokines attributable to Tat expression (Izmailova et al., 2003), leading to an overall cellular immune enhancement. An alternative explanation, however, is a general effect attributable to immunization with a mixture of antigens. We have previously reported immune modulatory effects in rhesus macaques immunized with Ad5hr-SIV recombinants encoding SIV env/rev, gag, and/or nef (Patterson et al., 2003). The cellular immune response to Env was significantly enhanced in monkeys that received both Ad5hr-SIVenv/rev and Ad5hr-SIVgag recombinants, while the cellular immune response to Nef was decreased in monkeys immunized with Ad5hr-SIVenv/rev, Ad5hr-SIVgag, and Ad5hr-SIVnef. Importantly, this immune modulation was only observed during the period of Ad-recombinant priming, and disappeared with time over the course of the immunization regimen during the period of protein boosting. Further, the initial immune modulation had no effect on vaccine-induced protection, as similar protective efficacy was observed among the macaque groups that received the multigenic Ad-recombinants following challenge with SIVmac251 (Patterson et al., 2004). The generality of Tat-induced enhancement of cellular immunity when administered as an Ad-recombinant and the eventual effect of any modulation on vaccine efficacy should be further evaluated in macaques which allow both replication of the Ad-recombinant immunogens and challenge with an appropriate SHIV isolate.

Extracellular Tat has been shown to inhibit proliferation of naïve and memory B cells triggered via the B cell receptor (Lefevre et al., 1999). This may explain the previously observed inhibition of anti-Tat and anti-Gag antibody responses following co-administration of native, but not oxidized, Tat with Gag protein immunogens (Cohen et al., 1999). Elicitation of a potent anti-Tat antibody response appears to be a desirable feature for an effective HIV/AIDS vaccine, in order to inhibit early effects of extracellular Tat released from infected cells. In fact, a correlation between the presence of anti-Tat antibodies and delayed progression to disease has been reported (Butto et al., 2003; Rezza et al., 2005). To achieve this, in combination with our Ad-recombinant priming strategy, boosting with Tat protein would likely be necessary. The induction of high-titered antibodies to HIV

and SIV envelopes by Ad5hr-recombinant priming followed by boosting with subunit proteins has been well documented (Buge et al., 1997; Lubeck et al., 1997). Similarly, priming of monkeys with DNA plasmids encoding Tat followed by boosting with Tat-protein together with ISCOM adjuvant has elicited strong anti-Tat responses (Mooij et al., 2004). Recent data suggest that most of the anti-Tat antibodies induced during natural infection are conformational, since major differences were observed when linear Tat peptides or oxidized Tat protein were used instead of the native Tat protein to detect specific antibodies (Butto et al., 2003). Further, a substantial cross-recognition of HIV-1 clade B Tat by sera from Ugandan and South African individuals infected mainly with HIV-1 from subtypes A, C, and D was found, indicating that both linear and conformational B cell epitopes are relatively well-conserved across different clades (Butto et al., 2003). Others have also reported significant serologic cross-clade Tat reactivity (Goldstein et al., 2001; Ruckwardt et al., 2004). Taken together, these data suggest that booster immunizations with native Tat protein rather than the oxidized form or peptides would be preferred. In contrast, cellular immunity to Tat was elicited to a similar extent by both Ad5hr-HIV*tat-wt* and -HIV*tat22*, suggesting that use of either immunogen would be suitable for priming. Whether immune modulation mediated by native Tat protein might lead to suppression of antibody responses following strong priming by the Ad-recombinants encoding Tat-*wt* or Tat22 remains to be determined.

Since the regulatory protein Tat plays a critical role in viral pathogenesis and infectivity, a Tat-based vaccine might achieve substantial control of infection by targeting extracellular Tat with specific antibodies and newly infected cells with anti-Tat CTLs. With this rationale, preventive and therapeutic phase I trials with the native Tat protein are ongoing in Italy. Nevertheless, the immunoregulatory and adjuvant functions of Tat (Fanales-Belasio et al., 2002; Gavioli et al., 2004) suggest that an AIDS vaccine combining Tat with other relevant antigens might be superior to vaccines based on single components (Mooij et al., 2004). In this regard, our observation that Tat, administered as an Ad-recombinant, enhances cellular immune responses to a co-administered immunogen in mice is important and relevant to vaccine design, and should be confirmed in primates. We have previously shown significant protective efficacy against the highly pathogenic SIV_{mac251} strain, achieved by priming with multigenic Ad5hr-SIV recombinants and boosting with envelope subunits (Patterson et al., 2004). The present results suggest that the incorporation of Tat into a similar vaccine strategy might enhance cellular immune responses elicited by other Ad-recombinants and at the same time elicit Tat-specific immunity, of potentially great value in targeting an HIV antigen that is expressed very early after HIV entry, even before virus integration (Wu and Marsh, 2001).

Materials and methods

Adenovirus type 5 host range (Ad5hr) recombinants

A replication competent Ad5hr-SIV recombinant carrying the SIV_{mac239} *gag* gene was described previously (Zhao et al., 2003a). Plasmids pBRAdΔE3 and pAd5tp15-18RD2 used for construction of Ad5hr-HIV*tat-wt* and Ad5hr-HIV*tat22* recombinants were obtained from Wyeth-Lederle Vaccines under a Cooperative Research and Development Agreement. pBRAdΔE3 contains the Ad5 sequence from 59.5 to 100 map units (mu) in which the E3 region from 78.8 to 85.7 mu is deleted. Plasmid pCI vector containing CMV and polyA was purchased from Promega (Madison, WI). Ad5hr-recombinants carrying HIV*tat-wt* and the transdominant mutant *tat22* (Cys22 to Gly) (Caputo et al., 1996) were constructed as follows. First, the CMV promoter, the *tp1* of the Ad5 serotype (*tp15*), *tat-wt* (or *tat22*), and polyA were amplified separately by PCR with four pairs of primers: CMV5 p1 (5' to 3'): CCTCTAGTTA-TAGTAATCAATTACGGGGTTCATT and *tp15*-CMV-R CGGAAGAGAGTCGAGCTCTGCTTAT; CMV-*tp15*-F: GCAGAGCTCGACTCTCTCCGCATCGC; and *tat-tp15*-R: CTACAGGCTCCATCTTGGCGACTGTGAC; *tp15-tat*-F: GTCACAGTCGCAAGATGGAGCCAGTAG and *Tat-polyA*-F: CCGAAGGAATAGTGATCCCCCGAC; and *Tat-polyA*-R: TCGGGGGATCACTATTCCTTCGG and CMV5p2 CCTCTAGATCTCCGAGGGATCTCGAC-CAAAT. Secondly, a hybrid expression cassette of CMV-Ad5*tp1-Tat-wt* (or *Tat22*)-polyA was assembled by PCR using a mixture of the above PCR products as template and the primer pair CMV5 p1 and CMV5 p2. The amplified expression cassette was then inserted into the XbaI site at 78.8 mu of pBRAdΔE3. The correct gene orientation and sequence of both pBRAdΔE3HIV*tat-wt* and pBRAdΔE3HIV*tat22* were confirmed by DNA sequencing. The Ad5hr-HIV*tat-wt* recombinant was generated by homologous recombination between Ad5hr viral DNA, 1 to 76 mu, and pBRAdΔE3HIV*tat-wt* as described previously (Zhao et al., 2003a). The Ad5hr-HIV*tat22* recombinant was similarly constructed by homologous recombination between Ad5hr viral DNA, 1 to 76 mu, and pBRAdΔE3HIV*tat22*.

Expression of the Tat protein in human 293 cells infected with the Ad5hr-HIV*tat-wt* or HIV*tat22* recombinant was evaluated by Western blot. The 293 cells were infected at an MOI of 10 with the recombinants or the Ad5hrΔE3 vector as negative control. When 90% of the cells exhibited a cytopathic effect, cell lysates were prepared with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% polyethoxyethanol (Sigma, St. Louis, MO), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride), separated on an SDS-polyacrylamide gradient gel of 4–20% (Bio-Rad), and then transferred onto a nitrocellulose membrane (Bio-Rad). Tat expression was

determined by incubating the membrane with anti-HIV-1 Tat monoclonal antibody (NT3 2D1.1; AIDS Research and Reference Reagent Program, NIAID, NIH) and subsequently visualizing bands using the ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ), as described by the manufacturer.

Immunization of mice and sample collection

Female Balb/C mice at 6–8 weeks of age were used in this study. Each group included 10 mice except groups VII and VIII that contained 5 mice each. The immunization schedule is outlined in Table 1. Each Ad-recombinant was administered intraperitoneally at 10^8 plaque-forming units (pfu) per immunization in a total volume of 500 μ l phosphate-buffered saline (PBS) regardless of the number of recombinants given. Five mice per group in groups I–VI were sacrificed 2 weeks after the first immunization and 5 mice per group in groups I–VIII were sacrificed 2 weeks after the second immunization at week 4. Spleens and blood were collected to evaluate immune responses. Mouse splenocytes were isolated by passing spleen fragments through a 70 μ m nylon cell strainer. After lysis of erythrocytes, the splenocytes were resuspended in R-10 medium (RPMI-1640 containing 10% FCS, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 μ g/ml streptomycin).

ELISPOT assay

IFN- γ secretion in response to SIV Gag, HIV-1 Tat, and Ad5 fiber peptides was evaluated by ELISPOT assay. Gag peptides included 50 20-mer peptides with 10-amino acid overlap, spanning the entire SIVmac239 Gag protein. HIV-1 Clade B consensus Tat peptides included 23 15-mer peptides with 11-amino acid overlap. Both SIV Gag and HIV-1 Tat peptides were obtained from the AIDS Research and Reference Reagent Program, NIAID, NIH. The Ad5 fiber peptides consisting of 142 15-mers overlapping by 11 amino acids were obtained from Advanced BioScience Laboratories, Inc. (ABL, Kensington, MD). The ELISPOT assay for the Th-1 cytokine IFN- γ was performed using commercial kits (U-Cytech, Utrecht, The Netherlands) according to the manufacturer's manual with slight modification. Briefly, 96-well flat-bottom plates were coated overnight with anti-IFN- γ monoclonal antibody MD-1 (U-Cytech), washed and blocked as described. Dilutions of splenocytes ranging from 2×10^5 to 0.25×10^5 per 100 μ l R-10 medium were transferred to triplicate wells, together with 2 μ g/ml of each peptide in the Gag, Tat, or Ad5 fiber peptide pools. The Tat peptides were used as a single pool. Responses to Gag and the Ad5 fiber were evaluated using 2 pools of Gag peptides (25 each) and 2 pools of Ad5 fiber peptides (71 each). Following subtraction of background spots, values for the Gag and Ad5 fiber pools were summed. Concanavalin A (Con A)

(Sigma) at 5 μ g/ml and R-10 medium alone were used as positive and negative controls. Following overnight incubation at 37 °C in 5% CO₂, the cells were removed, and the wells washed and incubated with biotinylated anti-IFN- γ antibody (U-Cytech). After further washing, bound anti-IFN- γ antibody was detected with a gold-labeled anti-biotin solution (U-Cytech). Spots were developed by incubating the plates with U-Cytech's activator mixture. The color reaction was stopped by washing with distilled water. The plates were air-dried, and spots were counted visually using an inverted microscope.

T cell proliferation

To account for possible effects of native Tat protein on splenocytes during the period of in vitro stimulation, both native and inactivated Tat proteins were evaluated in proliferative assays. Biologically active Tat protein (ABL), greater than 95% pure, was purified as previously described (Chang et al., 1997) followed by reverse phase HPLC to remove LPS. Assay for endotoxin showed 0.45 EU/ μ g Tat. Since Tat is easily oxidized and photo- and thermo-sensitive, the native Tat protein was reconstituted in degassed PBS containing 0.1% bovine serum albumin (BSA) immediately prior to use, and the handling of the protein was performed in the dark and on ice. Oxidized Tat protein was prepared by exposing the active Tat protein to air and light for 24 h. Cells were cultured in triplicate, at 2×10^5 /well in 200 μ l R-10 in the presence of active Tat or oxidized Tat proteins (5 μ g/ml), or SIV p27 (2 μ g/ml) (ABL). Con A (5 μ g/ml) and R-10 medium alone served as positive and negative controls, respectively. Following 5 days incubation at 37 °C in 5% CO₂, the cells were pulsed with [³H]thymidine (1 μ Ci per well) and further incubated overnight. Cells were harvested using a Mach IIM (Tomtech Inc.) cell harvester and counted on a Pharmacia (Wallac Inc.) beta-plate counter. Stimulation indices (SI) were calculated by dividing the mean counts per minute (cpm) with antigen by the mean cpm with medium alone.

Serological responses

Anti-Ad5 neutralization titers were determined by microtiter-neutralization assays on A549 cells as described previously (Chengalvala et al., 1991), using serial two-fold dilutions of mouse sera. Endpoint titers were defined as the reciprocal of the last serum dilution at which an Ad5 cytopathic effect was not observed.

Serum antibodies to SIV Gag were assayed by enzyme-linked immunosorbent assay (ELISA) using 25 ng of SIV p27 per microtiter well and horse radish peroxidase-conjugated goat anti-mouse IgG (1:25000). All sera were initially tested at a 1:50 dilution and then titered to endpoint as needed. The binding titer was defined as the reciprocal of the serum dilution at which the absorbance of the test serum was twice that of the negative control serum diluted 1:50.

Tat-specific antibodies were also evaluated by ELISA. Nunc-Immuno MaxiSorp 96-well plates were coated with Tat protein (100 ng/well in 0.05 M carbonate buffer, pH 9.6) for 12 h at 4 °C, washed 5 times with PBS without Ca²⁺ and Mg²⁺ containing 0.05% Tween 20 (PBS/Tween), and blocked with PBS containing 1% BSA and 0.05% Tween 20 (blocking buffer, BB) for 90 min at 37 °C. After extensive washings, aliquots of mouse sera, serially diluted in BB beginning at 1:100, were added to duplicate wells. Sera were also tested against wells coated with the buffer in which Tat had been resuspended to correct for any non-specific binding. After a 90-min incubation at 37 °C, the plates were washed extensively, and the wells were saturated with BB for 15 min at 37 °C. After further washing, 100 µl of horseradish peroxidase-conjugated anti-mouse IgG (Sigma) diluted 1:1000 in BB was added to each well. The plates were incubated an additional 90 min at 37 °C, washed, and developed with ABTS substrate solution (Roche Diagnostics) for 50 min at 37 °C. Absorbance was measured at 405 nm using a Sorin Biomedica microplate reader. Optical densities (OD) of the samples were normalized against the buffer-coated wells for each sample. The difference (Δ) in OD between the Tat and buffer coated wells was calculated. Positive and negative controls included one known Tat-antibody positive sample and 3 known Tat-antibody negative samples. The assay was considered valid if the both the Δ values and the absolute OD values of the positive and negative controls prior to normalization were within 10% of the values observed in 50 previous assays. A positive sample was defined as 3 standard deviations above the mean of both absolute OD and Δ values obtained from 50 assays of Tat antibody negative mouse sera. Antibody titer was defined as the reciprocal of the serum dilution at which the mouse serum still gave a positive value.

Statistical methods

Data in this study were analyzed using standard parametric and non-parametric methods, meaningful descriptive and graphical techniques, and followed up with standard post hoc parametric tests (e.g., Tukey's HSD method) and non-parametric tests (e.g., Wilcoxon). As our results satisfied normality and homogeneity of variance assumptions, and as all post hoc results were in agreement, interpretively, with results obtained from *t* tests, we report probability values from *t* tests (or Welch's modified *t* test) as appropriate. All tests were two-sided; probability values less than 0.05 were considered significant.

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