# Contribution of Nonneutralizing Vaccine-Elicited Antibody Activities to Improved Protective Efficacy in Rhesus Macaques Immunized with Tat/Env Compared with Multigenic Vaccines[1]

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**Previously, chronic-phase protection against $SHIV_{89.6P}$ challenge was significantly greater in macaques primed with replicating adenovirus type 5 host range mutant (Ad5hr) recombinants encoding HIV*tat* and *env* and boosted with Tat and Env protein compared with macaques primed with multigenic adenovirus recombinants (HIV*tat*, HIV*env*, SIV*gag*, SIV*nef*) and boosted with Tat, Env, and Nef proteins. The greater protection was correlated with Tat- and Env-binding Abs. Because the macaques lacked $SHIV_{89.6P}$-neutralizing activity prechallenge, we investigated whether Ab-dependent cellular cytotoxicity (ADCC) and Ab-dependent cell-mediated viral inhibition (ADCVI) might exert a protective effect. We clearly show that Tat can serve as an ADCC target, although the Tat-specific activity elicited did not correlate with better protection. However, Env-specific ADCC activity was consistently higher in the Tat/Env group, with sustained cell killing postchallenge exhibited at higher levels ($p < 0.00001$) for a longer duration ($p = 0.0002$) compared with the multigenic group. ADCVI was similarly higher in the Tat/Env group and significantly correlated with reduced acute-phase viremia at wk 2 and 4 postchallenge ($p = 0.046$ and 0.011, respectively). Viral-specific IgG and IgA Abs in mucosal secretions were elicited but did not influence the outcome of the i.v. $SHIV_{89.6P}$ challenge. The higher ADCC and ADCVI activities seen in the Tat/Env group provide a plausible mechanism responsible for the greater chronic-phase protection. Because Tat is known to enhance cell-mediated immunity to coadministered Ags, further studies should explore its impact on Ab induction so that it may be optimally incorporated into HIV vaccine regimens.** ***The Journal of Immunology,*** **2009, 182: 3718–3727.**

The HIV/AIDS pandemic remains a major public health concern, and development of an efficacious vaccine is still the best option to combat the disease. To date, at least 33.2 million people worldwide are infected with HIV; on average, 2.5 million new infections occur every year (www.who.int/mediacentre/news/releases/2007/pr61/en/index.html). One of the strategies being evaluated for AIDS vaccine development uses viral vectors to deliver viral subunits to the immune system. Our vaccine approach is based on replication-competent adenovirus (Ad)[3] recombinants (1) which have been shown to elicit better and more persistent cellular immune responses and prime higher titered Abs than nonreplicating Ad recombinants encoding the same HIV gene products (2). The Abs induced were able to neutralize primary isolates and showed Ab-dependent cellular cytotoxicity (ADCC) activity across HIV clades (3). In the rhesus macaque system, when replicating Ad-SIV recombinant priming was followed by envelope subunit protein boosting, the combination approach elicited potent protection against intrarectal challenge with virulent $SIV_{mac251}$ (4). This protection was durable 1 year later without intervening vaccination (5).

The ultimate goal of AIDS vaccine research is to provide sterilizing immunity, presumably by induction of neutralizing Abs (6) by candidate HIV envelope vaccines, thereby completely preventing HIV infection. Passive transfer studies using monoclonal or polyclonal HIV-neutralizing Abs have provided complete or partial protection against homologous viral challenge in nonhuman primate models (7, 8). However, in clinical trials, neutralizing Ab responses against HIV gp120 have not provided sufficient protective efficacy, primarily due to the inherent variability of the HIV envelope. Structural studies of the HIV envelope may yet reveal an envelope design able to elicit broadly reactive Abs able to neutralize across multiple HIV clades.

In the meantime, vaccine strategies are focused on limiting the initial viral burden during the acute phase of infection and lowering the viral set point during the chronic infection phase, thus reducing virus transmissibility and retarding disease progression. This strategy relies on induction of both humoral and cellular immune responses to a spectrum of HIV Ags. In addition to anti-envelope responses, Abs to early HIV regulatory gene products, including Tat, Rev, and Nef, might also be expected to impact HIV

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[3] Abbreviations used in this paper: Ad, adenovirus; ADCC, Ab-dependent cellular cytotoxicity; ADCVI, Ab-dependent cell-mediated virus inhibition; Ad5hr, Ad type 5 host range mutant; RFADCC, rapid fluorometric ADCC assay; MOI, multiplicity of infection; ABL, Advanced BioScience Laboratories; RT, room temperature; KPL, Kirkegaard and Perry Laboratories; huPBMC, human PBMC; IFA, immunofluorescence assay.

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acute infection. These Ags, in addition to Env and other viral structural proteins such as Gag, can also elicit cellular immune responses believed to exert greater control postacute infection.

Tat, an early gene product, is a potent *trans* activator of HIV gene expression and is essential for viral infectivity and pathogenesis (9–12). Additionally, the Tat released from infected cells and taken up by other infected or uninfected cells is capable of multiple functions (13). It can promote viral replication, *trans* activate *tat*-defective or latent proviruses, modulate cellular gene expression, up-regulate HIV coreceptors and induce or inhibit apoptosis (13–20). Thus, inhibition of Tat functions may be a good strategy to control viral replication. In fact, anti-Tat Abs can inhibit uptake of extracellular Tat by surrounding cells, thus limiting HIV replication and transmission (21, 22). In natural infection, the presence of Tat-specific CTLs and/or anti-Tat Ab were associated with control of viral replication and slow progression to AIDS (22–28). These findings suggest that immunization with Tat-based vaccines may impact both the acute and chronic phases of subsequent HIV infection (29). A HIV Tat vaccine has been successfully tested in a human therapeutic phase I trial, providing the basis for moving forward with phase II studies (30).

Recently, we investigated an Ad5 host range mutant (Ad5hr)-HIV*tat* recombinant as a vaccine candidate in rhesus macaques (31), alone or in combination with Ad5hr-HIV*env*, or together in a multigenic mixture including Ad5hr-HIV*env*, $SIV_{239}$*gag*, and $SIV_{239}$*nef*. After boosting with HIV Env, HIV Tat, and SIV Nef proteins, the animals were challenged i.v. with $SHIV_{89.6P}$. The protocol design was based on the hypothesis that the envelope components, compared with a solely Tat-based vaccine, would enhance acute-phase protection resulting from Ab induction, whereas the multigenic vaccine would enhance protection during the chronic phase due primarily to cellular immune responses. Unexpectedly, the macaques immunized with the Tat/Env regimen displayed the most potent protection, with a significant 4-log reduction in chronic viremia compared with controls, and a significant 1-log-greater reduction in chronic viremia compared with the multigenic group. Whereas the vaccines elicited cellular immunity, the significantly greater reduction of chronic phase viral load in the Tat/Env group compared with the multigenic group was correlated with Tat- and Env-binding Abs (31).

Here, we investigated anti-Env and anti-Tat Ab responses in greater depth to elucidate a possible basis for the better protection in the previous study. The prechallenge anti-Env Abs did not neutralize the $SHIV_{89.6P}$ challenge virus. Neutralizing Abs developed only postchallenge, exhibiting similar levels in both the Tat/Env and multigenic groups. Therefore, we examined other nonneutralizing, functional activities of envelope Abs, including ADCC and Ab-dependent cell-mediated viral inhibition (ADCVI). We also investigated whether anti-Tat Abs mediate ADCC activity.

## Materials and Methods

### *Vaccines, macaques, and immunization and challenge regimen*

This study makes use of sera, plasma, and secretory samples obtained from previously reported immunized and challenged macaques (31). The vaccine immunogens used for priming included Ad5 host range mutant (Ad5hr) recombinants separately encoding $HIV_{89.6P}$*gp140*ΔCFI (32), $HIV_{IIIB}$*tat* (33), $SIV_{239}$*gag* (34), and $SIV_{239}$ $nef_{\Delta 1-13}$ (35). The empty Ad5hrΔ E3 vector was used as control. Protein boosts included native $HIV_{IIIB}$Tat (Advanced BioScience Laboratories; ABL) $HIV_{89.6P}$gp140ΔCFI protein, and $SIV_{239}$Nef (ABL).

Nineteen juvenile Mamu A*01-negative Indian rhesus macaques (*Macaca mulatta*) were housed at the Washington National Primate Research Center (Seattle, WA) and maintained following the guidelines and protocols of the Animal Care and Use Committee, Washington National Primate Research Center, University of Washington (Seattle,WA). Eight macaques were included in each immunization group: four females and four males in the Tat/Env group; and five females and three males in the multigenic group. The control group was made up of two females and one male. The previously published immunization and challenge schedule, vaccine dosages, and routes of administration (31) are outlined in Fig. 1*A*.

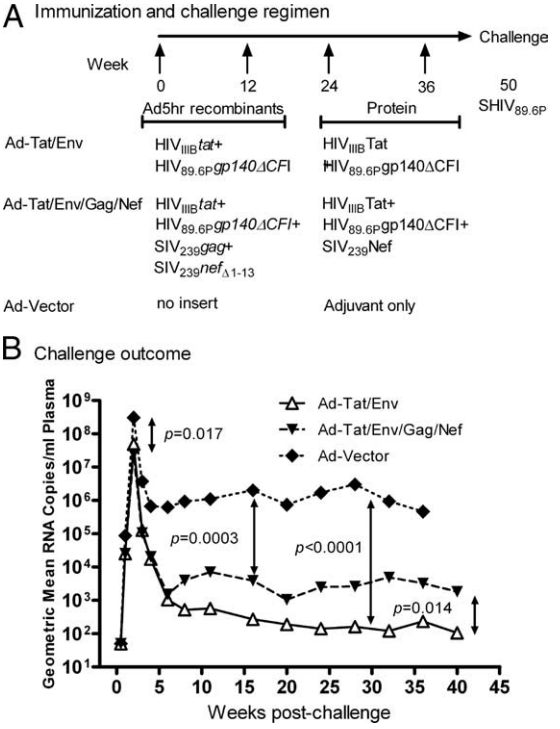


**FIGURE 1.** Immunization and challenge schedule and challenge outcome (from Ref. 31). *A*, Ad5hr recombinant dose: $5 \times 10^8$ PFU each, made up to a total Ad5hr dose of $2 \times 10^9$ PFU with Ad5hrΔ3 vector as necessary; wk 0 given intranasally; wk 12 given intratracheally; $HIV_{IIIB}$ Tat, 10 $\mu$g administered s.c. in alum; $HIV_{89.6P}$gp140ΔCFI protein, 100 $\mu$g administered i.m. in MPL-SE; $SIV_{239}$Nef, 20 $\mu$g administered i.m. in MPL-stable emulsion. $SHIV_{89.6P}$, thirty 50% monkey-infective doses administered i.v. in PBS. *B*, Geometric mean plasma viremia following i.v. challenge with $SHIV_{89.6P}$ for the three immunization groups.

### *Sample collection*

Plasma and sera were collected before and after challenge and stored at −70°C. Before use in functional assays, the samples were thawed at room temperature, diluted 10-fold with R-10 medium (RPMI 1640 containing 10% FCS, 2 mM L-glutamine, and antibiotics), and heat inactivated at 56°C for 30 min.

Rectal and vaginal secretions were obtained as previously described (36) by gently swabbing the mucous membrane surfaces with cotton-tipped applicator sticks, after which the applicators were stored at −70°C in PBS containing 0.1% BSA, 0.01% thimerosal, and 750 Kallikrein inhibitor U of aprotinin per ml. Any sample that contained blood was not analyzed.

### *Systemic binding Ab*

Serum Abs to HIV Tat, $SHIV_{89.6P}$gp140, and SIV Nef were assessed by ELISA as described previously (36). The titer of the Ab was defined as the serum dilution at which the OD of the test serum was 2 times greater than that of the negative control naive rhesus macaque serum diluted 1/50.

### *Mucosal binding Ab*

Specific IgG and IgA Abs in rectal and vaginal secretions were determined using a fluorescent bead-based flow cytometric assay (37). Briefly, 4–10 $\mu$g of each viral protein, $HIV_{89.6P}$gp140, $SIV_{239}$Gag, $SIV_{239}$Nef, and $HIV_{IIIB}$Tat, were covalently coupled to a specific carboxylated microsphere (bead) set (Bio-Rad Laboratories and Luminex). Each bead set is internally dyed with different ratios of two spectrally distinct fluorophores, making each bead set distinguishable by its fluorescent emission when excited by a laser. A mixture of the conjugated beads was deposited into

wells of a 96-well filter plate. After washing with PBS containing 0.1% Tween 20 (PBS-T), prediluted samples in 5% Blotto (5 g of nonfat dry milk in 100 ml of PBS plus 0.05% Tween) were added to the wells and allowed to react for 1.5 h on a plate shaker in the dark at room temperature (RT). After incubation, the beads were washed three times in PBS-T and reacted with a reporter fluorescent Ab for 40 min on a plate shaker at RT while protected from light. After further washing and resuspension in PBS, the samples were read on a Bio-Plex array reader. Ab titer is defined as the reciprocal of the sample dilution at which the mean fluorescent intensity of 100 counted beads in the test sample was greater than that of the negative control sample (preimmunization sample) diluted 1/40.

Total IgG and IgA Abs in rectal and vaginal secretions were determined by ELISA (36) with modifications as described here. Briefly, Maxisorp plates (Nalge Nunc) were coated with 100 $\mu$l of a 10-$\mu$g/ml solution of either affinity purified goat anti-monkey IgG or IgA (Kirkegaard and Perry Laboratories; KPL) for 1 h at 37°C in 5% $CO_2$. Plates were washed five times with wash solution (KPL) and subsequently blocked with a 1/10 dilution of milk blocking solution (KPL). Serially diluted mucosal samples were added to the plate and incubated at 37°C with 5% $CO_2$ for 1 h. After washing, the plates were reacted with the corresponding secondary Abs (KPL). After intensive washes, the plates were developed for 15 min with 100 $\mu$l of tetramethylbenzidine 2-component Microwell Peroxidase Substrate (KPL). The reaction was stopped using 100 $\mu$l of 1 M phosphoric acid, and the OD was read at 630 nm. Sets of monkey IgG or human secretory IgA standards were included on each plate. Concentrations of total IgG and IgA in test samples were determined based on standard curves of known Ab concentrations in the standards measured in the same plate.

The specific activity of rectal and vaginal IgG and IgA Ag-specific Abs was calculated by dividing the Ab titer by the total IgG or IgA concentration. Results are reported as fold increase in specific activity compared with specific activity of Ab in the preimmunization sample. Fold increases of $>2$ are considered positive.

### *ADCC*

CEM-NK$^R$ cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD) coated with $HIV_{89.6P}$gp140 were used as targets for the rapid fluorometric ADCC (RFADCC) assay as described (38). To evaluate ADCC mediated by anti-Tat Ab, the optimal concentration of $HIV_{IIIB}$Tat protein bound to the surface of CEM-NK$^R$ cells was first determined by immunofluorescence assay as described below. Subsequently, 10 $\mu$g of purified oxidized $HIV_{IIIB}$Tat protein were coated onto $1 \times 10^6$ CEM-NK$^R$ cells. The cells were double-stained with a membrane dye, PKH-26 (Sigma-Aldrich), and a viability dye, CFSE (Molecular Probes), before the addition of prediluted macaque plasma and human effector cells at an E:T ratio of 50:1. A duplicate of 50,000 nongated events were acquired within 24 h of the ADCC assay using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software, setting fluorescence 1 (FL1) as the CFSE emission channel and fluorescence 2 (FL2) as the PKH-26 emission channel. Percent killing was determined by back-gating on the PKH-26$^{high}$ population of targets and reported as the percentage of cells that lost the CFSE viability dye. Data were analyzed using WINMDI 2.9 software. Controls included were non-stained and single-stained target cells. ADCC titers are defined as the reciprocal dilution at which the percent ADCC killing was greater than the mean percent killing of the negative controls plus 3 SDs.

ADCC mediated by Nef-specific Abs was similarly evaluated using vaccinia-Nef-infected H9 cells. Briefly, H9 cells were infected with 1 multiplicity of infection (MOI) of vaccinia-Nef (vNef157; Ref. 39), kindly provided by Tilahun Yilma (University of California, Davis, CA) and incubated for 1 h at 37°C in 5% $CO_2$ with gentle agitation every 15 min. The cells were washed twice with R-10 medium and incubated for 18–24 h under the same conditions before using as targets. Cell viability was confirmed using trypan blue staining, and SIV Nef surface expression was examined by immunofluorescence staining as described below.

### *Immunofluorescence microscopy*

Viable CEM-NK$^R$ cells were enriched by Ficoll-Hypaque density centrifugation and washed with PBS. Cells ($1 \times 10^6$) were coated with varying amounts of purified, oxidized $HIV_{IIIB}$Tat protein or 10 $\mu$g of $HIV_{IIIB}$gp120 or mock coated with R-10 medium for 1 h at RT with gentle agitation every 15 min. Coated cells were washed twice with PBS, cytospun on poly-L-lysine-coated microscope slides (Tekdon), fixed with 4% (v/v) paraformaldehyde in PBS for 15 min, washed with PBS, and stained with rabbit anti-HIV Tat polyclonal serum (AIDS Research and Reference Reagent Program, National Institutes of Health) or Tat mAb, 1D9 (AIDS Research and Reference Reagent Program, National Institutes of Health). After 30 min of incubation at RT and three washes with PBS, the cells were reacted for 30 min at RT with corresponding secondary Abs conjugated to FITC (goat anti-rabbit IgG for polyclonal anti-Tat (Zymed); goat-anti-mouse IgG for monoclonal anti-Tat (ImmunoPure; PierceBio) and with phalloidin conjugated to Texas Red (Molecular Probes and Invitrogen) for actin staining. Slides were washed, and Vectashield with 4′,6′-diamidino-2-phenylindole (Molecular Probes) was used as the mounting medium. Cells were imaged by confocal microscopy.

H9 cells infected for 24 h with 0.1, 0.5, and 1 MOI of recombinant vaccinia-Nef were also analyzed for Nef surface expression. The infected cells were reacted with $SIV_{mac251}$Nef mAb 17.2 (AIDS Research and Reference Reagent Program, National Institutes of Health), washed with PBS, and subsequently reacted with goat anti-mouse IgG conjugated with FITC (ImmunoPure) and phalloidin conjugated to Texas Red. Cells were mounted and examined as above.

### *ADCVI*

The ADCVI assay was based on methods previously described (40, 41). Briefly, human PBMCs (huPBMC) which served as target cells were first stimulated with PHA (2 $\mu$g/ml) and rIL-2 (0.5 ng/ml) for 72 h, then washed and infected with 200 fifty percent tissue culture-infective doses of $SHIV_{89.6P}$. After adsorption for 1 h, the huPBMCs were washed and incubated further in R-10 medium at 37°C in 5% $CO_2$ for 48 h. Infected target cells ($5 \times 10^4$) were next plated onto wells of a 96-well round-bottom microtiter plate, and 1/100 dilutions of test plasma were added to the target cells along with huPBMC effector cells at an E:T ratio of 20:1. Plasma in the absence of effector cells was also tested. Target cells without plasma and effector cells were used as control. After 7 days of incubation at 37°C in 5% $CO_2$, supernatant fluids were collected and assayed for p27 by ELISA (ABL). Virus inhibition due to ADCVI was calculated as follows: % inhibition $= 100 \times \{[1 - ([p27_{E+}]/[p27c])] - [1 - ([p27_{E-}]/[p27c])]\}$, where $[p27_c]$ is the p27 concentration of control, $[p27_{E+}]$ and $[p27_{E-}]$ are the p27 concentration in the presence or absence of effector cells, respectively.

### *Statistical analysis*

The Wilcoxon rank-sum test and Wei-Johnson method were used to analyze differences between binding, ADCC and ADCVI titers in experimental and control groups at all time points evaluated. Analyses of percent ADCC killing and of differences in specific IgG and IgA Abs in mucosal secretions of experimental and control macaques were conducted using repeated measures ANOVA. Durations of enhanced ADCC killing in the two immunization groups following challenge were compared using the Cochran-Armitage test. The Spearman rank correlation coefficient test was used to determine the relationship between ADCVI activity and viral burden.

## Results

### *Systemic envelope-specific ADCC*

Previously, we reported that anti-envelope and anti-Tat Abs were correlated with the better protection seen in macaques immunized with a Tat/Env vaccine regimen compared with a multigenic regimen (31). The vaccine-induced binding Abs seen against $SHIV_{89.6P}$ envelope are summarized in Fig. 2*A*. At wk 48, 2 wk before challenge, the Tat/Env immunization group exhibited significantly higher titers against $SHIV_{89.6P}$gp140 ($p = 0.020$) than the multigenic group did. Additionally, anti-envelope binding Abs were also higher in the Tat/Env group over wk 6–11 postchallenge compared with the multigenic group ($p = 0.0044$). Although neutralizing Abs against $SHIV_{89.6P}$ were not elicited before the i.v. challenge, a neutralizing response did appear quickly postchallenge in the immunized macaques. However, as previously reported, no significant differences in titer were observed between the two immunization groups (31). Therefore, here we explored other functional Ab activities as a possible basis for the better protection seen in the Tat/Env group. As we have previously seen a correlation of Abs mediating ADCC with protection (42), we explored this functional activity first.

ADCC activity of macaque plasma using $SHIV_{89.6P}$gp140-coated targets was evaluated at wk 48, 2 wk before challenge, and at several time points out to 20 wk after challenge (Fig. 2*B*). Both

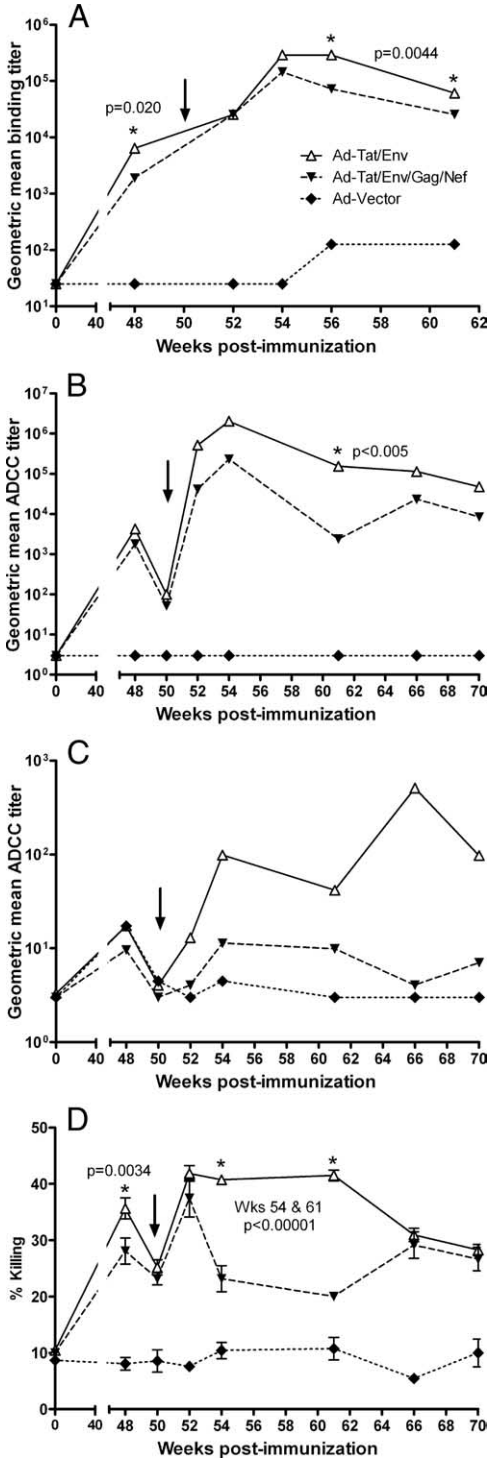


**FIGURE 2.** HIV envelope-specific Ab responses. *A*, Vaccine-induced binding Ab responses to $HIV_{89.6P}$gp140 before and after challenge. Geometric mean titers for each immunization group are shown (from Ref. 31). The arrow indicates time of i.v. $SHIV_{89.6P}$ challenge at wk 50. *B*, ADCC-mediating Ab titers in sequential plasma samples from immunized and control macaques against $HIV_{89.6P}$gp140-coated targets. *C*, ADCC-mediating Abs in sequential plasma specimens from immunized and control macaques against H9 target cells infected with $HIV_{IIIB}$. *D*, Percent ADCC killing mediated by Abs in plasma of immunized and control macaques using $HIV_{89.6P}$gp140-coated targets.

the Tat/Env and multigenic groups exhibited equivalent ADCC titers before and at the time of challenge; however, the Tat/Env group displayed a more pronounced anamnestic response 2 wk postchallenge. Higher ADCC titers were consistently seen in the Tat/Env group postchallenge from wk 52 to wk 70, although a statistically significant difference occurred only at wk 61, 11 wk postchallenge ($p < 0.005$ after correction for multiple times tested). Compared with the controls, which consistently had undetectable ADCC activity, ADCC titers of the two vaccinated groups were significantly elevated at all time points evaluated ($p \leq 0.012$ for the Tat/Env group and $p \leq 0.042$ for the multigenic group by the Wilcoxon rank-sum test).

ADCC activity was also evaluated using heterologous $HIV_{IIIB}$-infected targets (Fig. 2*C*). A similar pattern was observed, although ADCC titers were much lower. The Tat/Env group maintained consistently higher ADCC titers compared with the multigenic group over wk 52–70 (2–20 wk postchallenge), although significant differences were not reached.

In addition to the titer of Abs mediating ADCC activity, we also investigated the level of cell lysis (Fig. 2*D*). Using SHIV gp140-coated targets, the mean percent of ADCC killing at wk 48 before challenge was significantly higher in the Tat/Env group (36%) than in the multigenic group (28%; $p = 0.0034$; Fig. 2*D*). Equivalent anamnestic responses were seen 2 wk postchallenge (wk 52) in both groups. However, elevated percent killing persisted in the Tat/Env group compared with the multigenic group, with significant differences in killing levels seen at wk 54 and 61 ($p < 0.00001$ after correction for multiplicity). The duration of the elevated response, defined by the time to return to percent killing levels at time of challenge, was significantly longer in the Tat/Env group (wk 70 or later) than in the multigenic group (wk 61), $p = 0.0002$.

### *Systemic Tat-specific ADCC*

All macaques in both immunized groups developed strong binding Abs to Tat as published previously (31) and shown here (Fig. 3*A*). At 2 wk before challenge (wk 48), macaques in the Tat/Env group exhibited significantly higher Tat-specific binding Abs compared with the multigenic group ($p = 0.028$). Tat is expressed on the surface of ~6% of PBMCs obtained from HIV-infected patients, as shown by immunostaining and flow cytometric analysis, and is also released and can be taken up by neighboring uninfected cells (43). Therefore, we investigated whether anti-Tat Abs induced by the vaccine regimens could mediate ADCC activity.

Initially, we explored whether H9 cells infected in vitro with $HIV_{IIIB}$ would serve as appropriate target cells. We observed a low percentage of cells expressing Tat protein on the cell surface by indirect immunofluorescence assay (IFA), similar to that reported for cells in HIV-infected individuals (Ref. 43 and data not shown). Because this low level of positive cells would not provide sufficient sensitivity in the in vitro ADCC assay, we coated CEM-$NK^R$ cells with purified oxidized $HIV_{IIIB}$Tat protein, ranging from 10 ng to 10 $\mu$g, and examined them by IFA using both mAb 1D9 and a polyclonal anti-Tat Ab. A dose-dependent result was observed, with Tat cell surface expression varying from 0–1% (10 ng) to 99–100% (10 $\mu$g) with both Abs (Table I). Mock coated control CEM-$NK^R$ cells were negative for Tat cell surface expression at all concentrations tested. Representative IFA results showing mAb 1D9 staining of CEM-$NK^R$ cells coated with 10 $\mu$g of $HIV_{IIIB}$Tat or mock coated with R-10 medium alone are shown in Fig. 4, *A* and *B*. CEM-$NK^R$ cells coated with $HIV_{IIIB}$gp120 and stained with mAb 1D9 were also negative (data not shown).

Using CEM-$NK^R$ cells coated with 10 $\mu$g of Tat as targets, we analyzed plasma samples collected before and after challenge for Tat-specific ADCC activity (Fig. 3*B*). Results showed high-titer ADCC activity for both the Tat/Env and multigenic groups 2 wk

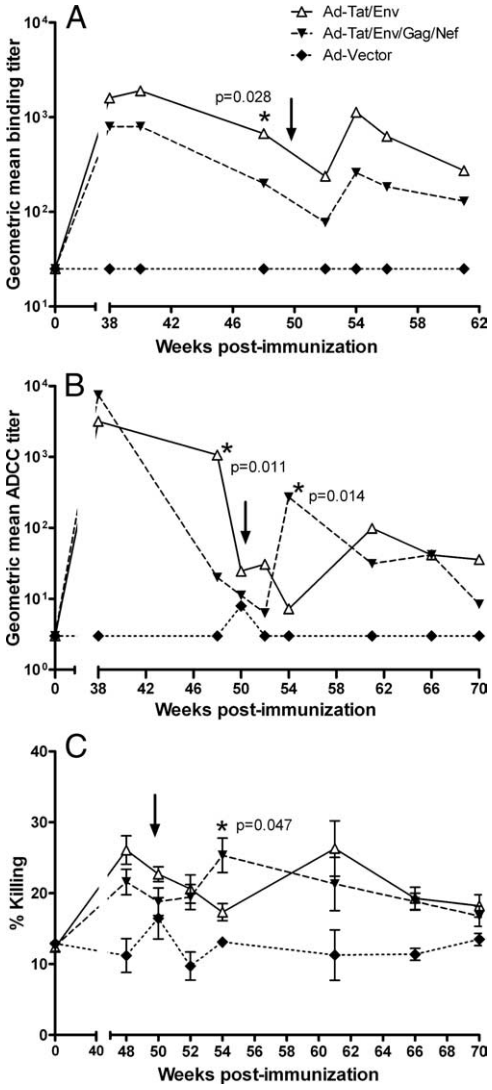


**FIGURE 3.** HIV Tat-specific Ab responses. *A*, Vaccine-induced binding Ab responses to HIV Tat before and after challenge (from Ref. 31). Arrows, time of i.v. $SHIV_{89.6P}$ challenge at wk 50. *B*, Titers of ADCC-mediating Abs in sequential plasma specimens from immunized and control macaques using HIV Tat-coated CEM-NKR cells as targets. *C*, Percent ADCC killing mediated by Abs in plasma of immunized and control macaques using HIV Tat-coated targets.

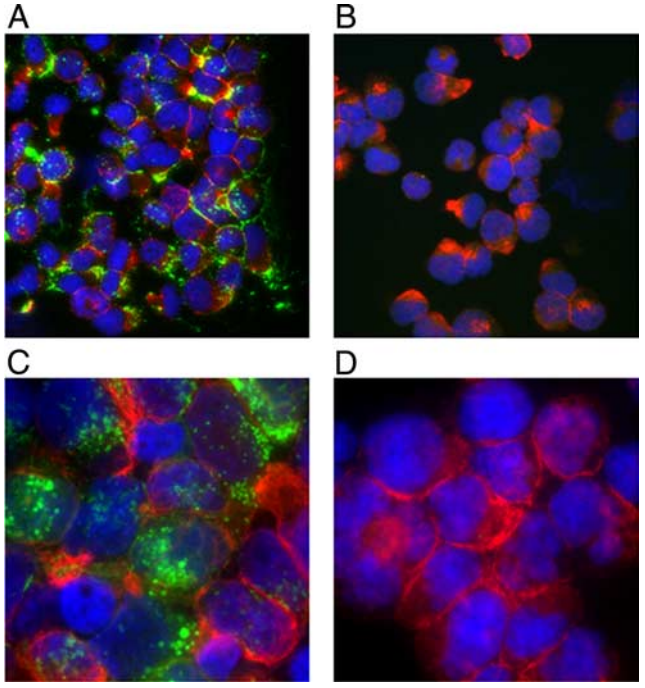


**FIGURE 4.** Indirect immunofluorescence staining of Tat-coated CEM-$NK^R$ and vaccinia-Nef-infected H9 cells. *A*, CEM-$NK^R$ cells coated with 10 $\mu$g of $HIV_{IIIB}$ purified oxidized Tat protein; *B*, CEM-$NK^R$ cells mock coated with R-10 medium. *A* and *B* are stained with 1D9 anti-Tat mAb. *C*, H9 cells infected with vNef157; *D*, mock infected H9 cells C and D are stained with 17.2 anti-Nef mAb. The presence of Tat or Nef on the surface of the cells is shown in green (FITC); actin is shown in red (Texas Red), and cell nuclei are shown in blue (4′,6′-diamidino-2-phenylindole).

after the second protein boost (wk 38). The titer was maintained for the Tat/Env group until wk 48, at which time a significant difference was observed compared with the multigenic group ($p = 0.011$) as well as to the controls ($p = 0.0061$). Both immunization groups exhibited low-level Tat-specific ADCC activity at the time of challenge. The Ad-multigenic group displayed a rebound in Tat-specific ADCC titer at wk 4 postchallenge (wk 54), significantly higher than the Tat/Env group ($p = 0.014$). Subsequent time points showed comparable ADCC titers between both immunization groups with no significant differences.

Similarly to the Env-specific ADCC activity, we also investigated whether differences existed in levels of cell lysis resulting from ADCC activity. As shown in Fig. 3*C*, overall Tat-specific percent killing was lower in comparison to Env-specific ADCC percent killing (see Fig. 2*D*). Further, no significant differences were seen between the immunization groups at any time point except wk 54 (4 wks postchallenge), where the modestly elevated percent killing by the multigenic group reached a marginal significant difference compared with the Tat/Env group ($p = 0.047$ after correction for multiplicity). This result coincided with the elevated ADCC titer exhibited by the multigenic group at the same time point (Fig. 3*B*).

### *Systemic Nef-specific ADCC*

HIV Nef has been reported to be partially expressed on the surface of HIV-infected cells and to serve as a target for ADCC (44–47). Therefore, we established a system to explore Nef-specific ADCC

Table I. *Dose-dependent surface expression of HIV Tat-coated and vaccinia-SIV Nef-infected cells*

| | Percentage of Positive Cells | | | | | | |
|---|---|---|---|---|---|---|---|
| Tat protein | 10 ng | 100 ng | 500 ng | 1 $\mu$g | 5 $\mu$g | 10 $\mu$g | Mock |
| Tat mAb 1D9 | 0–1 | 12–14 | 26–31 | 42–52 | 66–70 | 99–100 | Negative |
| Tat polyclonal serum | 0–1 | 10–14 | 32–36 | 47–58 | 59–62 | 100 | Negative |
| Vaccinia-Nef | 0.1 MOI | 0.5 MOI | 1.0 MOI | Mock | | | |
| Nef mAb 17.2 | 40–50 | 70–80 | 95–100 | Negative | | | |

activity. H9 cells were infected with vaccinia-Nef (vNef157) (39). From 18 to 24 h later, the cells that were 90–95% viable by trypan blue staining were assessed for surface expression of Nef by IFA. A dose effect dependent on the vaccinia-Nef MOI was observed (Table I). Representative IFA results showing staining with mAb 17.2 of H9 cells infected with vNef157 with an MOI of 1 and mock infected H9 cells are shown in Fig. 4, *C* and *D*. Subsequently, using 1 MOI of vaccinia-Nef to produce target cells, we analyzed the plasma of macaques from the Ad-multigenic group which showed Nef-specific binding Abs (31). All samples were negative for ADCC activity at a 1/10 dilution, the lowest dilution tested (data not shown). The Tat/Env group was not immunized with Nef and did not show detectable Nef-binding Abs as expected; therefore, plasma from these macaques were not included in the analysis.

### *Systemic ADCVI*

To explore possible effects of another nonneutralizing Ab function in the two immunization groups, we evaluated ADCVI activity in macaque plasma before and after challenge (Fig. 5*A*). A consistently higher ADCVI activity in the Tat/Env group compared with the multigenic group was seen at all time points analyzed. Nevertheless, only differences between the two groups at wk 61 and 70 approached statistical significance ($p$ values of 0.065 and 0.10, respectively). However, the Tat/Env immunization group exhibited a significant inverse correlation between ADCVI activity at the time of challenge (wk 50) and peak acute viremia which occurred at wk 2 postchallenge ($r = -0.74$; $p = 0.046$; Fig. 5*B*). A stronger inverse correlation was also observed for wk 50 ADCVI activity of the Tat/Env group and acute viremia 4 wk postchallenge ($r = -0.86$; $p = 0.011$; Fig. 5*C*). The inverse correlation between the Tat/Env group ADCVI activity at the time of challenge (wk 50) and acute plasma viremia did not persist at later time points (wk 58–70). No significant inverse correlation was observed between ADCVI activity and level of viremia in either the acute or the chronic phase of infection for the multigenic group.

### *Mucosal humoral immune responses*

Although in this study the $SHIV_{89.6P}$ challenge was administered i.v., we considered that vaccine-elicited mucosal Abs might have contributed to control of viral spread within the gastrointestinal tract. Therefore, we evaluated induction of mucosal Abs by the vaccination regimens. IgG-binding Abs were first assayed in rectal secretions as well as in vaginal secretions of the female macaques (Fig. 6). Induction of HIV gp140-specific IgG was initially detected in both the Tat/Env and multigenic groups at wk 26 after the first protein boost and was strongly enhanced by wk 38 after the second protein boost in both rectal and vaginal secretions (Fig. 6, *A* and *D*). Negligible levels were seen shortly before challenge (wk 48), but good anamnestic responses were observed 2 wk postchallenge (wk 52). Significant differences in Ab levels were not observed at any time point between the two immunization groups. Anti-envelope IgG Abs were not seen in secretions of control macaques.

The HIV Tat-specific IgG Abs exhibited the same pattern before challenge, although the IgG level detected in the vaginal secretions was higher than that seen in the rectal secretions of both immunized groups (Fig. 6, *B* and *E*). No difference in IgG level was observed between the vaccinated groups at all time points analyzed, and again, secretions of control macaques were Ab negative. In contrast to the strong envelope-specific anamnestic response observed in secretions of both immunization groups, only low-level increases in Tat Ab were detected postchallenge (wk 52).

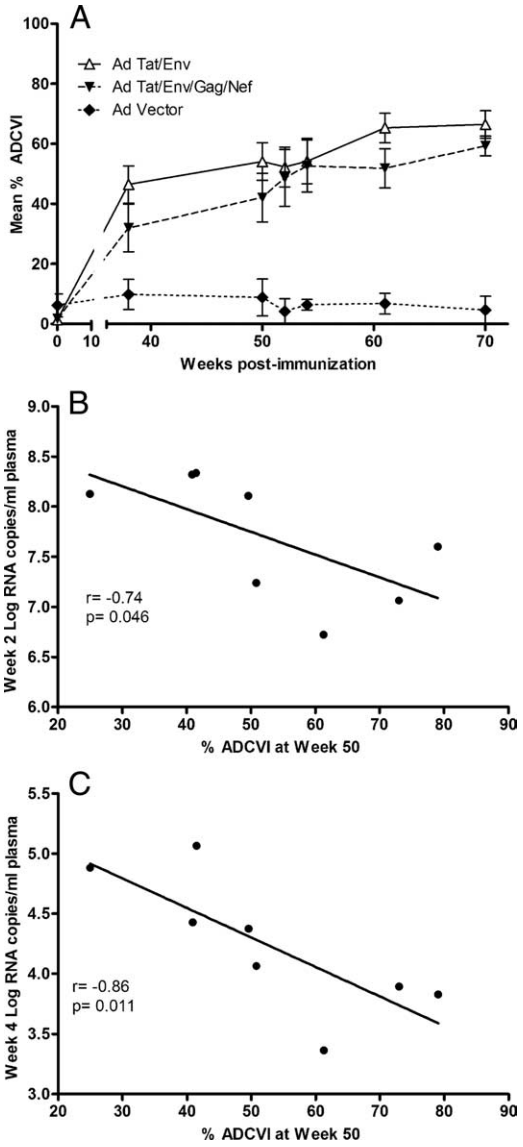


**FIGURE 5.** Evaluation of ADCVI activity in plasma of immunized and control macaques pre- and post-$HIV_{89.6P}$ challenge. *A*, Mean percent ADCVI ± SEM. *B*, Significant negative correlation between ADCVI activity at challenge (wk 50) and wk 2 viremia in the Ad-Tat/Env group. C, Significant negative correlation between ADCVI activity at challenge (wk 50) and wk 4 viremia in the Tat/Env group.

For completeness, we also evaluated mucosal secretions for Nef- and Gag-specific IgG Abs. As expected since only the multigenic group received Nef immunogens, SIV Nef-specific Abs of IgG isotype were observed only in rectal and vaginal secretions of the multigenic group (Fig. 6, *C* and *F*). As with the envelope-specific responses, a boost in specific Ab was observed at wk 38 after the second protein immunization. Further, a strong anamnestic Nef-specific response was observed postchallenge. The secretions of control macaques were negative. Neither the Tat/Env nor the multigenic group was boosted with Gag protein. Consequently, rectal and vaginal secretions of all macaques at the time points evaluated were negative for anti-Gag responses (data not shown).

The level of specific Abs of IgA isotype induced in rectal and vaginal secretions of all the macaques rarely reached the positive cutoff value of a 2-fold increase, and no significant differences

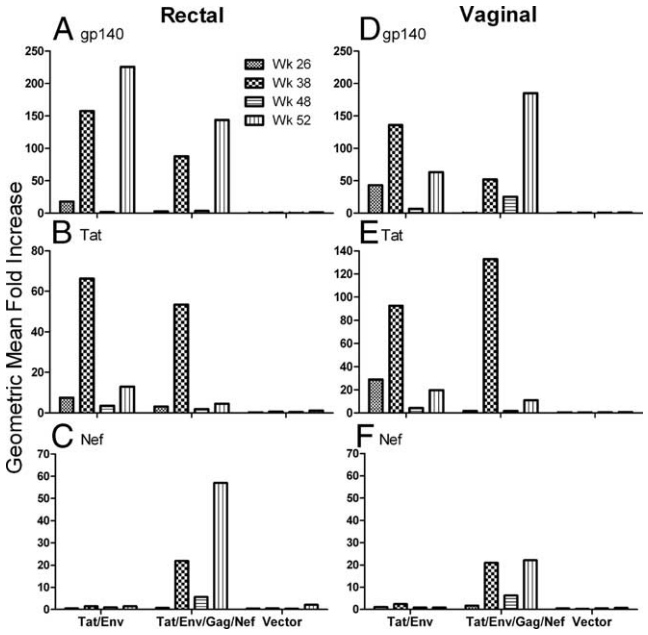


**FIGURE 6.** Induction of specific Abs of IgG isotype in immunized and control macaques in rectal and vaginal secretions at specified time points. Titers of each sample are normalized to total IgG present in the sample. Results are expressed as fold increases in Ab specific activity compared with the specific activity of a preimmunization sample from the same animal.

were observed between the Tat/Env and multigenic immunization groups (Fig. 7). The strongest Ab responses were seen postchallenge in the control group which exhibited Env-, Tat-, and Nef-specific IgA Abs.

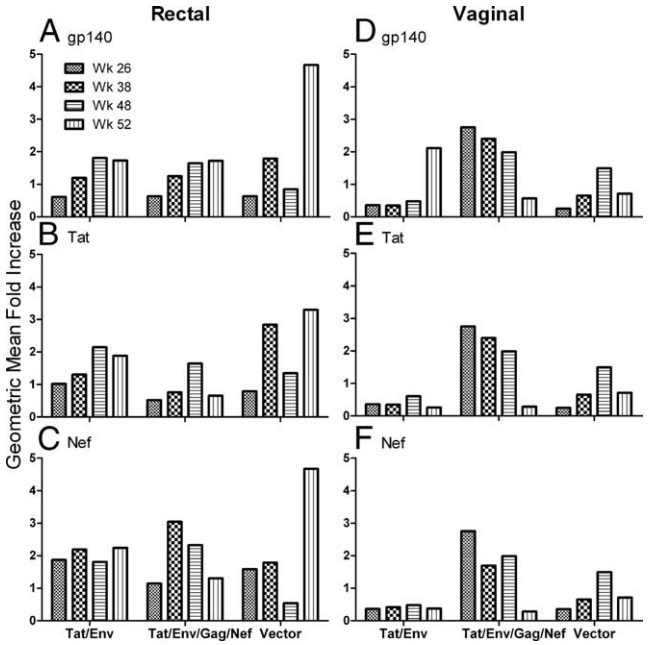


**FIGURE 7.** Induction of specific Abs of IgA isotype in immunized and control macaques in rectal and vaginal secretions at specified time points. Titers of each sample are normalized to total IgA present in the sample. Results are expressed as fold increases in Ab specific activity compared with the specific activity of a preimmunization sample from the same animal.

Overall, because significant differences between the Tat/Env and multigenic groups were not observed in either IgG or IgA mucosal binding Abs, further studies for functional activity were not conducted.

## Discussion

Abs play a pivotal role in protecting against viral infections and if present as a result of prior infection or vaccination act as a rapid first line of defense against invading pathogens. Neutralizing Abs can block viral entry, thus preventing infection, and can limit the spread of infection by blocking cell-to-cell viral transmission. In the macaques studied here, no neutralizing activity to $SHIV_{89.6P}$ was detected in sera of the immunized macaques before challenge (31). Further, the postchallenge neutralizing Abs that developed exhibited similar levels between immunization groups. Therefore, here we examined other nonneutralizing, functional Ab activities to elucidate the basis for the correlation of binding Ab with the significantly better protection exhibited by macaques in the Tat/Env group.

ADCC is a potent immune mechanism that bridges innate and adaptive immune responses. It can eliminate cells expressing viral Ags on the cell surface via Fc$\gamma$R-bearing effector cells, targeted by specific Ab bound to the Fc$\gamma$R. Abs mediating ADCC are one of the first immune responses to appear after HIV/SIV infection, often preceding neutralizing Ab (48, 49). In HIV-infected patients and SIV-infected rhesus macaques, ADCC activity has been associated with reduced viremia, slow disease progression, and better clinical outcome (42, 50–54). Among patients with progressive or late-stage disease, declining or low ADCC titer has been associated with low CD4 counts (55–58). A protective role for ADCC activity has not been established, however. Some HIV patients have not shown any correlation between ADCC activity and disease progression (59, 60). Further, passive transfer of nonneutralizing, ADCC-mediating IgG to neonatal rhesus macaques did not protect against $SIV_{mac251}$ oral challenge, although several variables might have affected the outcome (61).

Here, envelope-specific ADCC titers exhibited by the Tat/Env group were consistently higher compared with the multigenic group at all time points evaluated using gp140-coated targets. Moreover, the ADCC titer was significantly elevated at wk 11 postchallenge ($p < 0.005$). ADCC titers against heterologous $HIV\text{-}1_{IIIB}$-infected target cells were also higher compared with the multigenic group at all postchallenge time points evaluated. Importantly, when ADCC activity was evaluated based on level of cell lysis rather than titer, the Tat/Env group exhibited significantly greater killing just before challenge ($p = 0.0034$) and over wk 4–11 postchallenge ($p < 0.00001$) compared with the multigenic group (Fig. 2*D*). Furthermore, the elevated killing level of the Tat/Env group was maintained longer postchallenge compared with the multigenic group ($p = 0.0002$). These results are consistent with the higher Env-specific binding Ab titers induced in the Tat/Env macaques before and post challenge (Fig. 2*A*). Furthermore, they suggest a mechanistic basis for the significantly better chronic-phase protection (Fig. 1*B*) displayed by the Tat/Env-immunized macaques.

To date, HIV Ags reported to serve as targets for ADCC include the transmembrane (gp41) and extracellular (gp120) envelope proteins (62, 63) and Nef (44–47). Here, Nef-specific Abs did not mediate killing of target cells infected with vaccinia-Nef. It may be that the Nef epitope expressed on the surface of HIV-infected cells is not present on the surface of cells infected with vaccinia-Nef. Nef was not expressed on the surface of a sufficiently high percentage of SIV-infected cells to allow their use in the RFADCC assay.

Tat has also been reported to be expressed on the cell surface (43). Furthermore, Tat Abs have been associated with control of viral replication and slow progression to AIDS (22, 24, 26, 28, 64, 65), although others have reported no correlation between Tat Abs and disease progression (66). Here we investigated whether anti-Tat Abs induced in the immunized macaques could mediate killing of Tat-expressing target cells by the ADCC mechanism. Our results show clearly for the first time that Tat serves as a target for ADCC and that vaccine-induced Tat-specific Abs in the macaque plasma mediate ADCC killing. The Tat/Env group showed higher Tat-specific ADCC activity before challenge (wk 48, $p = 0.011$) compared with the multigenic group, but this was not maintained postchallenge and thus likely did not contribute to better chronic phase protection of the Tat/Env group. The higher Tat-specific ADCC anamnestic response at wk 54 of macaques in the multigenic group compared with Tat/Env group ($p = 0.014$) was not maintained at later time points. Based on the timing of this response, it did not seem to influence differences in acute- or chronic-phase viremia between the two immunization groups.

Whether Tat-mediated ADCC activity impacts natural infection is not known. Although the low level of Tat cell surface expression on infected cells is not sufficient for detection of ADCC activity in the RFADCC assay in vitro, they might nevertheless serve as in vivo targets. Furthermore, in natural infection, the soluble Tat released from infected cells that binds to neighboring infected and uninfected cells may facilitate Tat-specific ADCC-mediated bystander killing. This potentially deleterious effect of anti-Tat Abs could be prevented if high-titer vaccine induced anti-Tat Abs were present before infection and able to eliminate newly infected, Tat-expressing cells. Alternatively, the anti-Tat Abs might simply bind the released Tat and clear it, preventing its absorption to bystander cells. These possibilities should be further explored.

ADCVI, like ADCC, depends on interactions between Ab and Fc$\gamma$R-bearing effector cells. However, the readout is not target cell lysis but inhibition of viral replication, mediated not only by ADCC but also by soluble antiviral factors secreted by activated NK cells (40, 67) and other cell types, or by Fc$\gamma$R-mediated phagocytosis (68). ADCVI activity has been associated with reduction in viremia during acute HIV infection, a reduced rate of HIV infection, and protection in neonatal rhesus macaques passively infused with nonneutralizing Ab (40, 69, 70). Here, Ab titers mediating ADCVI activity of the Tat/Env group were consistently higher, although not significantly so, than those of the multigenic group at all time points analyzed pre- and postchallenge. ADCVI activity of the Tat/Env group at the time of challenge (wk 50) was inversely correlated with acute viremia at wk 2 ($r = -0.74$; $p = 0.046$) and 4 ($r = -0.86$; $p = 0.011$) postchallenge (Fig. 5, *B* and *C*), but not at later time points. Although this ADCVI activity was not directly associated with the stronger protection seen in the Tat/Env group during chronic infection, its impact on the initial viral burden perhaps facilitated chronic phase viremia control by other mechanisms. Overall, correlations of viral loads with ADCC killing were weaker than with ADCVI (not shown). This may reflect the several mechanisms, including ADCC, which can contribute to ADCVI activity, perhaps providing a greater impact on acute viremia levels. The significant differences in both percent ADCC killing and viral loads between the two immunization groups were seen during the chronic phase of infection when additional immune responses such as viral-specific CD8 T cell activity likely impacted viremia control. Under this circumstance, a direct correlation between viral loads and ADCC killing would be difficult to discern, despite the significantly higher, sustained ADCC activity in the Tat/Env group.

Taken together, our data indicate that nonneutralizing Ab activities had a significant impact on the challenge outcome, resulting in better chronic phase protection of the macaques immunized by the Tat/Env regimen. Anti-envelope Ab played a direct role by mediating Env-specific ADCC which exhibited sustained, higher titered activity during the chronic phase in the Tat/Env-immunized animals. ADCVI was also enhanced in the Tat/Env immunization group and correlated significantly with better acute phase protection. However, it cannot be attributed to any particular Ag, although Env is certainly involved. These results are consistent with the overall higher Ab titers elicited in the Tat/Env-immunized macaques. What remains to be determined is the mechanism leading to improved Ab induction in this group of macaques. Tat is known to enhance and broaden cellular immune responses to coadministered Ags including Gag and Env (33, 71). Whether it similarly enhances Ab induction is unknown. Here, as both immunization groups included Tat, perhaps competition in the multigenic group diminished overall Ab responses. Whether Ab avidity was somehow increased in the Tat/Env group as compared with the multigenic group is not known. Because this parameter could have affected ADCC and ADCVI activities, it should be explored in the future. It will be important to investigate all such possibilities, to make the best use of the Tat immunogen in vaccine strategies.

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## Disclosures

The authors have no financial conflict of interest.

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