Impact of antibody quality and anamnestic response on viremia control post-challenge in a combined Tat/Env vaccine regimen in rhesus macaques

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ABSTRACT

Previously, priming rhesus macaques with Adenovirus type 5 host range mutant-recombinants encoding Tat and Env and boosting with Tat and Env protein in MPL-SE controlled chronic viremia by 4 logs following homologous intravenous $SHIV_{89.6P}$ challenge. Here we evaluated Tat, Env, and Tat/Env regimens for immunogenicity and protective efficacy using clade C Env, alum adjuvant, and a heterologous intrarectal $SHIV_{1157ipd3N4}$ challenge. Despite induction of strong cellular and humoral immunity, Tat/Env group T and B-cell memory responses were not significantly enhanced over Tat- or Env-only groups. Lack of viremia control post-challenge was attributed to lower avidity Env antibodies and no anamnestic ADCC response or $SHIV_{1157ipd3N4}$ neutralizing antibody development post-challenge. Poor biologic activity of the Tat immunogen may have impaired Tat immunity. In the absence of sterilizing immunity, strong anamnestic responses to heterologous virus can help control viremia. Both antibody breadth and optimal adjuvanticity are needed to elicit high-quality antibody for protective efficacy.

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Introduction

Despite the success of highly active antiretroviral therapy (HAART) which can suppress HIV replication to undetectable levels and has shown 96% efficacy in preventing viral transmission (Cohen et al., 2011), an effective vaccine is still needed to fight the global HIV pandemic. To date, the most effective vaccine has been live attenuated virus (Koff et al., 2006), however, the possible reversion of the virus in vivo to a pathogenic form (Hofmann-Lehmann et al., 2003) has limited the use of this approach. Nevertheless, individually, all HIV structural, accessory and regulatory proteins may prove useful in vaccine design. Some studies have shown that co-administration of several HIV immunogens can lead to inhibition of immune responses against one or more of the co-administered immunogens (Toapanta et al., 2007), although other studies have shown positive modulatory

effects (Patterson et al., 2003). Overall, the selection and combination of HIV immunogens is critical for a successful HIV vaccine.

HIV structural proteins represent the main components in most HIV vaccines. Env, Gag and Pol elicit both cellular and humoral immunity, although in vaccine design Gag and Pol are intended to elicit mainly cellular immunity, while Env is included primarily to elicit antibodies, the key correlate of most successful vaccines. Accessory/regulatory proteins, such as Tat, Rev, and Nef, have also been incorporated into vaccines, primarily in combination strategies. Tat, in particular, has been a key vaccine target in numerous approaches due to its early expression in the viral life cycle and its indispensible contribution as a transactivator protein to viral infectivity and pathogenesis. Tat-specific immune responses elicited by prophylactic vaccines might therefore help prevent viral transmission and/or replication. As a vaccine immunogen, Tat has been shown to enhance cellular immune responses to co-administered antigens, and to elicit anti-Tat antibody in the absence of adjuvant. Tat-specific humoral and cellular immune responses have been associated with disease control in HIV-infected people, and SIV Tat-specific cellular immune responses have been associated with control of acute SIV

infection in rhesus macaques. These and other properties of Tat have been recently reviewed by Caputo, et al. (2009).

Previously we reported that strong protection, evidenced by a 4-log reduction in viremia in the chronic phase of infection, was elicited by combined immunization with Adenovirus type 5 host range mutant (Ad5hr)-recombinants expressing Tat and Env followed by boosting with Tat protein in alum and Env protein in monophosphoryl lipid A-stable emulsion (MPL-SE), using a homologous SHIV$_{89.6P}$ challenge model. In contrast, immunization with an Ad5hr-HIV*tat* prime/Tat protein boosting regimen conferred no protection at all (Demberg et al., 2007). In comparison with a multigenic regimen (incorporating Env, Gag, Nef and Tat immunogens) which reduced chronic viremia only 3 logs, the better chronic phase protection resulting from the Tat/Env regimen was associated with higher binding titers to Tat and Env and better antibody-dependent cellular cytotoxicity (ADCC) mediating antibodies (Florese et al., 2009). This result is in agreement with several other studies in non-human primate models of SIV and SHIV infection, in which vaccine-elicited high avidity antibodies mediating ADCC as well as antibody-dependent cell-mediated viral inhibition (ADCVI) are correlated with partial protection and control of viremia (Gomez-Roman et al., 2005; Hidajat et al., 2009; Xiao et al., 2010). Moreover, it

is believed that the ∼30% protection achieved in the recent clinical vaccine trial in Thailand (RV144) (Rerks-Ngarm et al., 2009) was conferred at least in part by ADCC- mediating antibodies. Ninety-nine percent of vaccinees exhibited binding antibodies to gp120 and 2/3 of them had detectable ADCC titers to gp120-coated target cells (Haynes et al., 2011).

The design of our previous Tat/Env study lacked an envelope only vaccine group, so we could not distinguish the contribution to protective efficacy of Env versus Tat. Moreover, the dual tropic SHIV$_{89.6P}$ challenge was homologous to the immunogens. Here we have addressed these issues, and report our findings from a study comparing immunogenicity and protective efficacy of a Tat plus Env immunization regimen to Tat only and Env only regimens followed by a heterologous R5 tropic SHIV$_{1157ipd3N4}$ challenge.

Results

Cytokines/chemokines induced by Ad-recombinant vaccination

To determine if Tat expressed by Ad5hr-HIV*tat* could potentially modulate immune responses, we examined induction by
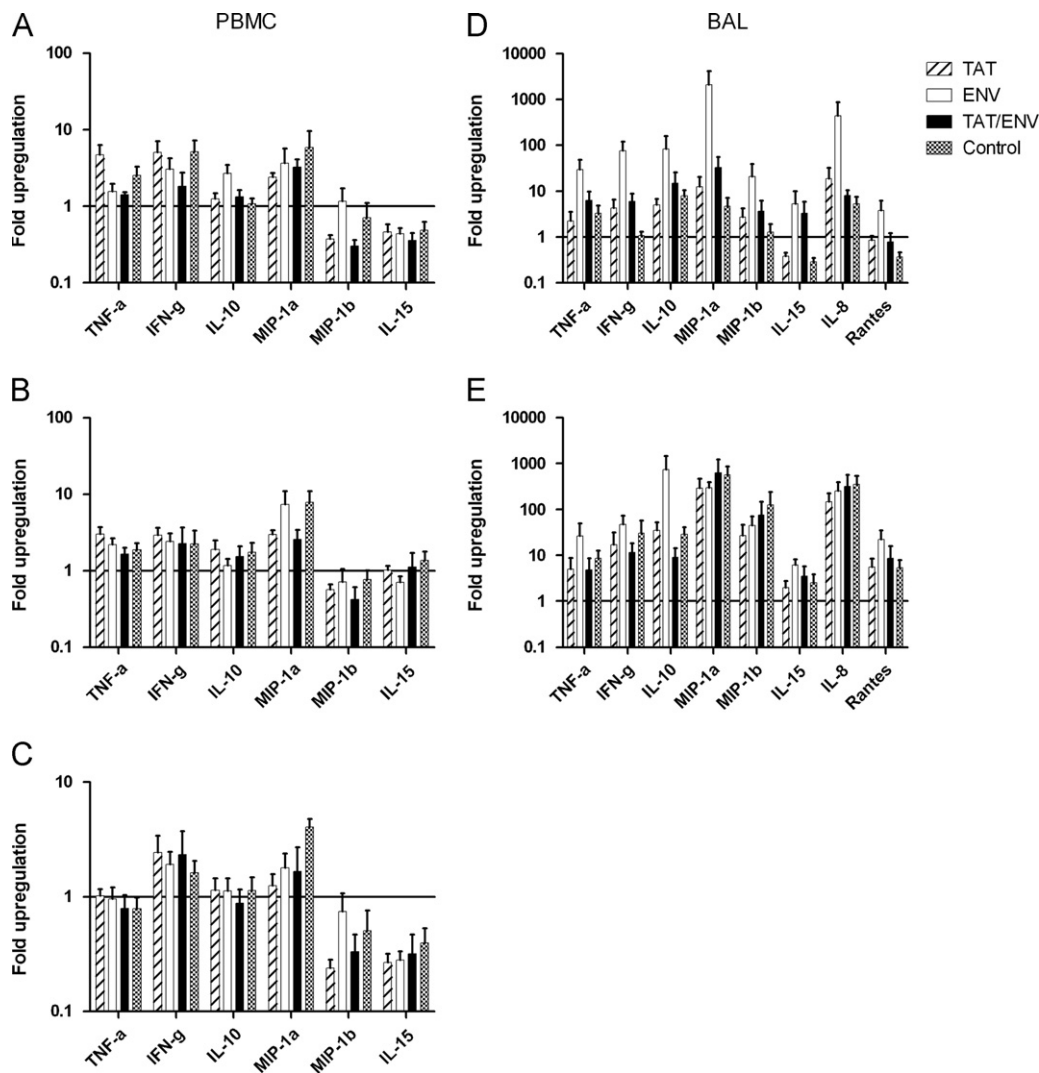
Fig. 1. Real time PCR evaluation of cytokine and chemokine responses after Ad priming in PBMC and BAL. Induction of cytokine and chemokine responses in PBMC 2 weeks after the first Adenovirus prime (A) and 3 (B) and 8 (C) days after the second Adenovirus immunization. Cytokine and chemokine responses in BAL 2 weeks after the first (D) and second (E) Adenovirus immunizations. Results are shown as the fold up-regulation in response relative to pre-immunization values. Mean responses ± the standard error of the mean (sem) are shown.

Ad-recombinant priming of cytokines and chemokines, representative of both innate and adaptive acute immune responses, in PBMC and bronchoalveolar lavage (BAL) cells. In PBMC, 2 weeks after the first Ad immunization, only MIP-1α was consistently up-regulated more than 2-fold in all groups including the control group which received Ad empty vector (Fig. 1A). MIP-1β and IL-15 were not up-regulated in any group. Up-regulation of the remaining cytokines/chemokines across the 4 groups was sporadic. Three days following the second Ad immunization, only IFN-γ and MIP-1α were consistently up-regulated more than 2-fold in all groups (Fig. 1B). By 8 days after the second Ad, only IFN-γ exhibited greater than 2-fold up-regulation in all but the controls (Fig. 1C).

BAL cells were examined as representative of a mucosal effector site. Two weeks after the first Ad immunization (intranasal) they showed cytokine/chemokine levels higher than those observed in PBMC after the first Ad prime (Fig. 1D). TNF-α, IL-10, MIP-1α and IL-8 were consistently up-regulated more than 2-fold in all 4 macaque groups, as were IFN-γ and MIP-1β in all but the controls. Rantes was only up-regulated in the Env immunization group. As with the PBMC, significant differences between responses by the Ad-recombinants compared to the Ad empty vector were not obtained.

Two weeks after the second Ad immunization (intratracheal) we observed stronger responses in the lung compared to responses following the first Ad administration (Fig. 1E), likely reflecting improved targeting of Ad-recombinants to the upper respiratory tract. All cytokines/chemokines measured were up-regulated more than 2-fold. Overall, data in both PBMC and BAL showed no evidence of modulation of cytokine/chemokine responses by the inserted genes in the Ad recombinants. Rather, results obtained were in response to the vector itself.

Cellular responses

In the previous Tat/Env study, vaccine-elicited cellular immune responses were not associated with the potent protection in the Tat/Env immunization group. Similarly, as summarized here, cellular immunity was not significantly enhanced in the Tat/Env group as assessed by IFN-γ ELISpot and CSFE-proliferation assays (Suppl. Fig. 1A–D). Central memory (CM; $CD28^+/CD95^+$), transitional effector memory (TEM; $CD28^+CD95^+CCR7^-$), and effector memory (EM; $CD28^-/CD95^+$) T cell subsets in PBMC were evaluated by intracellular cytokine staining following the second protein boost. In general, $CD4^+$ T cells secreted higher levels of cytokines compared to $CD8^+$ T cells (Suppl. Fig. 2A and B). Most of the CM Env- and Tat-specific responses occurred in TEM subpopulations (Suppl. Fig. 2C–F). Evaluation of polyfunctionality of peripheral blood CM, TEM, and EM cells showed the levels of both Env- and Tat-specific responses were greatest in the Tat/Env group, although Tat responses were much lower in magnitude and rarely detected among $CD4^+$ and $CD8^+$ TEM cells (Suppl. Fig. 3A–F). $CD4^+$ Env-specific triple positive cells were progressively elevated in the Tat/Env group as the cells matured from CM to TEM to EM (Suppl. Fig. 3G). Overall, however, enhanced levels and polyfunctionality of $CD4^+$ EM cells in the Tat/Env group did not reach statistical significance. In contrast to Env-specific $CD4^+$ memory cells, $CD8^+$ memory cells in PBMC contained very few triple positive cells, while Tat-specific polyfunctional cells were at the background level among $CD8^+$ EM and CM cells (data not shown).

We also analyzed total memory (TM) cells in BAL for cytokine secretion. Following the second protein boost, secretion of IFN-γ and TNF-α was greatest in both $CD4^+$ and $CD8^+$ populations of Tat/Env-immunized macaques compared to the Env-only group.
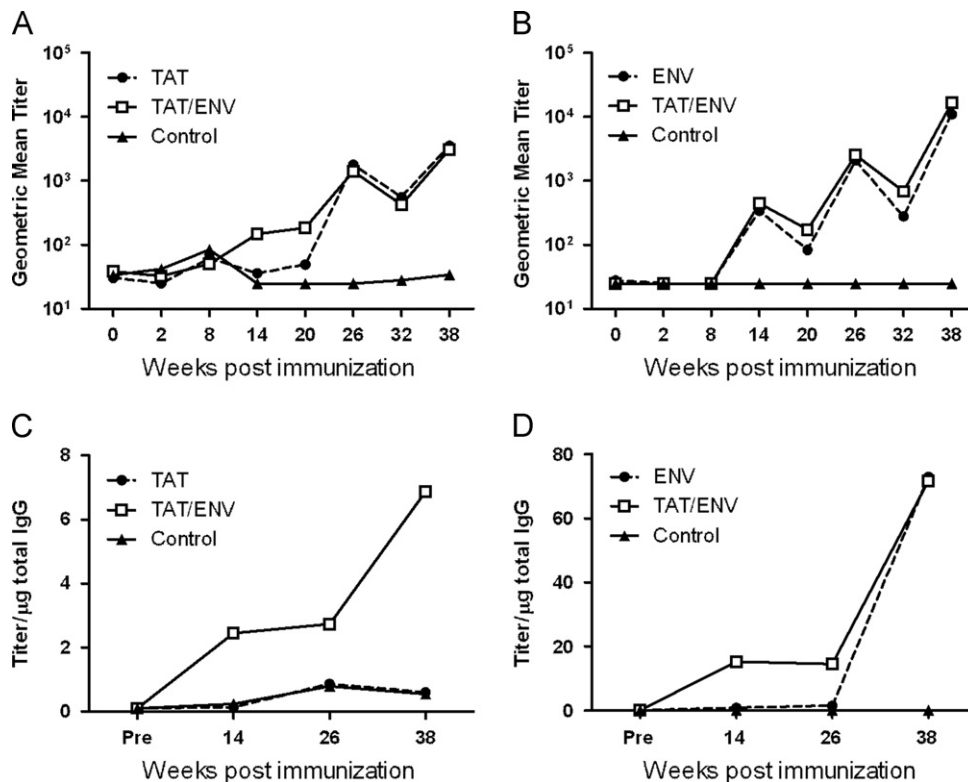
Fig. 2. **Pre-challenge humoral immune responses.** Geometric mean (A) Tat- and (B) Env-(HIV_{CZM} gp120)-specific binding antibody titers, respectively, are shown. (C) Tat- and (D) Env-specific binding antibodies, respectively, in rectal secretions.

Tat-specific cytokine secreting TM cells were seen at very low levels (Suppl. Fig. 4A). BAL Env-specific CD4$^+$ TM cells in the Tat/Env group exhibited a greater proportion of double and triple positive cells compared to those in the Env group (Suppl. Fig. 4B and D), although this did not reach statistical significance. In BAL CD8$^+$ TM cells, both the Env and Tat/Env groups were composed of mainly single-positive IFN-γ-secreting cells (Suppl. Fig. 4C).

Systemic binding antibody

As previous protection in the Tat/Env group was associated with humoral immune responses, here we carefully examined a number of antibody parameters. Serum binding antibody titers to Tat and Env proteins were first assessed. As purified SHIV$_{1157ipd3N4}$ Env was not available, we used HIV$_{CZM}$ Env, heterologous to both the challenge virus and the HIV$_{TV-1}$ immunogen, as well as HIV$_{TV-1}$ gp140. Tat antibody titers were in general low, moderately boosted after the first protein immunization and minimally boosted after the second (Fig. 2A). Peak titers did not differ between the Tat and Tat/Env groups. Good induction of antibody responses to HIV$_{CZM}$ gp120 were observed in both the Env only and Tat/Env groups after the second Ad-recombinant prime at week 14 (Fig. 2B), matching the appearance of ELISpot and CD4 proliferative responses at the same time point (Suppl. Fig. 1) and showing the effectiveness of Ad-recombinant priming. Anti-Env titers in both groups were further boosted by the protein vaccinations. Responses in both groups peaked after the second protein boost (week 38) with no significant difference in geometric mean titers of the Env (10910) and Tat/Env (16510) groups. As expected both the Env and Tat/Env groups exhibited higher geometric mean antibody titers (approximately 6-fold) to the TV-1 gp140 compared to the CZM gp120 at the week 38 time point: 74,700 for the Env only group and 105400 for the Tat/Env group (data not shown). However, this difference was not statistically significant.

We also assessed binding titers of IgG and IgA antibodies in rectal secretions to HIV Tat and HIV$_{CZM}$ gp120. Rectal IgG antibodies to Tat were initially detected after the second Ad-recombinant immunization only in the Tat/Env group and were boosted by the protein immunizations (Fig. 2C). Titers in the Tat-only group remained at control levels. Similarly, Env-specific rectal IgG antibodies developed in the Tat/Env group (Fig. 2D), but were increased in both the Env and Tat/Env groups to similar levels by the protein boosts. Env- and Tat-specific IgA antibodies in rectal secretions appeared sporadically. Geometric mean antibody titers remained close to control levels across the entire immunization regimen (data not shown).

Antibody dependent cellular cytotoxicity

ADCC activity was shown to be consistently higher in the original Tat/Env group in comparison to a multigenic group (Florese et al., 2009). We evaluated it here using HIV$_{CZM}$ gp120-coated target cells. Two weeks after the second protein immunization, the geometric mean antibody titer mediating ADCC was 520 for the Env only group and 2680 for the Tat/Env group (data not shown). As with the binding titers, this difference was not statistically significant.

Viral challenge

Fifty weeks after initial immunization, the macaques were challenged intrarectally with SHIV$_{1157ipd3N4}$. All animals became infected (Fig. 3A). No differences in peak or chronic viremia were observed between groups. The Tat/Env group showed somewhat lower viral loads than the other 3 groups during the first 10 weeks
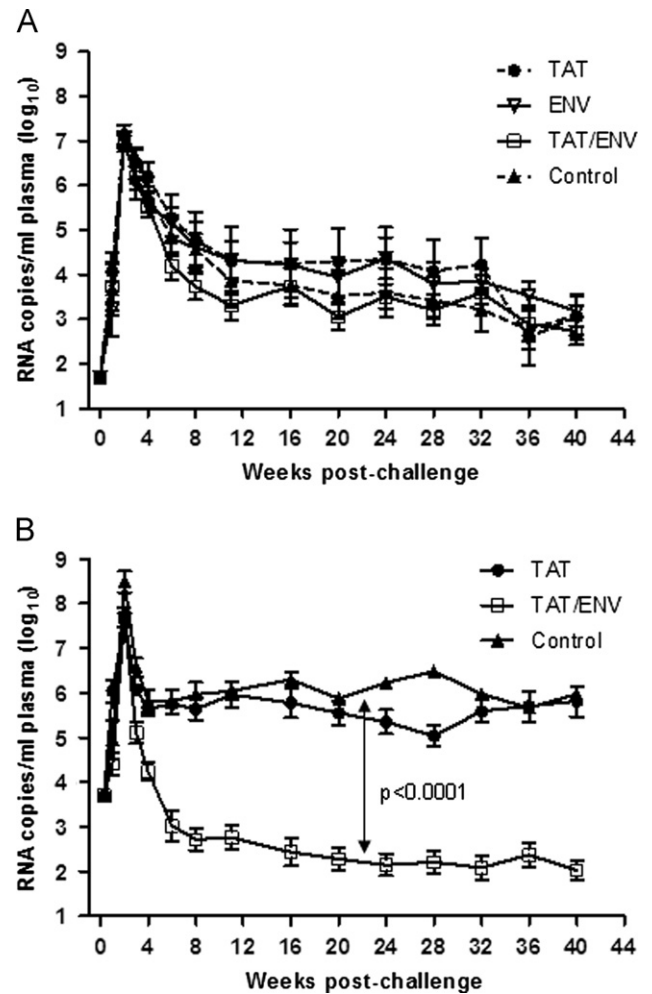

Fig. 3. Challenge outcome of the current (A) and previous (B) Tat/Env studies. Viral loads following challenge with SHIV$_{1157ipd3N4}$ (A) and SHIV$_{89.6P}$ (B) are expressed as geometric means ± sem. Data in panel B are taken from Demberg et al., 2007.

post-infection, but this trend did not reach significance. Overall there was no difference in CD4 T-cell counts between groups over the post-challenge monitoring period. All but the Tat group maintained approximately 52–83% of their initial CD4 T-cell levels while the Tat group tended to show somewhat increased numbers (data not shown). In keeping with the preservation of CD4 T cells, none of the animals progressed to AIDS.

The results of the heterologous challenge conducted here contrasted greatly with our previous Tat/Env study in which macaques received a homologous SHIV$_{89.6P}$ challenge (Demberg et al., 2007; Fig. 7B). In that first study, the Tat only group exhibited no reduction in chronic viremia, as seen here, while the Tat/Env group exhibited a strong, significant 4-log reduction in chronic viremia. In subsequent experiments we attempted to elucidate differences in vaccine-elicited immune responses that led to the strikingly different challenge outcomes.

Role of memory cells

As both CD4 and CD8 memory T cells were elicited by the current vaccine regimen, we initially assessed post-challenge Env and Tat-specific responses in PBMC. No evidence of either a Tat- or Env-specific cellular anamnestic response was observed (Fig. 4). Responses against both antigens declined continually from levels observed 4 weeks prior to challenge. The Env ELISpot
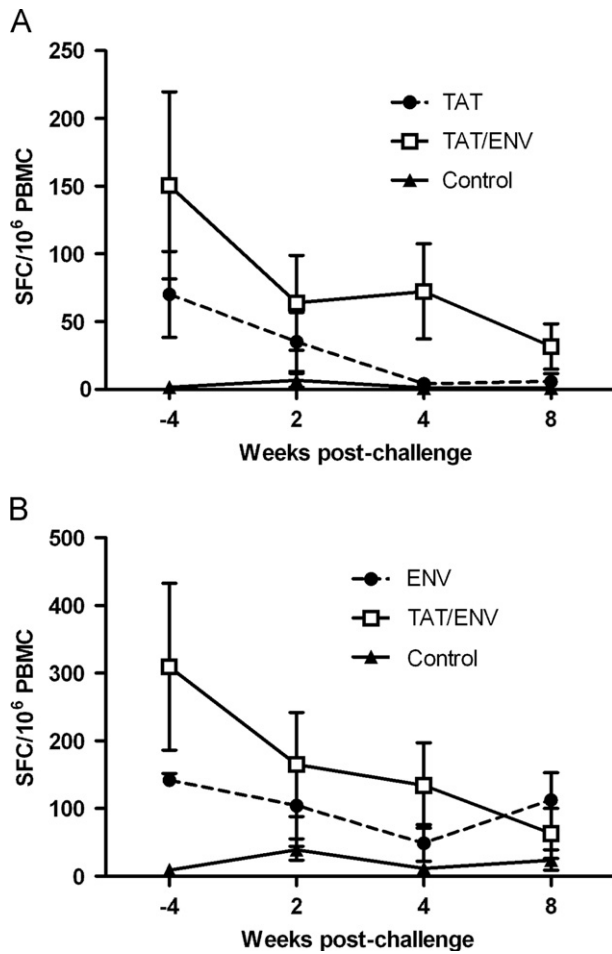
Fig. 4. Post-challenge ELISpot responses. Mean (A) Tat- and (B) Env-(HIV$_{TV-1}$) specific SFC $\pm$ sem are shown.

responses were assayed using HIV$_{TV-1}$ peptides, heterologous to the Env of the SHIV$_{1157ipd3N4}$ challenge virus, so the result might reflect in part envelope variability. However, an anamnestic response was not seen for Tat assayed using HIV clade B consensus peptides, closely related to the HXB2 Tat component of the challenge virus. Thus, although memory T cells were elicited by the vaccine regimen, no recall in cellular response to the challenge exposure was observed.

As the protection elicited by the previous Tat/Env vaccine regimen was associated with humoral immunity (Florese et al., 2009), we next assessed memory B cells in bone marrow of the immunized macaques. Env-and Tat-specific IgG memory B cells were most evident in the Tat/Env group in fresh cells assayed 2 weeks after the second protein boost (wk 38; Fig. 5A and B). Two weeks post-challenge (wk 52) Env-Specific antibody secreting cells (ASC) also appeared in the Env group. After 3 days of stimulation, both Env and Tat-specific ASC were observed in their respective immunization groups. A clear anamnestic response at 2 weeks post-challenge (wk 52) in Env-specific ASC was seen in both the stimulated Env and Tat/Env groups (Fig. 5A), but such a response for Tat was lacking (Fig. 5B). Env-specific IgA memory B cells were also observed, with anamnestic responses exhibited in both the Env and Tat/Env groups (Fig. 5C).

Post-challenge humoral immune responses

As memory B cells were recalled after the SHIV$_{1157ipd3N4}$ exposure, we next investigated several anti-Env responses
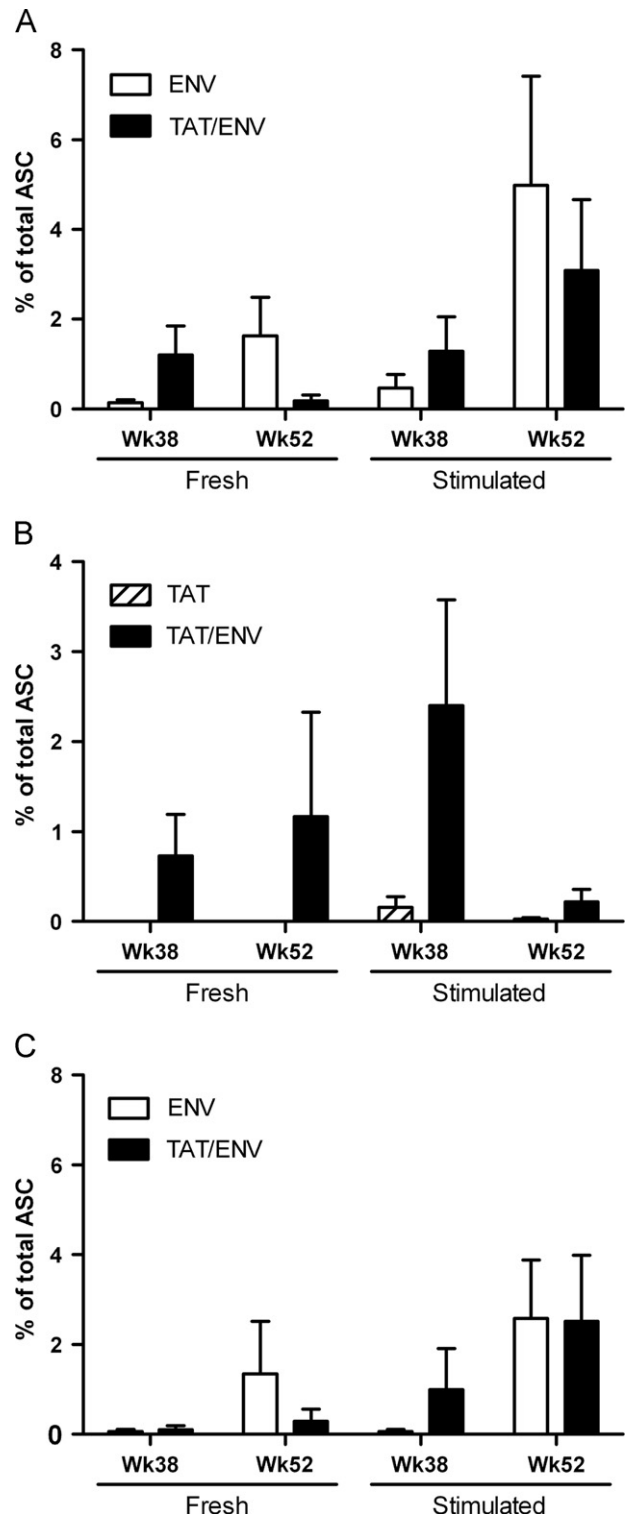
Fig. 5. Memory B cells pre and post-challenge. (A) Env (HIV$_{CZM}$ gp120)- and (B) Tat-specific IgG memory B cells, and (C) Env-Specific IgA memory B cells 2 weeks after the second protein immunization (wk 38) and 2 weeks post-challenge (wk 52). Results are presented for fresh cells and for cells after 3 days of stimulation as mean percent of total IgG or IgA ASC as appropriate, $\pm$ sem.

pre- and post-challenge. Anti-Env binding antibodies in serum exhibited an anamnestic response in both the Env and Tat/Env groups, with similar increases in titer by 4 weeks post-challenge (Fig. 6A). Serum anti-Tat binding antibodies were not assessed. Anamnestic antibody responses were also observed in rectal secretions for Env-specific IgG responses in both the Env and
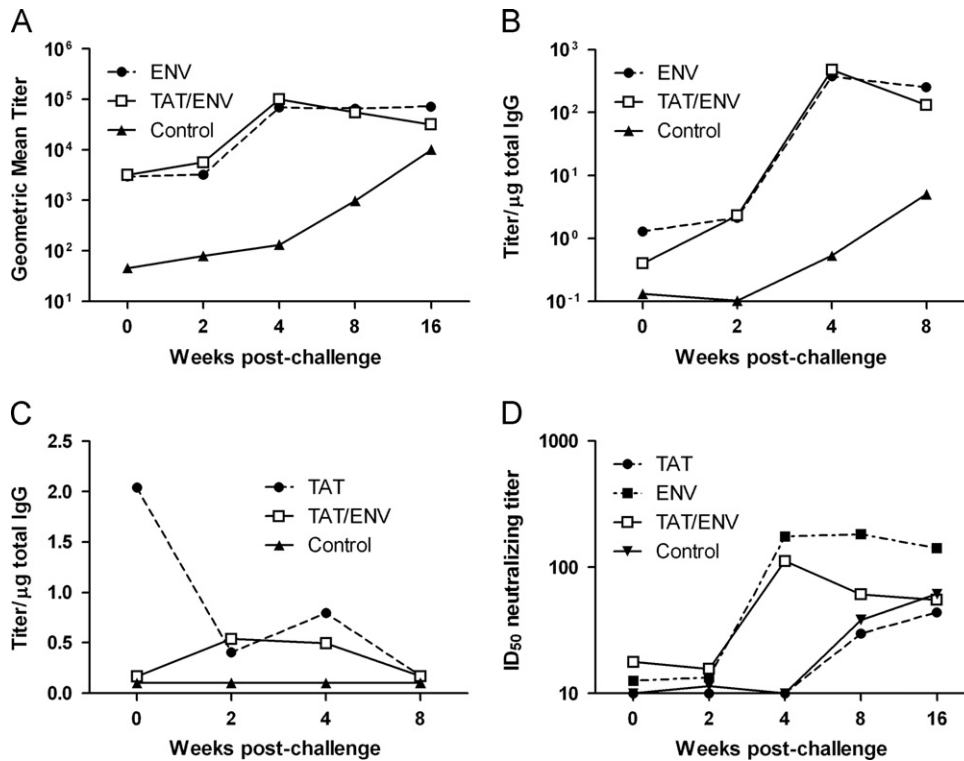
Fig. 6. Post-challenge humoral immune responses. (A) Env (HIV$_{CZM}$ gp120)-specific binding antibody. (B) Env- and (C) Tat-specific IgG antibody in rectal secretions. (D) Neutralization of SHIV$_{1157ipdEL-p}$. Geometric mean titers are plotted.
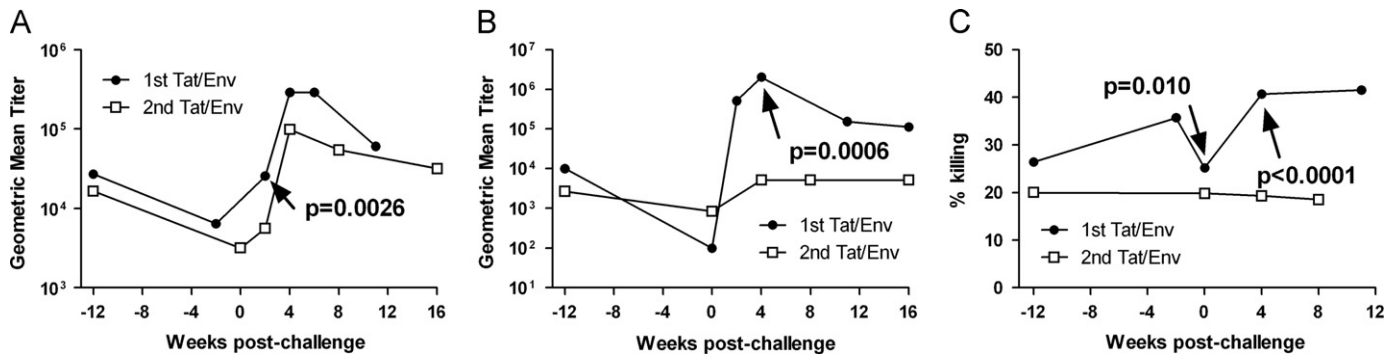
Fig. 7. Comparison of humoral immune responses between the first and second Tat/Env studies. (A) Serum binding antibody titer in the Tat/Env groups of the first and second studies. (B) ADCC titer and (C) % target cell killing in the Tat/Env groups of the first and second studies. Data for the first Tat/Env study were taken from Demberg et al., 2007.

Tat/Env groups (Fig. 6B), but not for Tat-specific responses in the Tat and Tat/Env groups (Fig. 6C).

Post-challenge neutralization titers were assessed against HIV$_{TV1.21}$, SHIV$_{1157ipd3N4}$ and the tier 1 virus, SHIV$_{1157ipEL-p}$ (Siddappa et al., 2010), on TZM-bl cells. Significant neutralizing titers developed only against SHIV$_{1157ipEL-p}$ (Fig. 6D), while titers against the other two viruses remained below 50 or at control levels. Both the Env and Tat/Env groups exhibited a more rapid appearance of neutralizing antibody compared to either the Tat or control groups as expected.

Comparative antibody responses between the two Tat/Env protocols

A direct comparison between antibody responses in the Tat/Env groups of the previous and current protocol revealed significant differences. Macaques in the first study exhibited a more rapid anamnestic binding antibody response compared to macaques in the second study (Fig. 7A). Prior to challenge, the geometric mean antibody titers of the first and second Tat/Env groups were similar: 12 weeks prior to challenge the macaques of the first study exhibited titers only 1.6-fold higher than those of the second, and at 2 (study 1) and 0 (study 2) weeks before challenge, titers were only 2-fold higher in the first Tat/Env group. However, by week 2 post-challenge, macaques in the Tat/Env group of the first study exhibited a 6.6-fold increase in titer over the pre-challenge level, whereas macaques in the second study exhibited an increase of only 2.6-fold. At this time point, titers of the first study were significantly elevated compared to titers of the second study (geometric mean titers of 25,600 and 5603, respectively, $p=0.0026$).

The anamnestic ADCC response of the first Tat/Env group was also much more striking than that of the second, with titers rising 3 logs by 2 weeks and 4 logs by 4 weeks post-challenge (Fig. 7B). In contrast, ADCC titers of the second Tat/Env group were only marginally elevated. At the time of challenge, ADCC titers in the two groups were similar (geometric mean titer of 100 for the first

study and 842 for the second study). However by 4 weeks post challenge the Tat/Env group of the first study had an ADCC titer of 20,20,490, while that of the second study was only 5179 ($p=0.0006$). This result was mirrored by the level of ADCC killing. The first Tat/Env group exhibited significantly higher percent killing at the time of challenge compared to the second study ($p=0.010$) and also exhibited a clear anamnestic response, rising to a percent killing of 40.7, whereas the response of the second group remained flat with only 19.3% killing ($p < 0.0001$; Fig. 7C).

The lack of a strong anamnestic ADCC response in the Tat/Env group of the current study prompted us to consider differences in antibody epitopes potentially associated with the activity. We therefore conducted pepscan analysis for sera of the Tat/Env group pre- (week 38) and post- (week 52) challenge against both HIV$_{TV-1}$ and SHIV$_{1157ipd3N4}$ Env peptides, and compared the results with pepscan analysis of the previous Tat/Env group against SHIV$_{89.6P}$ Env peptides. The Tat/Env group of the current study exhibited no or very low binding to SHIV$_{1157ipd3N4}$ peptides both pre-and post-challenge, with peak absorbance reaching no more than 0.2 for any given peptide. Responses to HIV$_{TV-1}$ peptides were somewhat higher, occasionally reaching an absorbance of 0.25. No significant boosting was observed post-challenge, and no distinct pattern of reactivity was observed (data not shown). In contrast, animals from the Tat/Env group of the previous study (Demberg et al., 2007) showed strong and broad responses to SHIV$_{89.6p}$ envelope peptides, both pre- and post-challenge, often exceeding OD values of 1.0, and with peak responses reaching OD levels of 3.0 to 4.0. Clear boosting of antibody reactivities was observed post-challenge (data not shown).

To further evaluate the quality of the antibody response induced, we evaluated avidity, comparing the serum binding antibody of the Tat/Env groups of the first and second studies at week 38 post-immunization. The mean half-maximal binding titers of sera from the first study against SHIV$_{89.6P}$ envelope and the second study against HIV$_{CZM}$ were 5393 ± 898 and 1405 ± 114, respectively ($p=0.0003$). The calculated avidity indices were marginally significantly different: 69.4 ± 4.7 for the first study and 53.1 ± 1.4 for the second study ($p=0.051$).

Discussion

Because Tat can stimulate production of Th1 cytokines and chemokines through its uptake into monocyte derived dendritic cells (MDDC; Caputo et al., 2009), we initially pursued the idea that priming with Ad5hr-HIVtat might modulate responses to Env in the Tat/Env group leading to enhanced immunity. We found, however, that overall responses were to the Ad vector itself, and the inserted genes had no additional impact. Responses in PBMC were relatively low, and remained low after the second Ad-immunizations (Fig. 1A–C), perhaps reflecting emergence of anti-Ad cellular and humoral responses (Peng et al., 2005; Qureshi et al., 2012). Responses in BAL were consistently higher even after the first Ad immunization, and were dramatically boosted by the second Ad administration (Fig. 1D and E). Replication-competent Ad in rhesus macaques is broadly distributed in macaque tissues, regardless of immunization route, and targets and persists in macrophages and mDC in BAL and rectal tissue (Patterson et al., 2012), perhaps explaining the increased expression of all cytokines/chemokines in BAL cells. Previous studies have addressed short term induction of cytokines and chemokines over hours to a few days after exposure to replication-defective Ad in humans and mice (Hartman et al., 2008; Higginbotham et al., 2002; Zsengeller et al., 2000). TNF-α, IL-6, MIP-2, and MIP-1α were strongly induced following pulmonary exposure to human Ad5 in the mouse model (Zsengeller et al., 2000), in agreement with our

TNF-α and MIP-1α results in BAL cells. Further, the induction of MIP-1α in PBMC seen here is similar to that described for wild type Ad5 in human PBMCs (Higginbotham et al., 2002).

We could not explain the altered challenge outcome by the cellular immune responses, which were strongly induced. In some cases CD4 responses to Tat and Env tended to be higher than CD8 responses, contrary to the notion that Ad vaccination targets mainly CD8 cells (Asmuth et al., 2010; Ganguly et al., 2011; Kaufman et al., 2008). CD8 responses may have been under-estimated due to induction of $\alpha 4\beta 7$ expression as observed previously by us (Zhou et al., 2007) and others (Ganguly et al., 2011; Kaufman et al., 2008), and presumed migration of the CD8 T cells to mucosal sites. Such gut homing of Ad-induced CD4 T cells occurs much less frequently (Sun et al., 2010).

Memory T cells in PBMC showed trends toward elevated Env responses and greater polyfunctionality in the Tat/Env group compared to the Env-only group, most evident for CD4$^+$ EM T cells. This was a promising result, as EM T-cell responses have been associated with protection in the SIV model (Hansen et al., 2011). Similarly, induction of B cell memory responses in bone marrow tended to show elevated levels in the Tat/Env group by the end of the vaccine regimen, although not statistically significant.

Considering the strong induction of cellular and humoral immune responses, including memory T and B cells, we were surprised to find no protection against the clade C SHIV$_{1157ipd3N4}$ challenge. Previously, although acute viremia was not reduced following vaccination with either the Tat-only or Tat/Env regimen (Demberg et al., 2007; Fig. 3), the Tat/Env regimen had provided a highly significant 4 log reduction in chronic phase viremia, correlated with binding antibody titers to Tat and Env (Florese et al., 2009). Here, the Tat-only vaccine was similarly ineffective, however, so were both the Env-only and the Tat/Env combined vaccine regimens. The reasons for this unexpected outcome were not immediately apparent. A recent study reports that Tat binds to the HIV envelope, thereby facilitating virus entry into MDDC via an integrin-mediated pathway (Monini et al., 2012). Anti-Tat antibody can block this integrin-mediated uptake, suggesting that both anti-Tat and anti-Envelope antibodies are needed for the most efficient blocking of HIV spread. Biologically active Tat is readily taken up by MDDC, but oxidized Tat, which undergoes conformational changes and multimerization (Fanales-Belasio et al., 2009) is not, suggesting that oxidized inactive Tat might not elicit antibodies able to block the integrin binding site. We retrospectively assessed the biologic activity of the Tat preparation used here for immunization, obtained from the same source as in the previous study, and found it to be inactive and unable to transactivate. Therefore, although anti-Tat antibodies were elicited to similar titers as in the first Tat/Env study, the lack of protection in the Tat/Env group in the current study may be in part attributable to failure to elicit Tat antibodies able to block HIV entry via the integrin mediated pathway. Future comparative studies characterizing Tat antibodies elicited in the two Tat/Env studies could address this possibility.

The experimental design of the current study did not replicate the previous successful study exactly, differing by the heterologous challenge and use of alum for both Env and Tat protein immunizations. We first considered that the SHIV$_{1157ipd3N4}$ challenge might have had a replication advantage compared to the previous SHIV$_{89.6P}$ challenge, due to greater fitness attributable to its possession of two NF-κB sites per long terminal repeat (LTR) (Song et al., 2006), whereas SHIV$_{89.6P}$ has only one. However, a comparison of the control groups in the two studies showed that the SHIV$_{89.6P}$ virus maintained greater chronic phase viremia levels than the clade C SHIV$_{1157ipd3N4}$,' suggesting that viral fitness was not a cause of the poor protective efficacy. Nevertheless, the

heterologous challenge is likely a major factor in the lack of protection. Although the dual-tropic SHIV$_{89.6P}$ used in the first Tat/Env study can utilize CCR5 for entry into CCR5-transfected cells, its preferential co-receptor for primary PBMC is CXCR4 (Zhang et al., 2000). Therefore, in vivo it targets and depletes CD4$^+$ naïve T cells that express CXCR4 (Nishimura et al., 2004). In contrast, R5 tropic viruses such as SHIV$_{1157ipd3N4}$ target CD4 memory T cells that express CCR5 (Grossman et al., 2006). The different outcomes for the two studies compared here might be related to these differences in tropism. Whereas the SHIV$_{89.6P}$ challenge of the first Tat/Env study should have left the CD4$^+$ memory T cells intact, providing help for elicitation of effective anamnestic responses, the R5 tropic SHIV$_{1157ipd3N4}$ challenge would have depleted CD4$^+$ memory T cells, effectively precluding vaccine-elicited memory responses. This possibility provides a further argument for use of CCR5-tropic viruses for challenge of non-human primates in pre-clinical studies, so that vaccine candidates can be appropriately evaluated, and strategies to surmount this problem can be developed.

Further, the heterologous challenge was apparently so dissimilar that it did not elicit an anamnestic response, in contrast to the first study, where the homologous challenge exposure significantly boosted immunity. Specifically, a rapid, high-level anamnestic ADCC response was observed. Subsequently, continued viral expression helped sustain this elevated functional activity, thus contributing to reduced chronic viremia. Here, lacking the initial boost in immune response, the persistent heterologous viremia was not able to maintain vaccine-elicited Env-specific ADCC activity. The lack of cellular anamnestic response suggests that SHIV$_{1157ipd3N4}$ T cell epitopes differed significantly from the immunizing antigens.

A degree of breadth was elicited in humoral immunity by the vaccine regimen, however, as anamnestic Env antibody responses were exhibited by binding antibodies in both serum and rectal secretions and in both Env and Tat-specific memory B cells. Nevertheless, we found the quality of vaccine-induced anti-Env antibodies was inferior in this study compared to the previous one. The functional antibody responses induced here were low titered. Neutralizing antibody to HIV$_{TV-1}$, the immunizing strain, never developed, although neutralizing antibody rapidly developed post-challenge against the tier 1 virus, SHIV$_{1157ipdEL-p}$ (Siddappa et al., 2010) compared to slow development in the control macaques. SHIV$_{1157ipd3N4}$ is a difficult to neutralize tier 2 virus (Song et al., 2006), but neutralizing activity against it did not appear even post-challenge. In contrast, neutralizing antibody against the SHIV$_{89.6P}$ challenge virus of the first study appeared soon after viral exposure. Moreover, in the previous study, the potent chronic phase protection seen in the Tat/Env immunization group was correlated with anti-Env binding antibody and ADCC activity (Florese et al., 2009). Here, ADCC activity was detectable, but of much lower titer compared to the previous Tat/Env group (Fig. 7 B and C). Additionally, while anti-Env IgG antibodies were detected in rectal secretions, rectal anti-Env IgA antibodies were induced at lower levels and only sporadically. We have reported a correlation of mucosal IgA antibodies with protection and delayed acquisition (Xiao et al., 2012, 2010). Their absence here may have contributed to the lack of protection against the intrarectal challenge.

Additionally, the Env antibody responses elicited here were of poorer quality compared to those of the previous study, as assessed by pepscan and avidity measurements. In the pepscan analysis, sera from the Tat/Env group did not strongly recognize homologous Env peptides of the immunizing strain in contrast to results of the earlier study. Moreover, in the previous study, binding antibodies exhibited higher avidity than those of the current study. Higher avidity has been associated with better protection (Xiao et al., 2012; Zhao et al., 2009) and is also directly correlated with functional antibody responses (Xiao et al., 2010). The low-titers and poor quality of antibody responses suggest the adjuvant used may have been suboptimal.

The change to use of alum adjuvant was made in order to correspond to an earlier Tat plus Env combination study in cynomolgus macaques (Ferrantelli et al., 2011), and to match a future Tat plus Env vaccine trial. Alum has long been used as an adjuvant and promotes immune responses independently of toll-like-receptor (TLR) mechanisms (De Gregorio et al., 2009). While initially believed to function by a "depot effect", promoting slow release of antigen, more recently it has been seen as an antigen delivery system (Morefield et al., 2005). As described by De Gregorio et al. (2009), alum promotes uptake of antigen by DCs and induces proinflammatory reactions leading to DC maturation and enhanced immune responses. In contrast, MPL-SE is a TLR-4 agonist (Ishii and Akira, 2007) that activates cells including DCs that express TLR-4. We did not directly compare alum versus MPL-SE in this study. However, studies in non-human primates are moving away from alum adjuvants and efforts to develop other, more potent adjuvants should continue.

Overall, our results suggest the need for a strong anamnestic response in control of chronic viremia. In general, viral heterogeneity is viewed as an issue related to elicitation of sterilizing immunity which can lead to prevention of HIV acquisition. However, as seen here, HIV heterogeneity can impact not only viral acquisition, but also can modulate the host's ability to respond and control subsequent viremia in the absence of sterilizing immunity. Moreover, to achieve a potent anamnestic response, vaccine strategies incorporating protein immunizations require use of adjuvants able to elicit high-quality antibody, characterized by strong memory incorporating functional antibody activities and high avidity.

Material and methods

Animals, immunization and challenge, and sample collection

Twenty-eight Indian origin rhesus macaques were housed at Advanced BioScience Laboratories, Inc. (ABL; Rockville, MD) and maintained according to institutional animal use committee guidelines and the NIH Guide for the Care and Use of Laboratory Animals. All animals were negative for SIV, STLV-1, and SRV and the Mamu-B*08 and B*17 MHC-class I haplotypes. They were not pre-screened for Ad5 antibody, as previous studies have shown little to no pre-exititng Ad5 antibody in this species. Four Mamu A*01 positive animals were evenly distributed among the groups. The immunization schedule was identical to that of our previous Tat/Env study (Demberg et al., 2007). Animals (7 per group) were vaccinated on week 0 via the intranasal (IN) and week 12 via the intratracheal (IT) route with replication-competent Ad type 5 host range mutant (Ad5hr) recombinants encoding HIV$_{IIIB}$ Tat, HIV$_{TV-1}$ gp160, or both Tat and Env (0.5×10^9 pfu per recombinant). For the Tat and Env only groups and the control group, empty Ad5hrΔE3 vector was added to make the total Ad dose 1×10^9 pfu. At weeks 24 and 36 the appropriate groups were boosted with 10 µg HIV$_{IIIB}$ Tat protein (ABL), 100 µg oligomeric HIV$_{TV-1}$ gp140ΔV2 envelope (Novartis Vaccines and Diagnostics, Cambridge, MA), or a combination of the Tat and Env proteins formulated in Alum adjuvant. The first boost was administered subscapulary, and the second subcutaneously. The controls received adjuvant only. Animals were challenged at week 50 intrarectally with a single high dose (25 AID$_{50}$) of the tier 2 clade C SHIV$_{1157ipd3N4}$ (Song et al., 2006).

Table 1
Real-time PCR primers.

Target	Forward primer 5′ to 3′	Reverse Primer 5′ to 3′	Amplicon size (bp)	Accession No.	Ref.
IFN-γ	GCAACAAAAAGAAACGGGATGAC	CTGACTCCTTTTTCGCTTCC	148	NM_001032905	Demberg et al. (2011)
IL-15	GAAGCTGGCATTCATGTCTTCA	ACATTCACCCAGTTGGCTTC	77	NM_001044731	
TNF-α	AGCCCATGTTGTAGCAAACC	GCTGGTTATCTGTCAGCTCCA	104	DQ902483	Demberg et al. (2011)
CCL-3 (MIP-1α)	CCTCCTGCTGCTTCAGCTAC	CTCCTTACTGGGGTCAGCAC	146	AF457195	Demberg et al. (2011)
CCL-4 (MIP-1β)	CTTCCTCGCAACTTTGTGGT	GCTTGCTTCTTTTGGTTTGG	88	NM_002984	Demberg et al. (2011)
CCL-5 (RANTES)	AGTGGCAAGTGCTCCAACC	CGAACCCATTTCTTCTCTGG	86	DQ913730	Hofmann-Lehmann et al. (2002)
CXCL8 (IL-8)	GAGTGGACCACACTGTGCCA	AAACTTCTCCACAACCCTCTGC	108	NM_001032965	Hardstedt et al. (2005)
IL-10	AGAACCACGACCCAGACATC	GGCCTTGCTCTTGTTTTCAC	119	DQ890063	Demberg et al. (2011)
18s	GCCCGAAGCGTTTACTTTGA	TCCATTATTCCTAGCTGCGGTATC	81	NR_003286	Medeiros et al. (2003)

BAL, blood and bone marrow samples were collected periodically over the immunization course. PBMCs were obtained by Ficoll-Paque PLUS gradient (GE Healthcare) separation, whereas BAL fluids were subjected to 35%/65% percoll (Sigma-Aldrich) gradient centrifugation. Cells were counted and checked for viability by trypan blue (Sigma-Aldrich) staining.

Cytokine/chemokine analyses by real time PCR

Primers were designed from human or rhesus reference sequences of the specific cytokine. Specific primers (Table 1) were selected using web based primer3 software (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi) and tested to be exon-spanning using Blat search (http://genome.ucsc.edu/index.html) to avoid amplicons from genomic DNA. Total RNA was isolated using Qiagen RNeasy kits (Qiagen) in combination with Qiagen Shredder columns. cDNA was synthesized using superscript III kits (Invitrogen) or Qiagen QuantiTect Kits. The manufacturers' protocols were followed with the following modifications: both poly-dT and random hexamers were used with the superscript III kit, and for both kits the reverse transcription step was extended to 1 h at 42 °C. All incubations were done on an Eppendorf Mastergradient cycler. cDNAs were frozen at −20 °C until further use. Real-time PCRs were performed on an ABI7000 (Applied Bioscience) PCR machine. The PCR reactions contained 25 µl of the Invitrogen Platinum SYBR Greener qPCR SuperMix-UDG with ROX, 1 ng of cDNA template, and 1 µl of 10 mM primer working stocks. Amplicons were generated as follows: 50 °C for 2 min, 95 °C for 5 min, followed by 45 cycles of 94 °C for 30 s, 59 °C for 15 s and 72 °C for 30 s. All samples were run in triplicate. To verify specific amplicon sizes, PCR products were mixed with loading buffer (MTR, Maryland) and 20 µl were loaded onto Invitrogen 4% 48-well or 4% 12-well E-gels. 10 bp and 25 bp ladders (Invitrogen) served as size markers.

Interferon-γ (IFN-γ) ELISpot

Nunc Maxisorb plates were coated overnight at 4 °C with 100 µl of the U-Cytech anti-monkey IFN-γ antibody in DPBS (Life Technologies, Carlsbad, CA), washed once on an automated plate washer (Molecular Devices), and blocked overnight at 4 °C with 200 µl of DPBS+2% BSA. PBMCs were adjusted to either 2×10^6 or 1×10^6 cells/ml in R-10 medium. After an overnight stimulation at 37 °C with a single pool of 23 clade B Tat peptides (AIDS Research and Reference Reagent Program, DAIDS, NIAID) or 214 HIV$_{TV-1}$ Env peptides (ABL) at a final concentration of 1 µg/ml for each peptide, cells were plated into triplicate wells of the coated and blocked plates at 2×10^5 and 1×10^5 cell/well. Concanavalin A (5 µg/ml) and R10 medium or R10 plus DMSO (0.7% final concentration) served as positive and negative controls. After a 5 h incubation at 37 °C, cells were removed by flicking the plates and remaining cells were lysed with 200 µl of ice cold distilled

water for 15 min. Plates were washed twice and 100 µl of DPBS+1% BSA containing the detection antibody (U-Cytech) were added. After a 1 h incubation at 37 °C the plates were washed again and 50 µl of DPBS+1% BSA containing the secondary antibody (U-Cytech) were added and the plates incubated as above. After 2 final washes, the plates were blotted dry and 30 µl of an activation solution (Activator 1 and 2 at a 1:1 ratio; U-Cytech) were added. The reaction was stopped by rinsing the plates with distilled water. Results are reported as spot forming cells (SFC)/million PBMC after subtraction of spots in negative control wells.

CFSE proliferation

PBMCs (5×10^6) were washed, and resuspended in 4 ml of pre-warmed DPBS containing 0.1% BSA in a 15 ml conical tube, and labeled with 1.5 µM CFSE (Invitrogen, Carlsbad, CA). The cells were gently vortexed, incubated for 15 min at 37 °C, and the staining reaction was quenched by adding 10 ml of cold (4 °C) R10 medium and incubating on ice for 8 min. The cells were pelleted by centrifugation (350xg, 10 min at RT), washed once with 14 ml of R10 medium, and resuspended in 4 ml of medium. One ml of cell suspension was plated into wells of a 24-well plate. Cells were stimulated with either HIV$_{IIIB}$ Tat protein (5 µg/ml), HIV$_{TV-1}$ Env gp120 (5 µg/ml) or both according to immunization group. Cells in R10 or stimulated with ConA (5 µg/ml) served as negative and positive controls, respectively. On day 6 post-stimulation cells were transferred into FACS tubes, washed once with PBS, and stained for 25 min in the dark at RT with the following antibodies: CD3 PE (clone SP34-2), CD8 PE-Cy7 (clone RPA-T8) and CD4 APC (clone L200) (all from BD Biosciences). Subsequently, the cells were washed in PBS and fixed in 2% formaldehyde (Tousimis) in PBS. Proliferation was evaluated by acquiring 100,000 cells in the lymphocytic gate on a BD FACSCalibur and is expressed as the percentage of cells which diluted out CFSE after subtraction of background proliferation in unstimulated controls.

Multiparameter flow cytometry

The antibody panel for flow cytometry analysis consisted of the following mouse or rat anti-human antibodies: CD3 Alexa Fluor 700 (Clone SP34-2), CD4 PE-TR (clone L200, custom conjugated), CD95 PE-Cy5 (clone DX2), IFN-γ APC (clone B27), IL-2 APC-Cy7 (clone MQ1-17H12, custom conjugated) (all from BD Bioscience); CD8 Qdot 655 (Clone 3B5, custom conjugated; Invitrogen); CD28 PE-Cy7 (Clone CD28.2; eBioscience, San Diego, CA), TNFα PerCP-Cy5.5 (Clone Mab11; Biolegend, San Diego, CA), and CCR7 FITC (clone 150503, R&D Systems, Minneapolis, MN). Aqua (Invitrogen) was used for exclusion of dead cells. Each antibody was titrated separately, and optimized amounts were used. One million cells in 400 µl of R10 medium were stimulated in FACS tubes for six hours with 1 µg/ml of either HIV$_{IIIB}$ Tat or HIV$_{TV1}$-Env gp120 peptides in the presence of 0.4 µl Golgi Plug

(Brefeldin A, BD Biosciences), 4 µl of purified anti-CD49d (clone 9F10, eBioscience), and 3 µl anti-CD28-PE-Cy7. BAL cells were stimulated with Tat and/or Env peptides according to immunization group except at the week 38 time point where cells from all animal groups were stimulated with both Tat and Env peptides. Unstimulated and SEB-stimulated samples were used as negative and positive controls, respectively. After stimulation, cells were washed twice with 1x DPBS, re-suspended in 95 µl of 1xPBS, and incubated at room temperature for 10 min with 5 µl of Aqua dye stock (1:40 dilution). After two washes with DPBS, the cells were incubated with CD4, CD8 and CD95 antibodies for 20 min at room temperature in 100 µl of FACS buffer (2% FBS in DPBS). After another two washes with FACS buffer, cells were incubated in 250 µl of fix/perm buffer (BD Bioscience) for 20 min at 4 °C. Cells were thereafter washed twice with perm/wash buffer (BD Bioscience), incubated with CD3, IFN-γ, IL-2 and TNF-α antibodies for 20 min at 4 °C, washed twice with perm/wash buffer, and re-suspended in 3.7% formaldehyde solution for FACS analysis on an LSRII flow cytometer (BD Bioscience) equipped with blue, red and UV lasers. The frequency of cytokine positive cells was determined using FlowJo version 8.8.6 (Tree star, Inc., Ashland, OR), and formatted and analyzed using Pestle and Spice software, respectively (both provided by Mario Roederer, VRC, NIAID, NIH).

Evaluation of binding antibodies

Serum binding antibodies specific for HIV_{IIIB} Tat, HIV_{CZM} gp120, and HIV_{TV-1} gp140 were evaluated by ELISA as previously described (Demberg et al., 2012). Antibody titer was defined as the reciprocal of the serum dilution at which the optical density of the test serum was at least twice that of a negative rhesus macaque serum diluted at 1:50.

Mucosal IgA and IgG titers were assessed in rectal secretions collected using WECK-CEL sponges (Medtronic Ophthalmics, Jacksonville, FL, USA) pre-moistened with 50 µl DPBS. The sponges were inserted into the rectum of the animals for 5 min and subsequently frozen at −70 °C. After thawing, sponges were transferred to 5 µm pore size centrifugal filter units (ultrafree-MC amicon, Millipore, USA) and clipped. Elution buffer (250 µl of 1% Igepal CA-630, 10x Sigma-FAST protease inhibitor cocktail in DPBS) was slowly added to each sponge. The filter units were spun at 1000 g for 30 min at 4 °C. The cartridge was discarded and 5 µl of each sample was transferred onto a Roche Chemstrip (Roche, USA) to test for blood contamination. Samples positive for blood were excluded from the analysis. An ELISA was performed by coating 96-well Microlon 600 half-area plates (Greiner Bio-one, USA) with either HIV Tat or HIV_{CZM} gp120 protein in carbonate-bicarbonate buffer (pH 9.6) (Sigma-Aldrich) over night at 4 °C. Plates were washed, blocked with a 1:10 dilution of BSA blocking buffer (KPL, USA) and washed again. Samples were serially diluted in the plates and incubated over night at 4 °C. After washing, the plates were incubated with HRP-conjugated anti-monkey IgG or IgA antibodies, washed and developed with TMB substrate (KPL), stopped with sulfuric acid, and read at 450 nm on an ELISA plate reader (Biotek Powerwave). Titers were defined as the reciprocal dilution of secretion at which the OD of the test sample was twice that of a negative sample at a 1:5 dilution. Total IgG and IgA in the secretions were determined as described previously (Xiao et al., 2010). Final results were expressed as specific activity: Env- or Tat-specific IgG or IgA titer divided by µg of total IgG or IgA in the sample.

ADCC

The ability of sera to mediate ADCC was assessed using the RFADCC assay as previously described using human PBMC as effectors and HIV clade C CZM gp120-coated CEM.NKr cells as targets at an E:T ratio of 50:1 (Gomez-Roman et al., 2006).

Ten-fold serial dilutions of sera starting at 1:10 were evaluated. ADCC titer was defined as the reciprocal of the serum dilution at which percent killing was greater than the mean plus three standard deviations of all negative control samples.

Neutralizing antibody

The ability of sera to neutralize $HIV_{TV1.21}$ (a pseudovirus grown on 293T cells) and primary isolates $SHIV_{1157ipd3N4}$ and $SHIV_{1157ipEL-p}$ grown on PBMC, was tested on TZM-bl cells as described previously (Montefiori, 2004). Results are expressed as ID_{50} values: the reciprocal of the sample dilution at which relative luminescence units were reduced 50% compared to virus control wells containing no macaque serum.

Avidity

Avidity of serum antibody was evaluated against HIV_{CZM} or $SHIV_{89.6P}$ gp120 proteins as previously described (Xiao et al., 2010). The avidity index was calculated as ratio of the NaSCN-treated serum dilution giving half-maximal binding to the PBS-treated serum dilution giving half-maximal binding, multiplied by 100.

PEPSCAN

PEPSCAN analysis against 214 HIV_{TV-1} and 214 $SHIV_{1157ipd3N4}$ envelope peptides was conducted by ELISA. Wells of Nunc Maxisorb 96-well plates were coated overnight at 4 °C with 500 ng peptide/well in 100 µl of sodium bicarbonate buffer (pH 9.6) and blocked with 200 µl of Pierce SuperBlock blocking buffer in PBS for 1 h at RT. Sera (100 µl of 1:50 dilutions) were added and incubated for 1 h at 37 °C. After 5 washes with PBS+0.05% Tween 20, 100 µl of horseradish peroxidise labelled goat anti-human IgG antibody (1:100,000 dilution) were added and plates were incubated for 1 h at 37 °C. After intensive washing, 100ul of K-Blue Aqueous TMB Substrate (Neogen) were added for 30 min at RT. Color development was stopped with 2 N sulfuric acid and plates were read at 450 nm on a Molecular Devices E-max plate reader.

B-cell ELISpot

Total and envelope-specific IgG and IgA ASC in bone marrow were assessed fresh and after 3-days stimulation with 1 µg/ml CpG (ODN-2006) (Operon), 0.5 µg/ml recombinant-Human sCD40L (Peprotech), and 50 ng/ml recombinant-Human IL-21 (Peprotech) as previously described (Brocca-Cofano et al., 2011). Resultant spots were counted using an automated ELISPOT reader (Axioplan 2 imaging; Zeiss, Munchen, Germany). Env-specific IgG or IgA ASC are reported as percent of total IgG or IgA ASC.

Statistical analyses

Differences in binding and ADCC antibody titers, and avidity indices and half maximal binding titers were evaluated using the Wilcoxon rank sum test. Differences in ADCC % killing were analyzed using repeated measures analysis of variance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.02.024.

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