Published in final edited form as:
J Acquir Immune Defic Syndr. 2009 December 1; 52(4): 433–442. doi:10.1097/QAI.0b013e3181b880f3.

Tonsillar application of AT-2 SIV affords partial protection against rectal challenge with SIVmac239

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Abstract

While mucosal responses are important for preventing infections with HIV, the optimal strategies for inducing them remain unclear. To evaluate vaccine strategies targeting the oral mucosal lymphoid tissue inductive sites as an approach to provide immunity at distal sites, we vaccinated healthy macaques via the palatine/lingual tonsils with aldrithiol 2 (AT-2) inactivated SIVmac239, combined with CpG-C immunostimulatory oligonucleotide (CpG-C ISS-ODN , C274) as the adjuvant. Macaques received 5 doses of C274 or control ODN C661 and AT-2 SIV on the tonsillar tissues every 6 weeks before being challenged rectally with SIVmac239, 8 weeks after the last immunization. Although no T or B cell responses were detected in the blood prior to challenge, Ab responses were detected in the rectum . Immunization with AT-2 SIV significantly reduced the frequency of infection compared to non-immunized controls, irrespective of adjuvant. In the vaccinated animals that became infected, peak viremias were somewhat reduced. SIV-specific responses were detected in the blood once animals became infected with no detectable differences between the differently immunized groups and the controls. This work provides evidence that vaccine immunogens applied to the oral mucosal-associated lymphoid tissues can provide benefit against rectal challenge, a finding with important implications for mucosal vaccination strategies.

Keywords

SIV; mucosal; vaccine; CpG ISS-ODN

Introduction

A quarter of a century after HIV-1 was shown to be the etiologic agent of AIDS, the pandemic has claimed tens of millions of lives. Despite a global scientific effort, an effective prophylactic vaccine for HIV/AIDS remains elusive. Hopes were dashed again with results from a recent a clinical trial not only showing a lack of efficacy in preventing HIV acquisition, but suggesting an increased risk of infection in some vaccinees [1]. These results led to recommendations for a

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renewed emphasis on basic preclinical research and the need to "make best use of animal, particularly monkey, models" [2]. The benefits of the SIV/macaque model as the principal animal model for studying HIV are well documented [3].

HIV infection occurs mainly via mucosal surfaces, suggesting that induction of mucosal immune responses may be an important property for an effective vaccine. Numerous studies in rodents have provided evidence that oral/nasal administration induces immunity at distal mucosal sites, as well as systemically. Oral or nasal immunization of mice against *Chlamydia* or HSV led to protective immunity in the vagina [4-7] and to memory CTL responses [8, 9]. Mucosal immunization of mice with Tat or Gag-containing vectors led to vaginal protection against challenge with recombinant HIV protein-expressing vectors [10, 11]. A number of mucosal immunization studies in macaques have also been shown to elicit mucosal and systemic immunity [12-17].

Aldrithiol 2 (AT-2) inactivated SIV and HIV are attractive vaccine immunogens as they contain all of the virion associated proteins in the absence of an infectious virus [18]. Previous work has shown that AT-2 viruses interact authentically with dendritic cells (DCs) [19] and mature DCs elicit both $CD4^+$ and $CD8^+$ T cell responses *in vitro* 20. Moreover, in therapeutic immunization regimens, injection of autologous mature DCs pulsed with AT-2 SIV or HIV were reported to boost immunity and reduce viral loads in infected macaques [21] and in a preliminary clinical study in humans [22], respectively.

CpG-C immunostimulatory oligonucleotides (ISS-ODNs) activate both plasmacytoid DCs (PDCs) and B cells [23, 24], to potentially augment innate and adaptive immunity elicited against a vaccine. Similar observations have been made in macaques where CpG-C ISS-ODNs induced PDC activation, IFNα and IL-12 production, and boosted SIV-specific T cell responses *in vitro* 25, as well as stimulating robust B cell proliferation, survival and activation [26]. Injection of CpG-C ISS-ODNs in macaque lymph nodes also activated both DCs and B cells, demonstrating the ability of CpG-C ISS-ODNs to work *in vivo* 27. While CpG ISS-ODNs have been shown to boost macaque immunity [28-30], there is no evidence for the activity of the more broadly acting CpG-C ISS-ODNs in this species.

We examined whether applying a combination of AT-2 SIV and CpG-C ISS-ODNs to the tonsillar mucosa (as a controlled way to model targeting the nasal lymphoid tissues) would protect against rectal challenge with infectious SIV. Whilst partial protection by tonsillar application of AT-2 SIV was observed, CpG-C ISS-ODNs did not augment this effect suggesting that alternative adjuvant strategies will be needed to optimize the efficacy of mucosally applied AT-2 SIV.

Materials and Methods

Reagents

CpG-C ISS-ODN C274 and the control ODN C661 were provided by Dynavax Technologies (Berkeley, CA). The sequences were: C274 5′-TCGTCGAACGTTCGAGATGAT-3′ and C661 5′-TGCTTGCAAGCTTGCAAGCA-3′. AT-2 SIV (AT-2 SIVmac239 lot numbers: P4001, P4146, P3876, P3778, P3782; AT-2 SIVmac239ΔV1V2 [31] lot number: P3956) and the no virus microvesicle (MV) controls (lot numbers: P3826, P3971), prepared from the same cell line in which the viruses were grown (SUPT1), were provided by the AIDS and Cancer Virus Program (NCI-Frederick, Frederick, MD). AT-2 inactivation of virus was performed as previously described [32]. AT-2 SIV was used at 300ng of p27/ml for all *in vitro* cultures. MV were normalized to SIV on total protein (300ng of p27 equivalent/ml). Concanavalin A (ConA; Sigma, St Louis, MO) was used at 1μg/ml.

Animals and Treatment

Adult male Chinese Rhesus macaques (*Macaca mulatta*) were housed at the Tulane National Primate Research Center (TNPRC; Covington, LA). All studies were approved by the Animal Care and Use Committee of the TNPRC. The animals' average age at the beginning of the study was 5 years and their average weight was 10kg. All animals tested negative for simian type D retroviruses, simian T cell leukemia virus-1, and SIV prior to use. Animals were anesthetized prior and during all procedures (10mg ketamine-HCl/kg), in compliance with the regulations detailed under the Animal Welfare Act and in the Guide for the Care and Use of Laboratory Animals [33, 34]. Animals were immunized a total of five times, at six week intervals, by application across the lingual and palatine tonsils of 1mg of CpG-C ISS-ODN C274 or the control ODN C661 mixed with 5μg of p27 of either AT-2 SIVmac239 or AT-2 SIVmac239ΔV1V2 [31] in a volume of 100μl. The treatment groups included animals immunized with the following: C274 and wild-type AT-2 SIVmac239 (C274/wt), C274 and AT-2 SIV mac239ΔV1V2 (C274/V1V2), C661 and wild-type AT-2 SIVmac239 (C661/wt), C661 and AT-2 SIVmac239ΔV1V2 (C661/V1V2), or nothing (non-vaccinated controls). Table 1 lists all study animals. Eight weeks after the final immunization, animals were challenged rectally with 10^3 $TCID_{50}$ of SIVmac239 (TNPRC stock virus propagated in SEB-stimulated rhesus PBMCs; "SIVmac239 RhPBMC 7/29/94"). Once the follow up period was completed, 5 months after the initial challenge, uninfected animals were re-immunized once more, re-challenged 8 weeks later and followed up for 6 months (as indicated). Immune responses were followed by collecting EDTA-anticoagulated blood and mucosal (oral and rectal) fluids throughout the immunization regimen and for up to 6 months after SIV challenge. Mucosal fluids were collected by insertion of a foam pad (approx. size 1×0.5cm) in the mucosal cavity for 5min, after which the swab was placed into a tube containing 1ml PBS/1% FCS/penicillin-streptomycin (Cellgro/Mediatech, VA). Blood, fluids and tissue samples were transported to the laboratory by overnight courier service. Blood was processed as described below and the mucosal fluids were spun at 805g for 10min, collecting the supernatant and storing at −80°C until analysis. Upon study termination, animals were sacrificed and standard full necropsy for SIV infected animals was performed. To further assess the *in vivo* activity of mucosally applied CpG-C ISS-ODNs, 1mg of C274 or C661 (50μl of 20mg/ml stock) were applied to the tonsils of healthy infected or uninfected macaques and tonsillar pinch biopsies were collected 24h later. Cellular activation was monitored by flow cytometry.

Cell isolation

Macaque peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density centrifugation (GE Healthcare, Sweden). Cells were cultured in complete RPMI 1640 (Cellgro, Springfield, NJ) containing 2mM L-glutamine (GIBCO Life Technologies, Grand Island, NY) 10mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; GIBCO Life technologies), 50μM 2-mercaptoethanol (Sigma), penicillin (100U/ml)/streptomycin (100μg/ml) (GIBCO Life Technologies) and 1% heparinized human plasma (Innovative Research, Southfield, MI).

Tonsillar biopsies were placed in RPMI (supplemented as above, but with 10% heat inactivated fetal bovine serum (Mediatech, Manassas, VA) instead of human plasma) containing 200μg/ml gentamycin (GIBCO) for 1 hour at 4°C. The tissue was washed by spinning at 244g for 10min and resuspending in medium (RPMI-10% FBS) containing 400U/ml Collagenase D (Roche, Indianapolis, IN) and 10μg/ml DNAse I (Roche) in a tissue-culture dish. The tissue was broken up using a forceps and a scalpel and incubated at 37°C for 1 hour. It was then filtered using a 70μm nylon filter (BD Falcon) and the suspension was spun at 340g for 10min. Cells were then resuspended in RPMI (1% human plasma) and counted.

Flow cytometry

Four color flow cytometry was used to characterize leukocyte subsets from macaque blood and tissue. DCs were identified as $Lin^{-}HLA\text{-}DR^{+}$ populations using FITC-conjugated anti-lineage marker Abs (CD3, CD8, CD11b, clones SP34, SK1 and F6.2); CD14, clone M5E2 (BD Biosciences); CD20, clone L27 (BD Biosciences)) and APC-conjugated anti-HLA-DR (clone G46-6, BD Biosciences). PDCs and MDC-containing fractions were identified as $CD123^{+}$ and $CD123^{-}$ subsets within the $Lin^{-}HLA\text{-}DR^{+}$ cells, respectively, using PE-conjugated anti-CD123 (clone TU27, BD Biosciences). DC activation status was examined using Cy-conjugated anti-CD86 (clone 2331 FUN1, BD Biosciences) and anti-CD80 (clone L307.4, BD Biosciences). B cells were identified using FITC-conjugated anti-CD20 and their activation status was monitored using PE-conjugated anti-CD86 (clone IT2.2, BD Biosciences), -CD80, and -CD40 (clone 5C3, BD Biosciences). T cell subsets were identified using APC-conjugated anti-CD28 (clone CD28.2, Biolegend) and PE-conjugated CD95 (clone DX2, BD Biosciences).

The appropriate isotype Ig controls were included in all experiments and typically gave MFIs of <1 log. Samples were acquired on a FACSCalibur (BD) and analyzed using FlowJo software (Tree Star, OR).

IFNγ ELISPOT

Numbers of IFN-γ spot-forming cells (SFCs) in peripheral blood responding to wild-type AT-2 SIVmac239, to AT-2 SIVmac239ΔV1V2 mutant or Gag/Env peptide pools (NIH AIDS Research and Reference Reagent Program) were measured by ELISPOT [25]. Each peptide pool was made by mixing 10 consecutive peptides (resuspended in 100μl DMSO) at 2μg/ml for each peptide (0.2% DMSO final concentration). 22 peptide pools were prepared for Env and 13 for Gag, covering the entire span of each protein. ConA was used at 1μg/ml as a positive control. Medium, MV, and 0.2% DMSO controls were included for the respective stimuli.

Viral Load and SIV Ab detection

Plasma samples were collected from all animals at all time points of the study by centrifuging whole blood at 805g for 10min, collecting the clear supernatant, centrifuging again and aliquots of the supernatants were stored at −80°C. SIV RNA was determined by quantitative RT-PCR [35] and SIV-specific Abs were measured by ELISA [36].

Neutralizing Ab activity against SIV was measured in monkey plasma samples using minor adaptations to published protocols [37, 38]. Plasma, from weeks 8-10 post-infection, was heat inactivated by incubating at 56°C for 1 hour and then clarified by centrifugation at 956g. Samples were then diluted 5-fold twice in flat-bottom 96-well plates (BD Falcon, NJ). 50 $TCID_{50}$ of SIVmac239 or SIVmac251 were added to each plasma-containing well and incubated for 1 hour at 37°C. 3×10^{5} 174×CEM cells (NIH AIDS Research and Reference Reagent Program) were added per well and the plates were incubated for 2 weeks at 37°C. 50μl of culture medium were exchanged for fresh medium after 7 days. No more plasma was added during this period. Samples were run in duplicate. The plates were monitored for cytotoxicity every 3 days and cell-free supernatants were collected at day 14 of culture for p27 ELISA (ZeptoMetrix Corporation, NY). Pooled heat-inactivated plasma from SIVmac239Δnef/wild type-infected animals (healthy, long-term infected) was used as a positive control. Negative controls included plasma from uninfected monkeys, as well as wells with no plasma added.

SIV-specific IgA was measured in rectal fluids collected at the beginning of the study, as well as at the last time point prior to challenge (week 26) or after challenge (week 36), by ELISA as previously described [17], with minor modifications. Briefly, 96 well plates (Costar, NY) were

coated overnight with lysed SIVmac239 (lot P4145) at 1μg/ml. Plates were blocked with 0.25% gelatin in PBS, the samples were added (100μl/well) and incubated for 2 hours at 37°C. Peroxidase-conjugated goat anti-monkey IgA (Alpha Diagnostic International, San Antonio, TX) was used as the secondary Ab at 1:10,000 and incubated for 2 hours at 37°C. TMB peroxidase substrate solution (KPL, Gaithersburg, MD) was then added (100μl/well) and incubated for 30min at RT. The reaction was stopped with 1M HCl (50μl/well) and the absorbance was read at 450/650nm. Pooled plasma from SIV mac239Δnef/wild type-infected animals (healthy, long-term infected) was used as a positive control. Plasma from uninfected monkeys was used as a negative control.

Statistical analyses

Viral load data were analyzed for statistical significance using the Mann-Whitney test. Frequency of infection data were analyzed using the Fisher's Exact test. *p* values <0.05 were taken as statistically significant.

Results

Tonsillar C274/AT-2 SIV vaccination partially protected against SIV challenge

Knowing that C274 activates macaque DC and B cells, and augments SIV-specific T cell responses [25-27], we set out to determine if combining C274 and AT-2 SIV would serve as a potent vaccine when applied to the oral mucosal-associated lymphoid tissues (MALT) of macaques. This allowed us to control that the vaccine was directly applied to the oral MALT (and not swallowed), to provide a model for future strategies that would target nasal MALT. In addition to comparing C274 as the adjuvant, we compared two vaccine antigens, wild-type AT-2 SIVmac239 and AT-2 SIVmac239ΔV1V2 [31], a mutant of the virus that lacks the hypervariable loops V1 and V2 from the viral envelope. We hypothesized that the deletion of V1 and V2 in the mutant virus might reveal neutralization sensitive epitopes that would be presented in this AT-2-treated vaccine, thereby inducing more potent neutralizing Ab responses, whilst inducing a similar cellular response to the wild-type virus.

SIV-naïve Chinese rhesus macaques were vaccinated 5 times by applying AT-2 SIV with the indicated ODNs to the tonsillar tissues every 6 weeks (Fig. 1). 8 weeks after the final immunization, the animals were challenged rectally with pathogenic SIVmac239. They were followed up for a period of 6 months. Vaccinated animals that remained uninfected after 5 months of follow up were then re-immunized once more and re-challenged 8 weeks later and followed for an additional 6 months to see if they continued to resist infection (Table 1). When comparing all challenges and infection outcomes, the vaccinated animal groups exhibited a significantly lower frequency of infection (average of 53%) compared to the non-vaccinated control group (83%), independent of the presence of C274 (Fig. 2, Table 1; $p<0.03$ when comparing all challenges for each group vs control).

Examination of the plasma viral loads (Fig. 3) revealed that all infected control animals, 6/6 infected C274/wt-vaccinated animals, and 2/4 infected C661/wt-vaccinated animals exhibited peak viremia 2 weeks post challenge. Peak viremias were delayed by 1-2 weeks in 4/6 infected C274/V1V2 and 1/2 infected C661/V1V2 vaccinated animals. Closer analysis of acute viremia revealed that wild type-vaccinated animals showed significantly lower viremia than controls (independent of C274) during the first week of infection (Fig.4, upper panel), but no difference in subsequent weeks. ΔV1V2-vaccinees also had significantly lower viremia than controls during the first 2 weeks of infection, with the exception of C661-vaccinees in the first week (Fig.4, lower panel). Set-point viremias (weeks 8 and 10 post-infection) and viremias at time of necropsy (4-11 months post infection) yielded no significant differences between any of the groups.

Blood $CD4^+$ T cell counts were measured over the length of the study, but there were no differences in the CD4 decline between the groups (Table 1). Full simian AIDS necropsies were performed when possible and the pathology results are summarized in Table 3. No dramatic pathology or striking differences were observed between groups. All animals, with one exception, survived the 6 month follow-up period. The animal (GJ66) that died during this period was in the C661/wt virus vaccination group and was sacrificed at 15 weeks post-infection due to excessive weight loss, a sign of simian AIDS. The CD4 decline observed in this animal (403 cells/μl blood at necropsy) was similar in magnitude to other animals.

C274 did not alter innate and adaptive immune responses *in vivo*

Paralleling the measurement of viral parameters, adaptive immune responses were monitored over time by measuring the presence of SIV-specific T and B cell responses in blood and mucosal fluids. No SIV-specific immune responses were detected in peripheral blood after tonsillar immunization, as measured by numbers of PBMCs producing IFNγ in response to stimulation with AT-2 SIV (ELISPOT) or the presence of SIV-specific Abs in plasma. However, following challenge, comparable SIV-specific T and B cell responses in blood were detected in most infected animals (Table 1). Both AT-2 SIV wild type and AT-2 SIV ΔV1V2 were used as stimuli *in vitro* and elicited comparable T cell responses in all animals (data not shown), indicating that there were limited T cell responses against the deleted V1V2 region. *In vitro* Ab neutralization activity against pathogenic SIVmac239 and SIVmac251 was also measured in plasma from the infected animals in each group. As reported by others [37, 38], limited neutralization of SIVmac239 was observed, but SIVmac251 was neutralized more effectively (Fig. 5). There appeared to be no difference between any ofthe groups.

Mucosal SIV-specific IgA was measured in the rectal fluids of animals at the beginning of the study, as a baseline and again, prior to challenge, in order to determine whether there were vaccine-induced adaptive responses in the mucosa. 5/7 animals in the C274/wt group, 3/4 animals in the C661/wt group, 5/6 animals in the C274/V1V2 group and 3/3 animals in the C661/V1V2 group exhibited positive vaccine-induced IgA responses, although the actual titers were low in all cases. For the animals with a positive post-vaccination rectal SIV-specific IgA response, the average fold increases, compared to baseline, ranged from 2.2 to 3.9-fold (Fig. 6) and there was no significant difference between the differently immunized groups. IgA levels were also measured at 4 weeks post-challenge. The average fold increases ranged from 2.4 to 4 (Fig. 6), and were similar to those seen post-vaccination, suggesting that mucosal IgA is primarily a vaccine-induced response. However, a 2.7-fold increase in mucosal IgA was also observed for control animals, suggesting that infection also induces this type of humoral response in naïve animals.

To further characterize the SIV-specific immune responses, IFNγ responses to Env and Gag peptide pools were measured and the percentages of effector vs central memory T cell subsets were determined at the time of necropsy in some animals (5-10 months post infection). At this late time point, the average IFNγ response to AT-2 SIV was only 22 SFC/2×10^5 cells. Not singly, minimal Env or Gag peptide-specific responses were seen in all animals tested (1-20 SFC/2×10^5 cells), with no preferential responses to any peptide pool being detected (data not shown). Similarly, there were no significant differences in the numbers of effector memory ($CD28^-CD95^+$), central memory ($CD28^+CD95^+$), or naïve ($CD28^+CD95^-$) $CD4^+$T cell subsets in the blood or lymphoid tissues of the differently immunized animals (see Figure A, Supplemental Digital Content 1, http://links.lww.com/QAI/A23) no non-vaccinated controls were tested in this experiment). In blood and lymphoid tissues, the percentage of central memory T cells was higher than that of effector memory cells. This was more pronounced in lymphoid tissues and blood contained a bigger percentage of naïve $CD28^+CD95^-$ T cells.

Notably, the $CD28^{-}CD95^{+}$ effector memory subset (as well as $CD28^{-}CD95^{-}$ naïve cells) was detected in the ileum and these levels were comparable to those seen at other sites.

To investigate the lack of significant C274 enhancement of the protective effect of AT-2 SIV in the vaccine observed at the virologic and immunologic levels, we applied C274 or C661 *in vivo* on the tonsils and monitored local cellular activation. DCs ($Lin^{-}HLA\text{-}DR^{+}$) and Lin^{+} cells (containing B cells) within the suspensions isolated from pinch biopsies taken 24 hours after ODN application were then monitored for CD80 and CD86 expression (see Figure B, Supplemental Digital Content 1, http://links.lww.com/QAI/A23). CD86 and CD80 expression remained unchanged in both PDC ($CD123^{+}$) and MDC ($CD123^{-}$) subsets after application of C274 to the tonsils. A small increase in CD86 expression was observed in the $Lin^{+}HLA\text{-}DR^{+}$ B cell-containing fraction after C274 application (not significant, p=0.5), whereas CD80 expression remained unchanged. This was not due to the timing, since cells from the tonsils of animals receiving C274 vs C661 48 hours prior to biopsy (n=3 each) similarly showed no change in DC or B cell CD80/CD86 expression (data not shown). Thus, the limited activity of C274 in stimulating local DC/B cell activation following tonsillar application might contribute to its suboptimal ability to boost immunity and the protective effects of AT-2 SIV.

Discussion

This study aimed to test the efficacy of a CpG-C ISS-ODN/AT-2 SIV based vaccine applied to oral MALT in preventing rectal SIV infection. Earlier studies have described the effectiveness of oral/nasal vaccines in inducing responses at distal mucosal sites [4, 5, 10, 12]. We used the accessible palatine/lingual tonsils to model pharyngeal tonsils likely targeted by nasal vaccines for this proof of concept approach against SIV. CpG-C ISS-ODNs are effective activators of B cells and DCs [23, 24] and boost SIV-specific T cell responses *in vitro* 25. AT-2 inactivated virus contains all of the virion proteins, but is non-infectious [18]. Mature DCs presenting AT-2 SIV/HIV stimulate $CD4^{+}$ and $CD8^{+}$ T cells *in vitro* 20 and AT-2 virus-loaded mature DCs showed promise as a therapeutic vaccine [22]. Since animals become infected after tonsillar application of infectious SIV [39, 40] and AT-2 SIV interacts authentically with target cells [19], we hypothesized that AT-2 SIV applied to the tonsils would cross the epithelial barriers, enter the underlying MALT, and induce virus-specific immunity. Two types of AT-2 inactivated SIVmac239 virus were used here: the wild-type and the V1V2 mutant, where the hypervariable loops V1 and V2 of the viral envelope protein have been deleted. The deletion in the latter reveals neutralization sensitive epitopes [41-43]. We postulated that the exposed neutralization face might be presented within the AT-2-treated form to induce a superior Ab response compared to the wild-type form , whilst also inducing similar T cell responses, thereby leading to greater control of infection than the wild-type AT-2 SIV.

Targeting the MALT by vaccinating across the palatine/lingual tonsils provided a controlled way in which we could test the immunogenicity of CpG-C ISS-ODN/AT-2 SIV as a mucosal vaccine. Although SIV-specific T and B cell responses were not detected in the blood after immunization, application of AT-2 SIV to the tonsils protected 53% of the animals from infection by subsequent rectal challenge with pathogenic SIVmac239. It has been previously reported that no nasal vaccine-induced PBMC IFNγ responses were measured prior to significant post rectal challenge protection [44]. Also, the value of the IFNγ ELISPOT as a sole measure of immune activation has been debated in the past [45].

Given the rectal route of challenge used here, SIV-specific IgA were measured in the rectal fluids, prior to challenge. Low-level vaccine-induced SIV-specific Abs were identified in most vaccinees, even though no such Abs were seen in the plasma prior to infection. Rectal SIV-specific IgA were increased 2.2-3.9-fold in all groups, compared to baseline, levels in line with previous publications [17]. As with most other data presented here, no differences were observed

between vaccination groups, suggesting that the vaccine effect observed is due to the AT-2 SIV, irrespective of adjuvant or form of the virus. The presence of these Abs in the pre-challenge mucosal fluids of the vaccinated animals may contribute to the vaccine protective effect observed, although no direct correlation between Ab levels and infection was determined. SIV-specific IgA responses were not boosted when vaccinated animals became infected, although comparable responses were detected in non-vaccinated controls after infection.

SIVmac239 is commonly used in pre-clinical vaccine trials, but has so far been shown to be very difficult to protect against as a homologous or heterologous intravenous or rectal challenge [37, 46-49]. The magnitude of the viral inocula for mucosal challenge with our SIVmac239 stock was comparable to what was used previously [46, 50, 51] and has also been historically very effective in our lab in infecting naïve animals via the rectal route (frequency of infection >90% ; 29 out of 32 challenged monkeys infected). Notably, we observed that 53% of the AT-2 SIV-vaccinated animals were protected from homologous mucosal challenge with SIVmac239. AT-2 SIV vaccinated animals had lower rates of infection than controls, irrespective of C274 or C661 as an adjuvant and irrespective of whether AT-2 wild-type or V1V2 virus was used as the vaccine. The discordance between previously published *in vitro* activities of C274 [25, 26] and the apparent lack of vaccine enhancement by C274 observed herein can be due to a number of reasons. Firstly, differences between *in vitro* and *in vivo* observations are not uncommon [52-54] and can be due to numerous factors, e.g. the active component perhaps cannot efficiently cross the epithelial cell layer to activate the underlying leukocytes. Another possibility is that CpGs, via their effect on PDCs, induce regulatory T cells (Tregs) that suppress further immune activation [55, 56]. It has been recently shown that HIV-stimulated human PDCs can also induce Treg generation [57]. Even though Tregs were not measured here, the lack of differences in the immune responses measured suggests that Treg activation by C274 is unlikely to have played a significant role. A recent study using CpG-B and AT-2 SIV as a therapeutic vaccine in SIV-infected macaques also showed a lack of enhancement of the AT-2 SIV effect by the CpG [58].

AT-2 SIVmac239 (wild-type or V1V2 mutant) applied on the tonsils confers a significant protective effect against pathogenic challenge at the distal rectal mucosa. Many previous macaque vaccine studies, using varied vaccination techniques, such as DNA prime/MVA boost or adenoviral prime/boost, show development of humoral and cellular post-immunization immune responses in the blood, which have not been observed here. In these studies though, all animals became infected upon pathogenic SIV challenge [51, 59-63]. On the other hand, vaccine studies with live attenuated viruses [64-66] or other replication competent viruses [44] seem to show significant protection against pathogenic SIV challenge, but safety concerns may limit translating such research to humans. Not only was the AT-2 SIV vaccine partially effective, it is safer than replicating vaccines. The possibility that cellular components in the vaccine virus preparation [67] might have an effect was not addressed by this study, nevertheless the presence of SIV-specific responses (especially the rectal IgA responses post vaccination) suggest that the vaccine effect is due to SIV antigens. Also, the genetic background of the animals was not examined. However, it seems unlikely that the random assignment of animals to vaccine vs control groups would have divided them into groups with different genetic susceptibilities [68].

Although there was no difference in the frequency of infection between the vaccinated groups, most of the V1V2-vaccinated animals exhibited delayed peak viremia. Significant differences were also observed between the average viral loads of vaccinees, compared to controls, during the first two weeks of infection, suggesting a slight delay in viral replication which is then overcome by the third week of infection. We had hypothesized that the V1V2 mutant might induce more neutralizing Abs [43], which could contribute to improved control of infection, but

there was no difference in the neutralizing Ab activity (in plasma) nor the rectal IgA Ab responses detected between the different groups.

Despite the reduced infection frequency in the AT-2 SIV-vaccinated animals, no considerable differences were observed between groups once the animals were infected, as measured by plasma viremia, CD4 counts, numbers of IFNγ-producing cells and disease progression. There were also no differences in the distribution of effector and central memory $CD4^+$ T cells between vaccinated groups, with central memory cells dominating in the blood and lymphoid tissues and effector memory cells predominating in the gut, as expected [69, 70].

This study suggests that tonsillar immunization with a non-replicating immunogen can help protect against rectal challenge with a highly pathogenic SIV, although we could not correlate SIV-specific immune responses with protection. In addition to reducing the frequency of infection, AT-2 SIVΔV1V2 appeared to better limit the initial amplification of infection in some animals. While C274 appeared to have no boosting effect on the AT-2 SIV vaccination under this regimen, future studies using this and/or other TLR ligands to augment oral/nasal vaccines represent an exciting strategy to tackle HIV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

174×CEM cells were obtained from the NIH AIDS Research and Reference Reagent Program, courtesy of Peter Cresswell. SIV Env and Gag peptide pools were obtained from the NIH AIDS Research and Reference Reagent Program. We thank William Bohn, Jeremy Miller, Terra Schaden-Ireland, Rodman Smith, Robert Imming and Elena Chertova for producing, inactivating, purifying and characterizing AT-2 SIV and MV preparations. We thank Jason Marshall and Gary Van Nest from Dynavax Technologies for the ODNs. We would like to acknowledge the Population Council Cell Core for flow cytometry assistance and the veterinary staff at the TNPRC for their continued support. We thank Irving Sivin (Population Council) for his assistance with statistical analyses and R. Paul Johnson (New England Primate Research Center, Harvard University) for advice on the Env/Gag peptide pools. We thank members of our laboratory for their assistance in editing the manuscript and continued help during the course of this study.

Work presented at the International Congress of Immunology, Rio de Janeiro, Brazil, August 2007 and at the Modern Mucosal Vaccines, Adjuvants and Microbicides conference, Porto, Portugal, October 2008. This project was funded by the National Institutes of Health grant R01 DE016256, the base grant RR00164, and in part with federal funds from the National Cancer Institute, NIH, under contract N01 CO 124000. MR was a 2002 Elizabeth Glaser Scientist.

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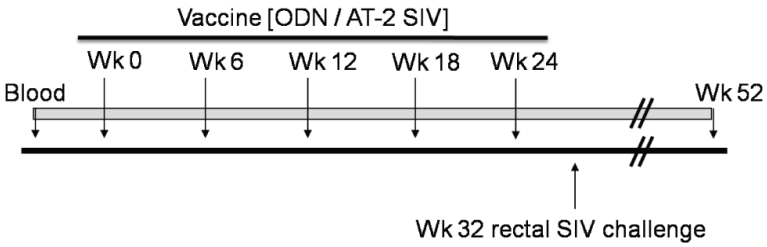

Figure 1. Schematic of vaccination and challenge regimen

Animals were vaccinated 5 times at 6 week intervals. Blood and mucosal fluids were collected at each time point. 8 weeks after the final immunization, all animals were challenged rectally with SIVmac239. Animals were followed up for a further 6 months.

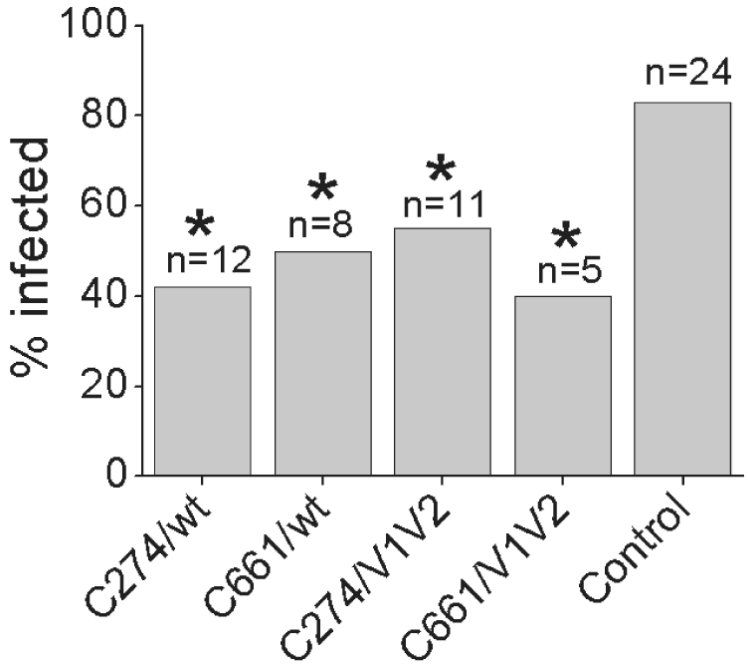

Figure 2. Vaccinated animals have lower frequency of infection

The frequency of infection per vaccination group is shown for all challenges (summarized in Table 1). Asterisks indicate statistically significant difference to control group ($p<0.03$ for each). The numbers above each column indicate the total number of challenges for that group.

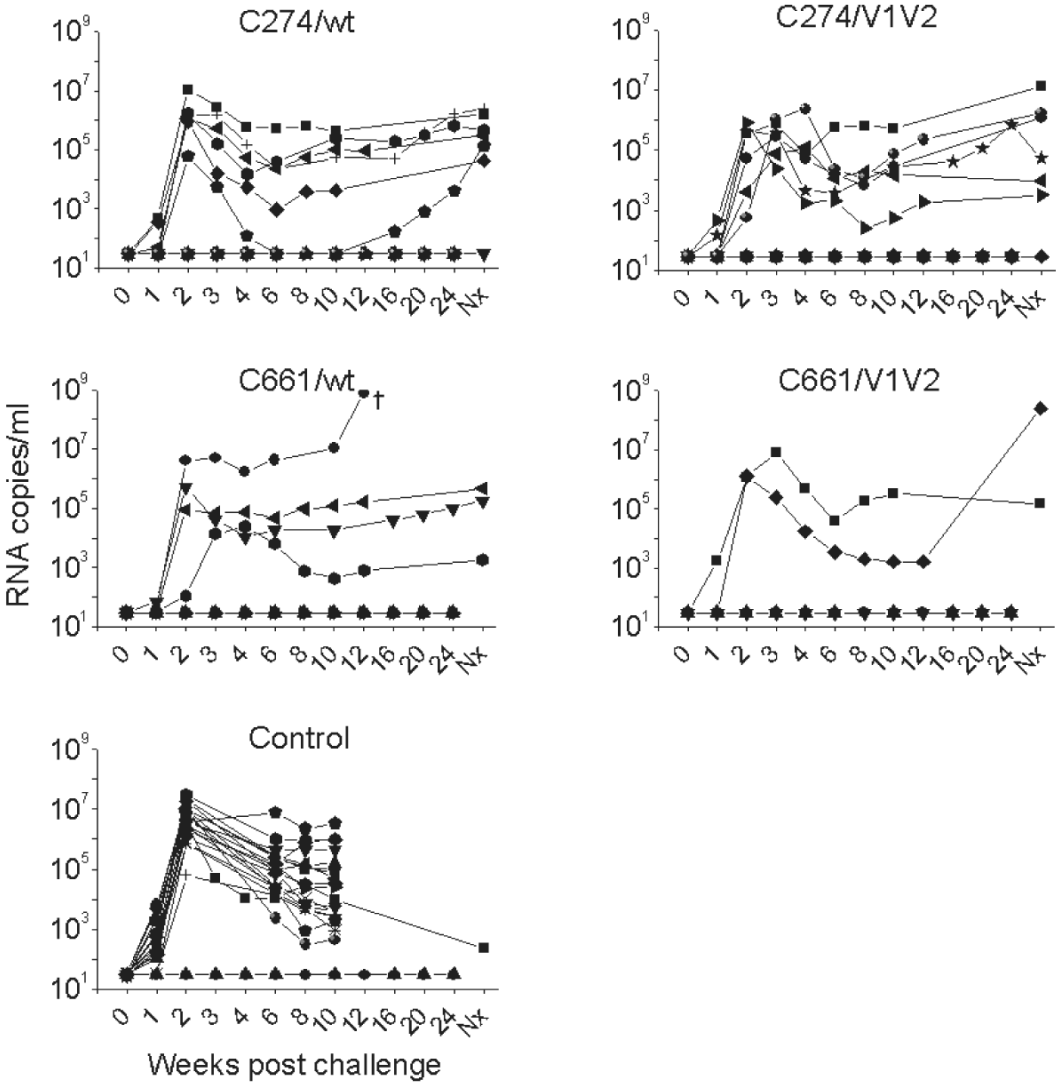

Figure 3. Individual viral loads of vaccinated and control groups

(A) Plasma viral loads (SIV RNA copies/ml) of vaccinated and control monkeys were measured by RT-PCR. Each symbol denotes a different macaque challenge in each group. Nx, necropsy (5-10 months post infection). Profiles end at 10 weeks for 18 control animals that were moved to a different study at this point, having established the outcome of rectal challenge for infection status as well as peak and set point viremia.

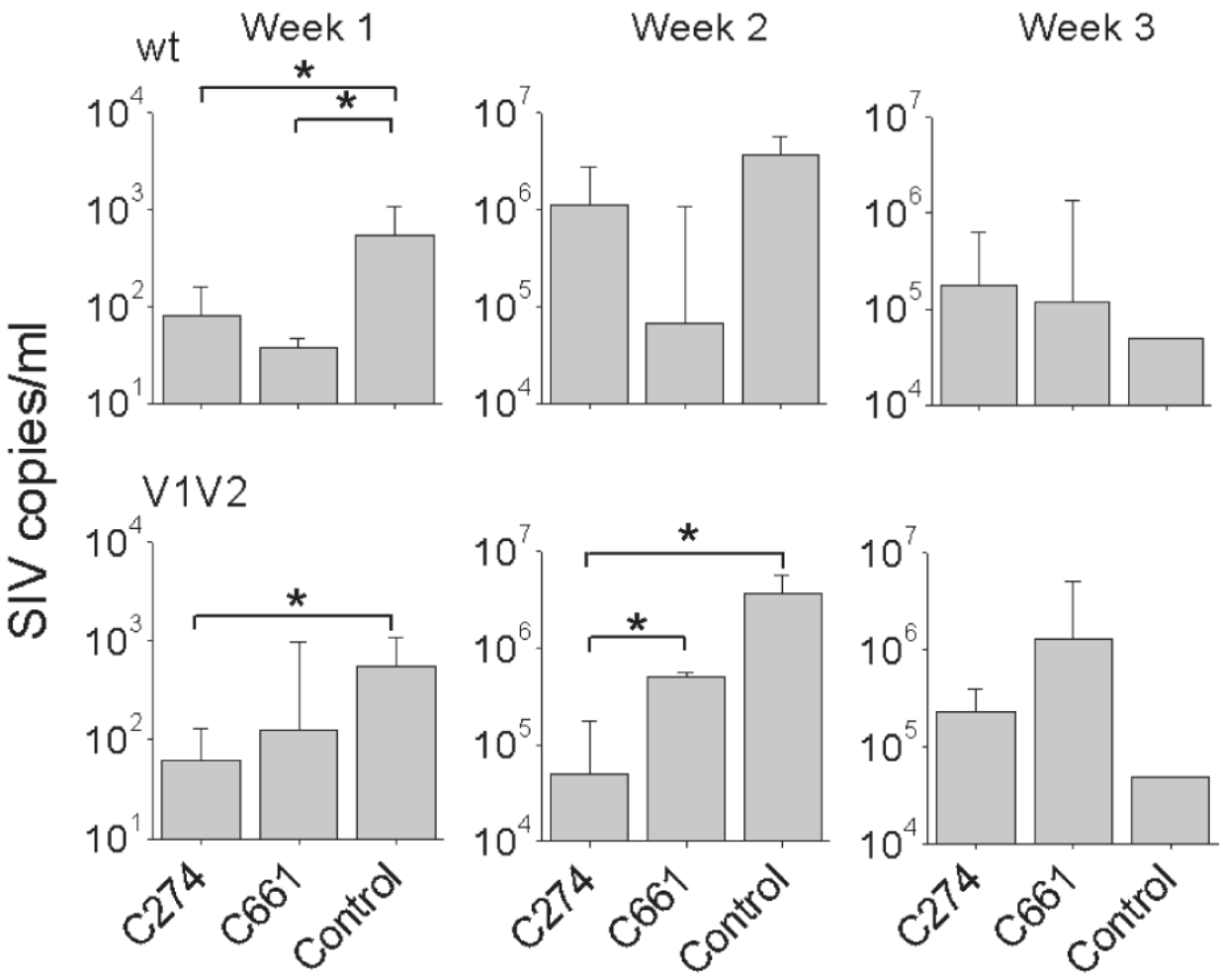

Figure 4. Vaccine reduces early viremia

The geometric means of viral loads (±SEM) are shown for the infected animals in each group, for weeks 1, 2 and 3 post-infection. Wild type-vaccinated animals exhibited significantly lower viremia compared to controls at week 1 (upper panels, $p<0.04$). C274/V1V2-vaccinees had significantly lower viremia than controls at week 1 ($p<0.02$) and both C274 and C661/V1V2-vaccinees had significantly lower viremia than controls at week 2 ($p<0.05$, lower panels). No significant differences between vaccinees and controls were observed at week 3.

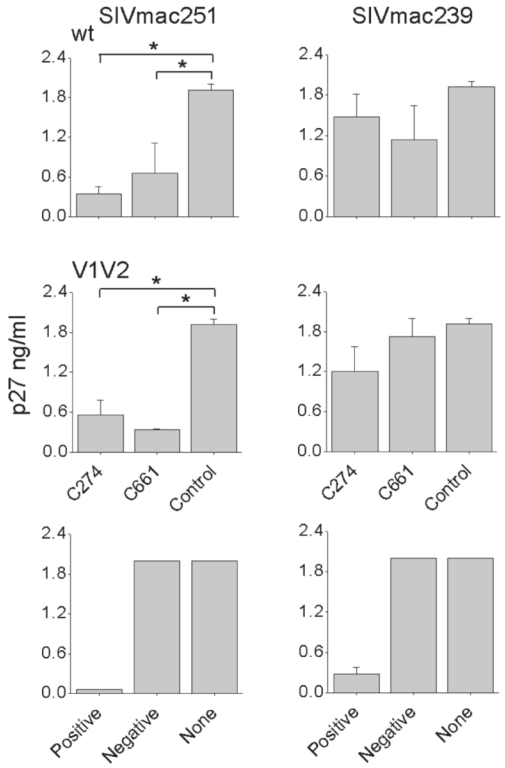

Figure 5. Comparable neutralization Ab responses in vaccinated and non-vaccinated animals

Heat inactivated plasma (diluted 1:25 in medium) was incubated with pathogenic SIVmac251 (left) or SIVmac239 (right) before addition to 174×CEM cultures. SIV p27 production was measured by ELISA after 14 days of culture. Wild type-vaccinated animals are shown in the upper panels and V1V2-vaccinated animals in the middle panels. Assay controls (lower panels) included positive plasma (pooled plasma from SIVΔnef/wild-type long-term infected animals), negative plasma (pooled plasma from SIV-naïve animals) and no plasma (None). Mean p27 values are shown per group (±SEM); n=7 for C274/wt, n=4 for C661/wt, n=7 for C274/V1V2, n=3 for C661/V1V2 and n=23 for Control. Significantly higher neutralizing activity was observed vs. SIVmac251 ($p<0.0003$) for all vaccinees.

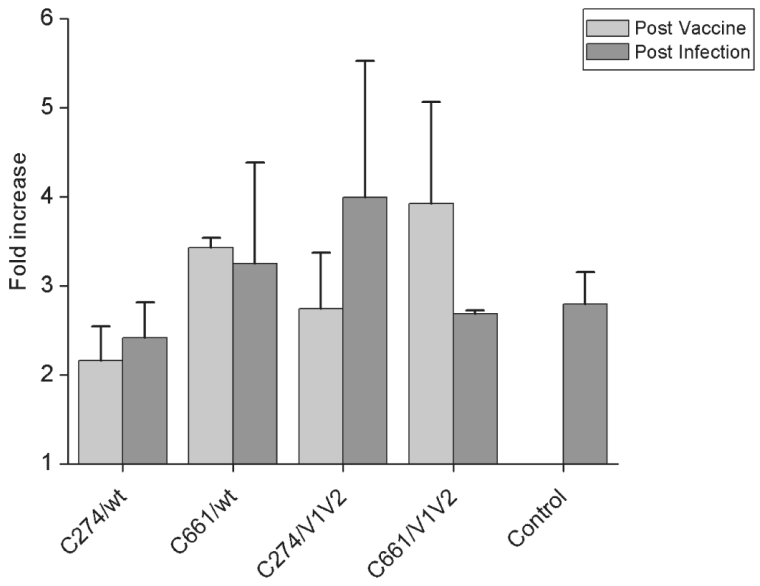

Figure 6. Vaccine-induced mucosal Ab responses

Levels of secreted IgA in rectal fluids were measured by ELISA at baseline, at the last time point prior to challenge and at 4 weeks post-challenge. 5/7 C274/wt animals, 3/4 C661/wt animals, 5/6 C274/V1V2 animals and 3/3 C661/V1V2 animals were positive for post-vaccine rectal IgA. Control animals did not receive vaccinations and are not included in the post-vaccine fold difference determination. 6/7 C274/wt animals, 3/4 C661/wt animals, 4/7 C274/V1V2 animals, 2/3 C661/V1V2 and 14/20 control animals were positive for post-challenge rectal IgA. The mean fold increases per group, relative to baseline, are shown for positive animals.

TABLE 1

Summary of animal treatments, infection and immune status

Immunization	Animal ID	Infection		IFNγ	SIV Ab	CD4 counts	
		1st Challenge	2nd Challenge			Initial	Final
C274/wt	GE89	+		+	+	1486	582
	GE90	−	−	−	−	1312	624
	GE93	+		+	+	1039	401
	GJ88	+		−	+	886	168
	GJ89	−	+	−	+*	394	162
	GJ96	−	+	+*	+*	580	399
	GJ61	−	+	+*	+*	395	123
C274/V1V2	GF02	+		+	+	1461	390
	GF03	−	+	+*	−	1996	385
	GE94	+		+	+	1005	335
	GE95	−	−	−	−	651	559
	GJ62	+		−	+	861	446
	GJ63	+		−	+	603	88
	GJ64	−	+	+*	+*	1385	697
C661/wt	GE91	−	+	+*	+*	1251	204
	GE96	−	+	+*	+*	1327	401
	GJ66	−	+	−	+*	509	460
	GJ67	−	+	+*	+*	1179	464
C661/V1V2	GE97	+		+	+	1107	480
	GE99	−	+	+*	+*	2379	818
	GJ68	−	−	−	−	647	538
Control	GF01	+		+	+	1874	560
	GJ52	−	−	−	−	640	471
	GJ39	+		+	+	302	n/d
	GJ47	+		+	+	508	n/d
	GJ48	+		+	+	842	n/d
	GJ49	+		n/d	n/d	n/d	n/d
	GJ50	+		+	+	1088	n/d
	GJ53	+		+	+	1357	n/d
	GJ55	+		+	+	192	n/d
	GJ57	+		+	+	368	n/d
	CC48	+		+	+	440	n/d
	EL02	+		+	+	285	n/d
	DV67	+		+	+	537	n/d
	DD94	+		+	+	394	n/d
	DA47	+		+	+	510	n/d
	CM96	+		+	+	585	n/d
	CK25	+		+	+	290	n/d
	CL68	+		+	+	627	n/d
	BG93	+		+	+	538	n/d
	GJ65	+		+	+	686	n/d
	GJ97	+		+	+	744	n/d
	CF57	−		n/d	n/d	n/d	n/d
	CN06	−		n/d	n/d	n/d	n/d

SIV-specific (AT-2 SIVmac239 and AT-2 SIVmac239ΔV1V2) IFNγ-producing cells measured by ELISPOT post infection; a positive is given to animals with more than 10 SIV-specific SFC/ 2×10^5 cells at more than one time-point. To obtain SIV-specific values, the number of spots induced by SupT1 MV was subtracted from the SIV value. SIV Ab positivity measured by ELISA on plasma collected at 4 and 8 weeks post-infection.

* animal became IFNγ or SIV Ab positive after 2nd challenge. Infection status was confirmed by RT-PCR on plasma samples (supported by the detection of infectious virus in PBMC-174×CEM co-cultures). Two animals (GE90 and GE95) were challenged a 3rd time (not shown) and remained negative; these data are included in later calculations. CD4 counts are given at baseline (initial) and necropsy (final) as CD4 cells/μl blood (measured by FACS at TNPRC). n/d=not determined.

J Acquir Immune Defic Syndr. Author manuscript; available in PMC 2010 December 1.