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Comparative study of Tat vaccine regimens in Mauritian cynomolgus and Indian rhesus macaques: influence of Mauritian MHC haplotypes on susceptibility/resistance to $SHIV_{89.6P}$ infection

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Abstract

Protection afforded by HIV Tat-based vaccines has differed in Indian rhesus and Mauritian cynomolgus macaques. We evaluated native Tat and Ad-HIV*tat* priming/Tat-boosting regimens in both species. Both vaccines were immunogenic. Only the Ad-tat regimen modestly reduced acute viremia in rhesus macaques after $SHIV_{89.6P}$ challenge. Confounding variables uncovered in Mauritian macaques included significant associations of susceptibility to infection with MHC class IB and class II H2 and H5 haplotypes, and resistance to infection with class IB haplotypes H3 and H6. Although protection here was limited, Tat-based vaccines incorporating other HIV components have shown greater efficacy. Combination strategies should be further explored.

1. Introduction

The HIV pandemic is a major and urgent public health concern. At least 40 million people worldwide are infected with the virus. Thus, development of an effective vaccine continues to be a critical need. Among target HIV antigens for vaccine development is Tat, the potent transcriptional transactivator of HIV gene expression. Tat is produced early after infection [1,2] and is indispensable for viral replication, transmission, and AIDS pathogenesis [3-6]. Release of Tat from infected cells and its uptake by infected and uninfected cells is critical to the biology of the virus [5,7-10]. In infected cells, Tat promotes viral replication or

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transactivates the replication of *tat*-defective or latent proviruses [11]. In uninfected cells, Tat can modulate cellular gene expression [3,12,13], up-regulate HIV co-receptors [14,15], and induce or inhibit apoptosis [16,17]. Early inhibition of Tat function should contribute to control of viral replication and slowing of AIDS progression. In fact, Tat-specific CTLs are associated with control of virus replication early in infection [18] and both anti-Tat antibody and Tat-specific CTLs have been correlated with reduced viremia and slow progression to AIDS [19-21].

Conflicting results have been reported following vaccination with Tat-based vaccines. Immunizations of rhesus macaques with Tat protein, vectored *tat*, Tat toxoid or Tat peptides have elicited no protection [22,23] or partial protection [24,25] against $SIV_{mac239,}$ $SHIV_{33,}$ or $SHIV_{89.6P}$ challenges, while immunizations of cynomolgus macaques with native Tat protein or DNA encoding *tat* have shown strong, long-term protective efficacy against $SHIV_{89.6P}$ [26-29].These contrasting results might reflect species differences with regard to immunogenicity or host resistance factors, or differences in vaccine characteristics, vaccination routes, delivery systems, timing of immunizations or challenge protocols. Here we addressed these issues, eliminating the latter variables by conducting two identical immunization and challenge protocols in Indian rhesus and Mauritian cynomolgus macaques. The first approach replicated previous studies in cynomolgus macaques in which multiple immunizations with native HIV Tat protein were shown to elicit long-term protection against $SHIV_{89.6P}$ in Mauritian cynomolgus macaques [26,29]. The second approach was based on a replication-competent Ad-recombinant vaccine strategy [30]. These replicating vaccines have been shown to elicit better cellular immune responses and prime higher titered antibodies, including functional antibodies, compared to replication-defective Ad-recombinants encoding the same HIV genes [31,32]. When combined with envelope subunit boosting, the vaccine strategy has shown potent protection against virulent SIV_{mac251} challenge [33] and durable protective efficacy with no intervening boost [34].

Studies using both vaccine regimens were conducted in the two non-human primate models, and immunogenicity and protective outcomes following challenge with $SHIV_{89.6P}$ were compared. As the entire repertoire of MHC alleles can now be predicted for essentially all Mauritian cynomolgus macaques [35-37], we also determined the MHC genotypes of the study animals. These investigations revealed a new association of Mauritian MHC haplotype and susceptibility/resistance to $SHIV_{89.6P}$ infection. The association of particular MHC alleles with resistance of rhesus macaques to SIV and SHIV infection is well established [38-43]. Our results here extend the phenomenon to cynomolgus macaques of Mauritian origin. The significantly higher peak Tat-specific T cell proliferative responses seen in vaccinated macaques with the resistant haplotypes prior to challenge suggest cellular immunity should be further explored as a possible mechanism for the observed resistance. Results of the haplotype analysis and vaccine evaluations, together with reports showing that vaccines targeting Tat in combination with other viral proteins elicit good protective efficacy in non-human primates [44-46], suggest that HIV Tat vaccines might be best exploited in combination with other viral antigens.

2. Materials and methods

2.1. Vaccines

Escherichia coli- expressed HIVIIIB Tat protein (Advanced Bioscience Laboratories, Inc., (ABL) Kensington, MD), greater than 95% pure and retaining full biological activity [7], was lyophilized and stored at -70°C prior to use. To retain activity, Tat for immunizations was freshly reconstituted at 4 μg/μl in degassed phosphate buffered saline (PBS; Invitrogen) containing 0.1% BSA (Sigma-Aldrich) and 0.1mM dithiothreitol (DTT), capped, covered with foil, and kept on ice. All plasticware was pre-rinsed with PBS-BSA buffer. For subcutaneous

administrations, Tat was diluted in cold PBS, mixed with an equal volume of alum and inoculated (10 μg/500 μl final concentration). For intradermal administrations, Tat was diluted in PBS to a concentration of 6 μg/250μl. Tat-immune stimulating complexes (ISCOMS) [47] were prepared by adding 200 μl of the ISCOM matrix to 50 μg lyophilized Tat, mixing, and incubating with slow stirring at room temperature for 30 minutes. The mixture was cooled on ice, diluted with PBS to 600 μl and administered (200 μl/dose) intramuscularly as soon after preparation as possible.

Replication-competent Ad5hr-HIV*tat* has been described [48]. Control immunogens included an empty Ad5hr E3-deleted vector, alum, and ISCOM matrix.

2.2. Animals, immunization and sample collection

Indian rhesus (*Macaca mulatta*) and Mauritian cynomolgus (*Macaca fascicularis*) macaques were maintained according to guidelines and protocols of the Animal Care and Use Committee, Washington National Primate Research Center, University of Washington (Seattle, WA). Identical immunization regimens were followed for both cynomolgus and rhesus macaques (Table 1). The schedule of Tat protein immunizations was published previously [26]. The experimental cynomolgus immunization groups contained 9 animals each, however, 2 macaques in the Ad5hr-HIV*tat* group died from anesthesia complications prior to challenge. Pre-challenge data are reported for all nine macaques and post-challenge data for the remaining seven. Control cynomolgus groups contained 3 macaques each. All cynomolgus macaques were males. The rhesus experimental immunization groups contained 8 macaques each; 3 macaques were in each control group. Five of eight macaques in the Tat protein and four of eight macaques in the Ad5hr-HIV*tat* groups were females. Control groups each contained two females and one male. All rhesus macaques were negative for Mamu A*01, but two were positive for Mamu B*17 (A02005 in the Ad5hr-HIV*tat* group and A02023 in the adjuvant control group).

Peripheral blood mononuclear cells (PBMCs), collected before, during, and after immunization, were purified using lymphocyte separation medium (ICN Pharmaceuticals, Inc.) for rhesus and Ficoll-Paque^{TM} PLUS (Amersham Biosciences) for cynomolgus samples and used fresh for immunological assays. Plasma and sera were aliquoted and stored at -70°C until use.

2.3. Challenge virus

All macaques were challenged intravenously (IV) at week 50 with $SHIV_{89.6P}$. Rhesus macaques received 30 MID_{50} of a $SHIV_{89.6P}$ stock [49] kindly provided by Drs. Norman Letvin and Keith Reimann, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA. Cynomolgus monkeys received 15 MID_{50} of a $SHIV_{89.6P}$ stock derived from a cynomolgus macaque inoculated with the original $SHIV_{89.6P}$ rhesus stock and termed $SHIV_{89.6Pcy243}$ [26].

2.4. ELISPOT Assay

PBMCs secreting gamma interferon (IFN-γ) in response to overnight stimulation with a single pool of Tat 15-mers (1 μg/ml each) were enumerated using ELISPOT kits (U-Cytech, Utrecht, The Netherlands) as described [50]. Assays were performed in triplicate; background spots in wells containing only medium (RPMI 1640 containing 5% fetal calf serum, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) were subtracted. A positive response is defined as at least 10 spot forming cells (SFC) per million PBMC after subtraction of the mean SFC of control macaques plus two standard deviations at each time point evaluated.

2.5. T-Cell Proliferation Assay

Oxidized Tat was used for proliferative assays. Lyophilized Tat was reconstituted with degassed buffer as described above, exposed to light and air for 2 hours at room temperature, capped and exposed to light overnight, then aliquoted and stored at -70°C until use. Freshly isolated PBMC (10^5 cells/well) were cultured for five days in triplicate in 200 μl of RPMI 1640 medium containing 10% fetal calf serum (FCS), 1mM L-glutamine and 100 U/ml penicillin, 100μg/ml streptomycin (R-10) with 1μg of oxidized Tat /well at 37°C. On the fifth day, cells were pulsed overnight with ^{3}H-thymidine (1μCi/well), harvested and counted as described [50]. Stimulation indices (SI) were calculated by dividing mean counts per minute (cpm) with Tat by mean cpm with R-10 plus degassed buffer. A positive response is defined as an SI of 2 or more after subtracting the mean SI of control animals + 2 standard deviations at each time point tested.

2.6. Antibody Assay

Serum binding antibodies to HIV Tat were determined by enzyme–linked immunosorbent assay (ELISA) [51]. Antibody titer was defined as the reciprocal of the serum dilution at which the absorbance of the test serum was twice that of a serum from a naïve macaque diluted 1:50.

2.7. Viral RNA and proviral DNA detection

Viral RNA in plasma was determined by nucleic acid sequence-based amplification (NASBA) as described [52]. Sensitivity of the NASBA assay is less than 2000 viral copies/input volume. A real time assay with a sensitivity of <50 copies/input volume [34] was used when plasma samples exhibited viral loads below the NASBA sensitivity level. For proviral DNA analysis, cellular DNA was purified using QIAamp DNA mini kits (QIAGEN Inc., Valencia, CA USA). SIV *gag* DNA was detected by nested PCR and confirmed by Southern blotting and hybridization to a ^{32}P-labeled SIV *gag* probe as described [51]. The first PCR reaction consisted of 500 ng of purified DNA, 25 μl of 2X ready mix Go Taq^R Green Master Mix (Promega, Madison, WI), 10 pmoles of each outer SIV *gag* primer, and distilled water to a final 50μl reaction volume. The second PCR reaction used a 10 μl aliquot of the first PCR product as DNA template and the inner primer pair. Thirty amplification cycles (1 minute denaturation at 94°C, 1 minute of primer annealing at 58°C, and 1.25 minute of extension at 72°C) were performed for each reaction followed by a final primer extension of 7 minutes. Positive controls (plasmid pCMV SIV-gag DX and a proviral-positive DNA sample) and negative controls (distilled water and DNA extracted from pre-challenge PBMC samples) were run concurrently with test samples.

2.8. MHC Microsatellite Haplotype Analysis

Microsatellite PCR assays were performed with genomic DNAs and a panel of 16 markers spanning the 5-Mb MHC region essentially as described previously [37,53]. MHC haplotype predictions were generated based on the microsatellite profiles, inferring alleles for the class I and class II regions based on previously established haplotype-allele associations [36,37].

2.9. Mafa-B*510101 sequence-specific PCR

PBMC RNA was isolated with a MagNA Pure LC RNA Isolation kit (Roche Applied Science, Indianapolis, IN). Complementary DNA (cDNA) was synthesized using a Superscript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed on a LightCyler 480 (Roche Applied Science, Indianapolis, IN) with cDNA templates and SYBR Green PCR Master Mix (Applied Biosytems, Foster City, CA) in a 10ul final volume. Amplification of a 140 bp cDNA-PCR product from the Mafa-B*510101 allele was achieved with the primer pair Mafa-B*510101-SPPF, 5'-CAAGGACGCCGCACAGT, and Mafa-B*510101-SPPR, 5'- GATACCCGCGGAGGAGGT. The thermal cycling conditions used

were: activation at 95°C for 10 min, amplification between 60°C for 30 sec and 95°C for 30 sec x 40 cycles, and a final denaturation between 60°C and 95°C (30 acquisitions/sec) to generate melting profiles (PCR product Tm = 87.1 °C).

2.10. Statistical Analysis

Differences in peak and chronic phase viremia between immunization groups, species, and CD4 counts among the macaques pre- and post-challenge, were evaluated using the exact two tailed Wilcoxon rank sum test. CD4 decline between groups of cynomolgus macaques was compared using the two tailed Student's t test. The Cochran-Armitage trend test was used for the comparisons of antibody titers and analysis of MHC haplotype distribution among macaques grouped by chronic viremia outcomes. Analysis of peak viremia levels, MHC haplotypes, and proliferative responses used the Kruskal-Wallis test and the Wilcoxon rank sum test.

3. Results

3.1. Pre-challenge immune responses to Tat

Strong humoral immunity was elicited in both species by both vaccine regimens. In cynomolgus monkeys (Fig. 1A), anti-Tat titers first appeared in the Tat protein group at week 4. By week 8, the titers were significantly higher than those in controls, and the difference persisted until the time of challenge ($p = 0.0045$). In contrast, Tat–specific antibody in the Ad5hr-HIV*tat* group first appeared after the second Ad5hr-HIV*tat* immunization and became significantly increased above control levels after the second Tat protein boost at week 36 ($p = 0.018$). This difference was maintained until week 48. The Tat protein group had significantly higher Tat-specific binding titers than the Ad5hr-HIV*tat* group from weeks 14 through 34 ($p<0.0004$). This higher titer was also observed at week 48, two weeks prior to challenge ($p = 0.0056$; Fig. 1A).

A similar pattern was observed in the rhesus macaques (Fig. 1B). The Tat protein group showed significantly higher Tat antibody titers compared to the adjuvant controls beginning at week 4 until the time of challenge (weeks 4-38, $p = 0.0061$; week 48, $p = 0.012$) The Ad5hr-HIV*tat* group first exhibited elevated titers compared to controls at week 34, and the difference was maintained until week 48 ($p = 0.024$). Compared to the Ad5hr-HIV*tat* group, the Tat protein group had consistently elevated anti-Tat titers (weeks 4 – 30, $p<0.0015$). The higher titers were maintained, although the significant difference disappeared by week 48, $p = 0.14$; Fig. 1B).

Overall, the Tat protein vaccine elicited higher anti-Tat titers in cynomolgus compared to rhesus macaques, beginning at week 14 and over the immunization course (p values from 0.039 to 0.0023). The difference at week 48 prior to challenge was significant at the $p = 0.018$ level. The Ad5hr-HIVtat regimen also elicited slightly higher anti-Tat titers in cynomolgus compared to rhesus macaques, reaching a significant difference at week 48 prior to challenge ($p = 0.037$).

Cellular immunity was elicited by both vaccine regimens in both animal models, although less potent relative to the induced humoral immunity (Table 2). Numbers of Tat-specific IFN-γ secreting cells induced were low in both species, as was the frequency of positive responses. The Ad5hr-HIV*tat* regimen elicited two- to five-fold higher mean peak ELISPOT responses than the Tat protein regimen in the cynomolgus and rhesus models respectively, but similar percentages of responding macaques were seen in both species. Most macaques of both species also exhibited T cell proliferative responses induced by both vaccine regimens, although with a low frequency similar to the ELISPOT results (Table 2). Overall, neither non-human primate model displayed a consistently better cellular immune response to the vaccines.

3.2 Outcome of $SHIV_{89.6P}$ challenge

Following $SHIV_{89.6Pcy243}$ challenge, all cynomolgus macaques became infected (Fig. 2A-D). The majority of animals exhibited high peak viremia followed by a decline in the chronic phase of infection. However, one adjuvant control (04020, Fig. 2B) and two macaques each in the Tat protein (04016, 04017, Fig. 2A) and the Ad5hr-HIV*tat* groups (04022, 04023, Fig. 2C) never exhibited detectable viremia, although PBMC from these five macaques were positive for SIV *gag* proviral DNA at one or more time points (Fig. 2A-C). The aviremia in adjuvant control 04020 and the rapid viremia control in Ad5hr control 04026 (Fig. 2D) made it impossible to attribute aviremia in the immunized macaques to the vaccine or a host control mechanism.

Following $SHIV_{89.6P}$ challenge, all rhesus macaques became productively infected (Fig. 3A-D). In both the Tat protein group and adjuvant controls high peak viral burdens declined to variable set points. Two controls and one immunized macaque rapidly controlled viremia to undetectable levels (Fig. 3A, B), but overall no protection was observed. However, the Ad5hr-HIV*tat* regimen resulted in significant reduction in geometric mean peak viremia compared to controls (4×10^7 versus 3×10^8 SIV RNA copies/ml plasma; $p = 0.024$). This modest protective effect was not maintained in the chronic phase (Fig. 3C, D).

The CD4+ T cell counts in the two species reflected the viral burdens (data not shown). No differences were observed between the counts of immunized macaques of either species and their respective control groups. Overall, the cynomolgus macaques maintained higher CD4 counts over weeks 3-18 post-challenge compared to the rhesus macaques, whether they were immunized with Tat protein ($p = 0.0025$) or the Ad5hr-HIV*tat* regimen ($p = 0.0037$).

3.3 Analysis of MHC haplotypes

The characterization of six common MHC haplotypes in feral Mauritian cynomolgus macaques [37] allowed determination of MHC genotypes for the cynomolgus macaques studied here. Microsatellite allele profiles were used to infer haplotypes spanning the 5–Mb MHC region and deduce complete genotypes for class I and class II alleles (Fig. 4). All MHC haplotypes observed were consistent with those previously reported for feral Mauritian-origin cynomolgous macaques except for two macaques (04022 and 04019) that shared a novel microsatellite profile for the MHC class I region of one of the paired haplotypes. Additional genotyping of several hundred feral Mauritian cynomolgus macaques has confirmed that this rare MHC haplotype, designated H7, is present in approximately 1% of the feral Mauritian population (RWW, JAK & DHO, unpublished results). In several cases, MHC alleles for two alternative haplotypes could not be distinguished with current microsatellite markers for ambiguous chromosomal regions flanking recombination breakpoints (hatched areas in Fig. 4). However, two of the four ambiguous class IB regions were tentatively resolved using a sequence-specific cDNA/PCR assay for Mafa-B*510101, an allele encoded on the H3 haplotype. cDNA from animal 04020 contained the Mafa-B*510101 allele but this allele was not detected in animal 04010, suggesting the presence and absence of the class IB H3 haplotype, respectively (Fig. 4).

An intriguing MHC genotype/phenotype correlation emerged when the animals were grouped according to virological outcome after $SHIV_{89.6P}$ challenge rather than their immunized or control status. Three clear categories were observed: A) those that never exhibited detectable viremia, B) those that exhibited acute viremic but controlled chronic viremia to below 50 $SHIV_{89.6P}$ RNA copies/ml, and C) those that maintained high chronic viremia. All seven cynomolgus macaques possessing the H6 class 1B MHC haplotype remained aviremic or controlled chronic viremia (Fig. 4). Likewise, six of seven animals that possessed the H3 1B haplotype were aviremic or controlled viremia, while only 1 remained viremic. Thus, 100%

of macaques that remained aviremic and 80% of those that controlled viremia possessed either an H3 or H6 IB haplotype, while only 14 % of macaques that remained viremic had either of these haplotypes (significant trend for resistance, p = 0.0014; Fig. 4). Conversely, macaques that possessed either the H2 or H5 class IB haplotype appeared more susceptible to $SHIV_{89.6P}$ infection: 0 of 5 were aviremic and 2 of 10 controlled viremia, while 6 of the 7 remaining viremic animals carried one of these two haplotypes (trend analysis: p = 0.0014; Fig. 4).

These correlations of MHC haplotype with susceptibility/resistance to $SHIV_{89.6P}$ challenge were supported by longitudinal analysis of viral loads (Fig. 5A). Macaques with the H2/H5 haplotype exhibited peak viral loads significantly elevated 2 to 3 logs compared to H1/H4 and H3/H6 macaques respectively (peak acute viremia weeks 1-4 for H2/H5 macaques versus all others: p = 0.0006). The peak acute viral load for H3/H6 macaques vs all others has a p value of 0.015. These differences persisted during chronic infection (weeks 8 – 52) where H2/H5 macaques maintained higher viral loads than the others (p = 0.013) and H3/H6 macaques exhibited lower viral loads than the others (p = 0.023).

An effect of the MHC class IB haplotypes on pathogenesis was seen by a greater CD4 decline in animals that became productively infected after $SHIV_{89.6P}$ challenge. Cynomolgus macaques with H3 and H6 haplotypes had a smaller drop in CD4 counts over weeks 0 and 28 (621 ± 93) compared to non-H3 and H6 macaques (911 ± 69; p = 0.027). The CD4 decline of H2 and H5 macaques (904 ± 79) vs all others (655 ± 90) was not statistically significant (p = 0.067).

These correlations suggested that the H2/H5 and H3/H6 haplotypes had a strong effect on susceptibility/resistance to $SHIV_{89.6P}$ infection and may have obscured a protective effect of the vaccine regimens on challenge outcome. After challenge, 69% of the 16 immunized cynomolgus monkeys were aviremic or controlled viremia. Similarly, 67% of the 6 controls were aviremic or controlled viremia. A retrospective analysis of a larger cohort of vaccinated and control Mauritius cynomolgus macaques is underway to further examine the role of MHC class 1B haplotypes on $SHIV_{89.6P}$ infection and vaccine-induced protection (Ensoli et al, in preparation).

3.4 Analysis of cellular immunity by haplotype

The correlation of viral burden with MHC haplotype implicates cellular immunity in chronic viremia control. ELISPOT responses of macaques grouped by MHC haplotype revealed no differences pre- or post-challenge in Tat-specific IFN-γ secretion with respect to MHC haplotypes in the vaccinated macaques or post-challenge in the controls (data not shown). However, peak Tat-specific T cell proliferative responses pre-challenge were higher in vaccinated macaques with the H3 or H6 haplotype compared to all others (Table 3; p = 0.011). Post-challenge the H3 and H6 macaques continued to display higher peak SI (Table 3) although not significantly different from non-H3/H6 macaques, even when the aviremic macaques which lacked continual stimulation *in vivo* were excluded. Further, post-challenge the control macaques did not exhibit differences in SI with respect to MHC haplotype. These results suggest that vaccination against Tat of H3/H6 macaques rather than non-H3/H6 macaques might elicit Tat-specific T cell proliferative responses and better control of chronic viremia, a hypothesis currently being explored in a larger cohort of animals (Ensoli et al, in preparation).

Viral loads by haplotype groupings were examined after omitting aviremic macaques to eliminate reduced acute phase viral burdens mediated by unknown mechanisms. H2/H5 macaques continued to display elevated chronic viremia, 0.5 to 1.5 logs higher than H1/H4 and H3/H6 macaques respectively (Fig. 5B). During acute infection (weeks 1 – 4) the H2/H5 macaques still displayed higher peak viremia compared to all others (p = 0.0079). Viremia of

H2/H5 macaques two-weeks post-challenge was higher than that of all others ($p = 0.0010$), while that of H3/H6 macaques was lower ($p = 0.036$). Three of the six H3/H6 macaques vaccinated with Tat exhibited delayed peak viremia, and two of these three exhibited an anamnestic T cell proliferative response (data not shown) implying a vaccine effect.

T cell proliferation is a surrogate for MHC class II-restricted CD4 T helper cell responses [54]. The majority of Mauritian macaques with H3 or H6 MHC class IB haplotypes also had H3 and H6 class II haplotypes (5 of 7 for both), and all macaques with H2 or H5 class IB haplotypes also had H2 and H5 class II haplotypes (Fig. 4). Ten of eleven macaques with H3 or H6 class II haplotypes were aviremic or controlled viremia, while 1 of 11 remained viremic, a non-significant trend for resistance ($p = 0.15$). But macaques with H2 and H5 class II haplotypes exhibited a significant trend for susceptibility ($p = 0.0014$): none were aviremic, 2 of 8 controlled viremia, and 6 of 8 remained viremic. Analysis of longitudinal viral loads by MHC class II haplotypes showed that macaques with the H2 or H5 class II haplotype had higher viremia levels (data not shown). Acute viremia in macaques with class II H2/H5 haplotypes vs non-H2 and H5 macaques was higher when all macaques were included ($p = 0.0006$) and when aviremic macaques were excluded ($p = 0.0079$), as was chronic viremia: ($p = 0.025$ with all macaques included; $p = 0.015$ with aviremic macaques excluded).

4. Discussion

In this study identical vaccine protocols in Mauritian cynomolgus and Indian rhesus macaques addressed previously reported disparate outcomes of Tat-based vaccine regimens in these animal models. Strong anti-Tat antibodies were elicited in both species, with the highest titers seen in the cynomolgus macaques immunized with Tat protein. In contrast, weak cellular immunity was elicited in both species by both vaccine regimens. As strong induction of IFN-γ secreting cells by the Ad5hr-HIV*tat* recombinant was previously seen in mice [48], fewer Tat T cell epitopes may be recognized in non-human primates. Epitope mapping could resolve this question. Tat-specific proliferation was also low compared to results of previous cynomolgus monkey studies [26], but no basis for this difference could be discerned.

Following the $SHIV_{89.6P}$ challenges only rhesus macaques vaccinated with the Ad5hr-HIV*tat* regimen showed a transient 1 log reduction in acute viremia. When corrected for multiple comparisons in a multi-arm vaccine study, this protection was no longer statistically significant [44]. In the cynomolgus model, however, MHC class IB haplotypes were seen to influence the course of $SHIV_{89.6P}$ infection. Animals carrying the H6 or H3 class IB haplotypes displayed chronic phase viral loads near the limit of detection after $SHIV_{89.6P}$ challenge, while animals with H2 or H5 class IB haplotypes, maintained chronic viremia ~20-fold higher than the cohort as a whole. Higher viremia was also seen in macaques with H2 and H5 class II haplotypes. As most of the cynomolgus macaques in this study were concordant for class IB and class II haplotypes (Fig. 4), it will be important to examine whether both contribute to $SHIV_{89.6P}$ control and the immunologic mechanisms responsible. Recently, class II alleles have been shown to influence SIV viremia levels in rhesus macaques [55]. Here, vaccinated macaques with H3 or H6 haplotypes exhibited higher peak proliferative responses to Tat prior to challenge, suggesting that cellular immunity may contribute to the resistant phenotype. The remarkably simple MHC genetics of the geographically isolated Mauritian cynomolgus population [37] can be exploited in prospective studies to further explore the relationship between MHC haplotypes, immune response, and susceptibility to infection with $SHIV_{89.6P}$ and other SHIV isolates or SIV strains.

The lack of protection in the cynomolgus macaques immunized with Tat protein contrasted with earlier results in which the identical vaccine regimen protected against the same $SHIV_{89.6Pcy243}$ stock [26]. The reason for this difference is not known, but the 10 MID_{50}

challenge dose instead of the 15 MID_{50} used here might have played a role. Challenge dose effects will be explored in depth in a large retrospective cohort study (Ensoli et al, in preparation).

The association of MHC class IB haplotypes with viremia control in Mauritian cynomolgus macaques is not surprising. In humans, HLA-B alleles exert a dominant influence on the outcome of HIV infection, with particular HLA-B allele expression associated with control of viremia, CD4 count, and rate of disease progression [56]. HLA-B*27 and B*57 are associated with delayed AIDS progression, while HLA-B*35 is associated with accelerated AIDS onset [57]. In rhesus macaques, Mamu-B*17 is associated with reduced plasma viremia and slowed disease progression following infection with SIV_{mac239} [40,42], although by itself it does not guarantee better disease outcome [58]. Mamu-B*08 positive rhesus macaques display reduced chronic phase viremia following SIV_{mac239} infection, and the allele is overrepresented in elite controllers [38]. The particular *Mafa-B* sequences within the Mauritian H3 and H6 class IB haplotypes associated with resistance to SHIV infection and the identity of epitopes recognized remain to be identified. The basis for the association of the H2 and H5 class IB haplotypes with greater susceptibility to SHIV infection also needs elucidation. The HLA-B*35 allele has been reported to actively exert a negative effect [57]. *Mafa-B* sequences of the H2 and H5 class IB haplotype may behave similarly.

Susceptibility/resistance phenotypes of the Mauritian cynomolgus macaques may also be influenced by interactions of the highly polymorphic killer immunoglobulin-like receptors (KIR) present on natural killer (NK) cells and their equally polymorphic ligands, MHC class I molecules. NK cells provide a rapid initial defense against invading pathogens, and KIR by recognizing specific MHC class I molecules on target cells regulate their inhibition or activation. Specific interactions between distinct KIR3DL1 alleles and HLA-B loci have been shown to delay AIDS progression, contain HIV replication, and protect against opportunistic infections [59-62]. An absence of specific HLA ligands for inhibitory KIR has also been associated with the resistance of highly-exposed persistently seronegative individuals to HIV infection [63]. Similar interactions may be uncovered in the Mauritian cynomolgus macaques, a task that should be facilitated by the simple genetics of this population.

Our findings suggest an explanation for results reported earlier in which a majority of naïve Mauritian cynomolgus macaques naturally controlled SIV or SHIV replication [64]. The control was associated with early IFN-γ responses to Gag and Env peptides post-challenge. Here, only Tat responses were evaluated, so further studies are needed to examine other cellular responses by haplotype in depth. Confirmation of our findings will be important for future vaccine trials to allow selection of macaques that will exhibit susceptibility to SHIV infection, thus providing the sensitivity needed for low-dose challenge studies, while avoiding resistant animals that naturally control viral infection and confound vaccine experiments.

While little protection was elicited here by the Ad5hr-HIV*tat* or Tat protein regimens, protection was observed in previous Tat vaccine studies [26,28] in which a lower challenge dose (10 MID_{50}) was used. Tat combined with other HIV antigens might better confer protection at higher challenge doses. Immunizations with Tat plus Rev and Tat plus other non-structural HIV gene products have shown protection against SIV [45,46]. A potential synergy between Tat and Env leading to enhanced protective efficacy in rhesus macaques against $SHIV_{89.6P}$ was recently reported [44]. Prospective studies using Tat-based vaccine strategies are being conducted in non-human primates typed to control for host susceptibility/resistance factors. Further, human phase II trials of the HIV Tat vaccine and a phase I trial combining HIV Tat and HIV Env are about to begin. The outcome of these studies will determine the value of Tat as an HIV vaccine candidate.

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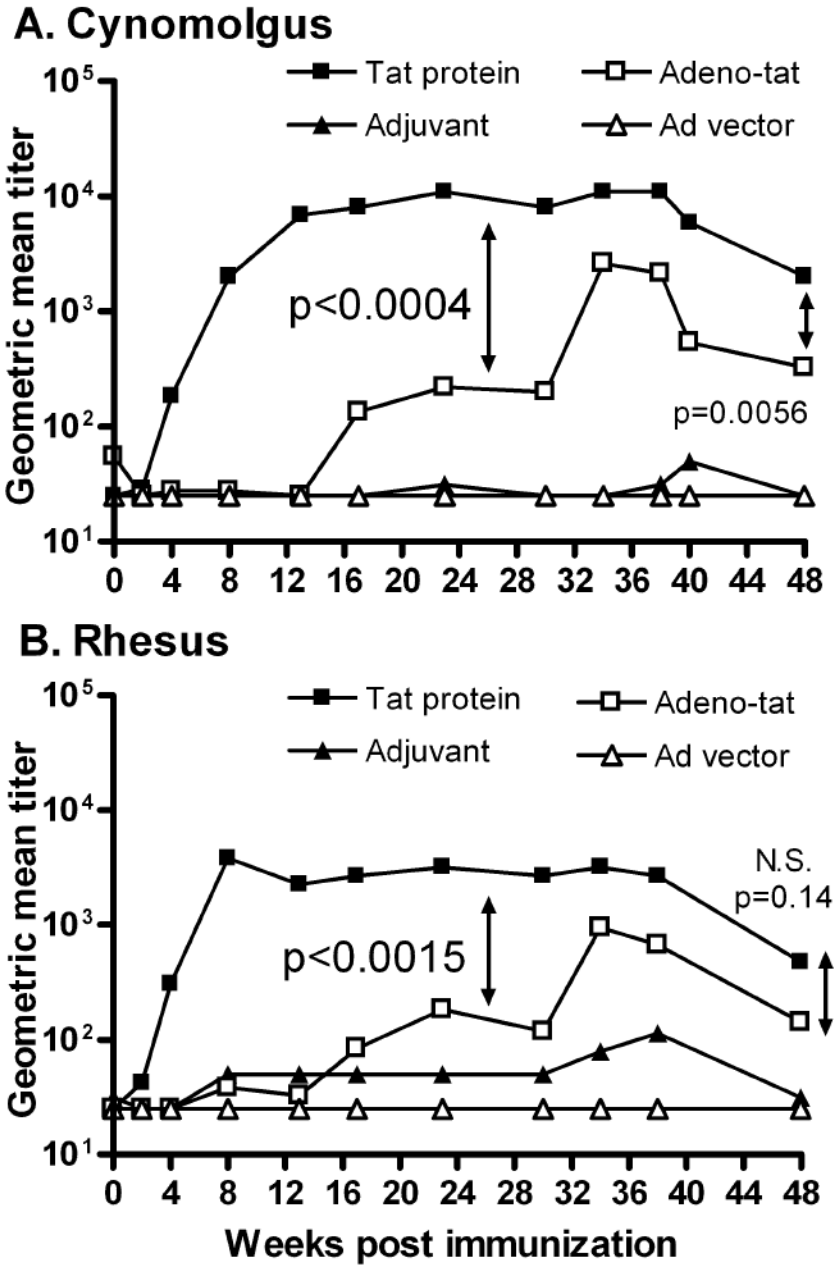

Figure 1. Tat-specific binding antibody induced in cynomolgus and rhesus macaques by vaccination prior to challenge

Geometric mean titers for each immunization group are shown for Mauritian cynomolgus (Panel A) and Indian rhesus (Panel B) macaques. P values shown in panels A and B represent the significant difference in antibody titer between Tat protein and Ad5hr-HIV*tat* groups for weeks 14-34 (panel A) and for weeks 4-30 (panel B). P values at week 48 represent differences prior to challenge at week 50.

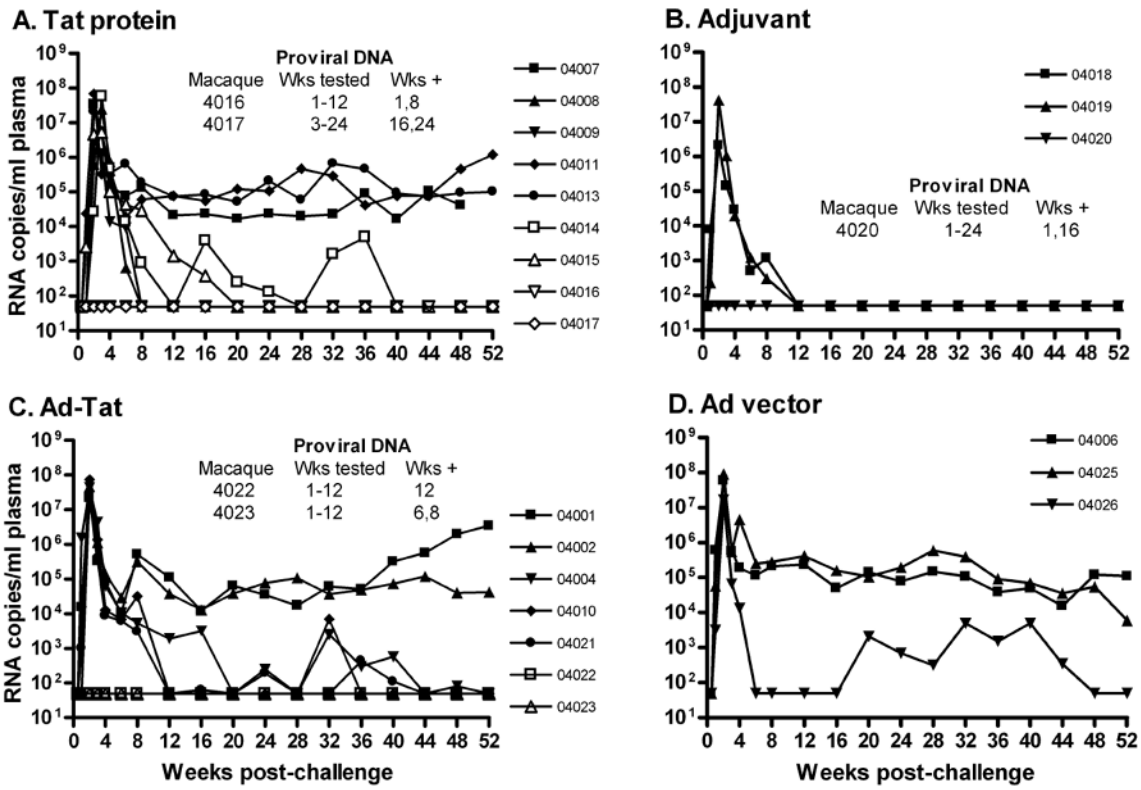

Figure 2. Level of plasma viremia following intravenous challenge of Mauritian cynomolgus macaques with $SHIV_{89.6P}$

Panels A-D show viral loads for individual macaques in each immunization group. Proviral DNA (results shown in panels A,B,C) was evaluated on available PBMC samples collected post-challenge at weeks.1, 2, 3, 4, 6, 8, and monthly thereafter. PBMC obtained at 6 time points were assayed for each macaque where weeks tested are listed as 1-12. PBMC at 7 and 9 time points were assayed for each macaque where weeks tested are listed as 3-24 and 1-24, respectively.

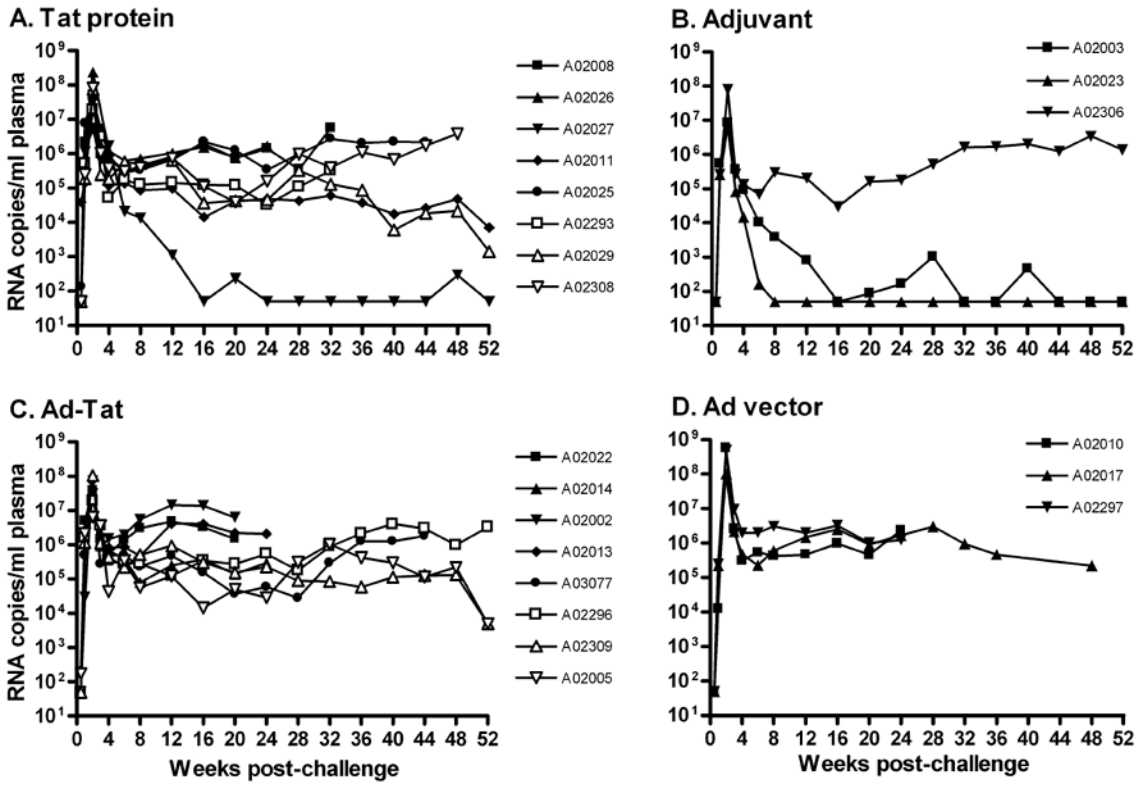

Figure 3. Level of plasma viremia following intravenous challenge of Indian rhesus macaques with SHIV$_{89.6P}$

Panels A-D show viral loads for individual macaques in each immunization group.

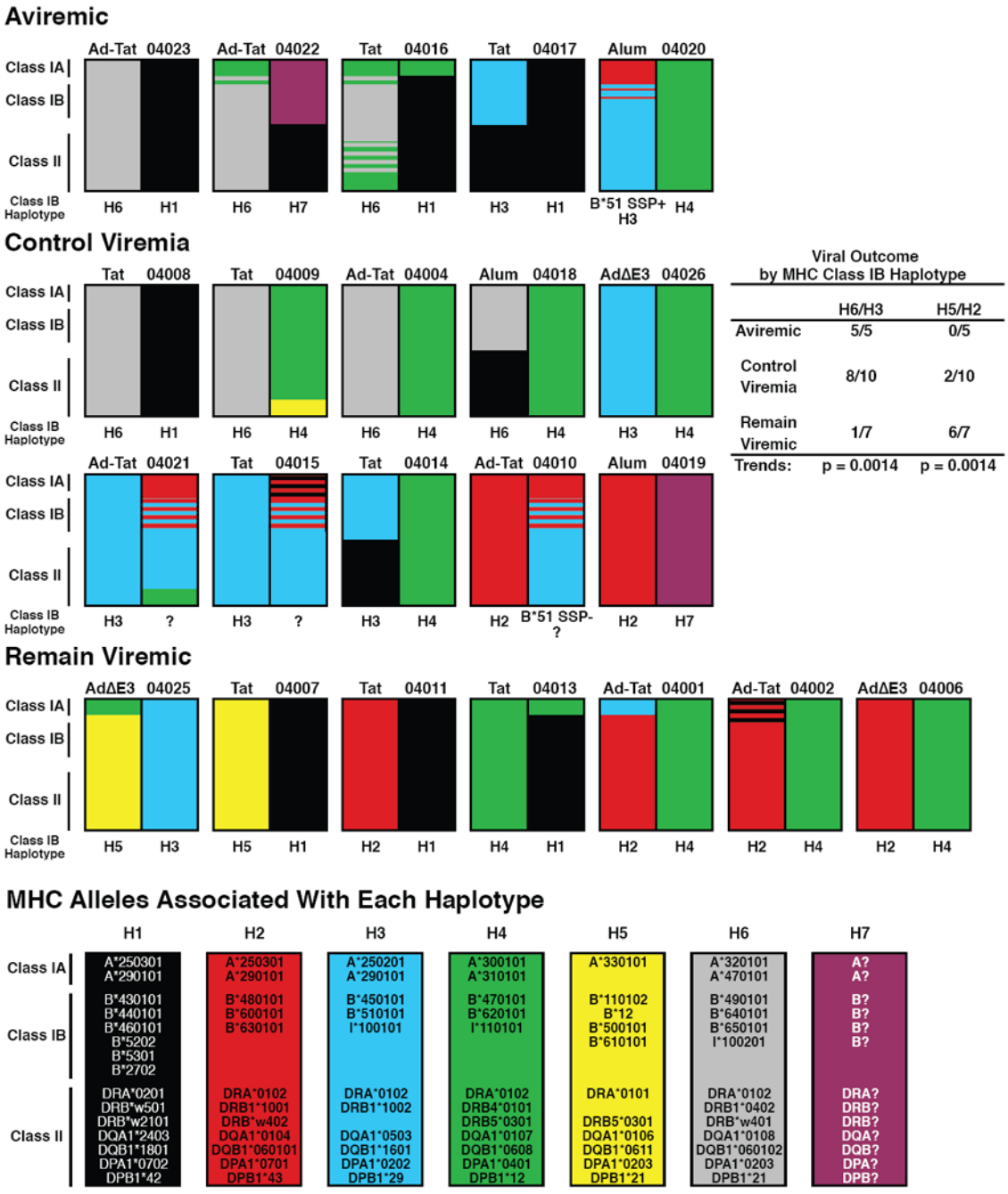

Figure 4. Susceptibility and resistance to SHIV$_{89.6P}$ infection correlates with MHC class IB haplotypes of Mauritian cynomolgus macaques

Viral outcomes were defined as aviremic: never exhibiting a plasma viral load greater than 50 SHIV$_{89.6P}$ RNA copies per ml over the entire 52 week observation period; control viremia: viremia level during the chronic phase of infection dropped to an undetectable level (<50 copies/ml) on two or more occasions; remain viremic: plasma viremia persisted above detectable levels over the entire observation period.

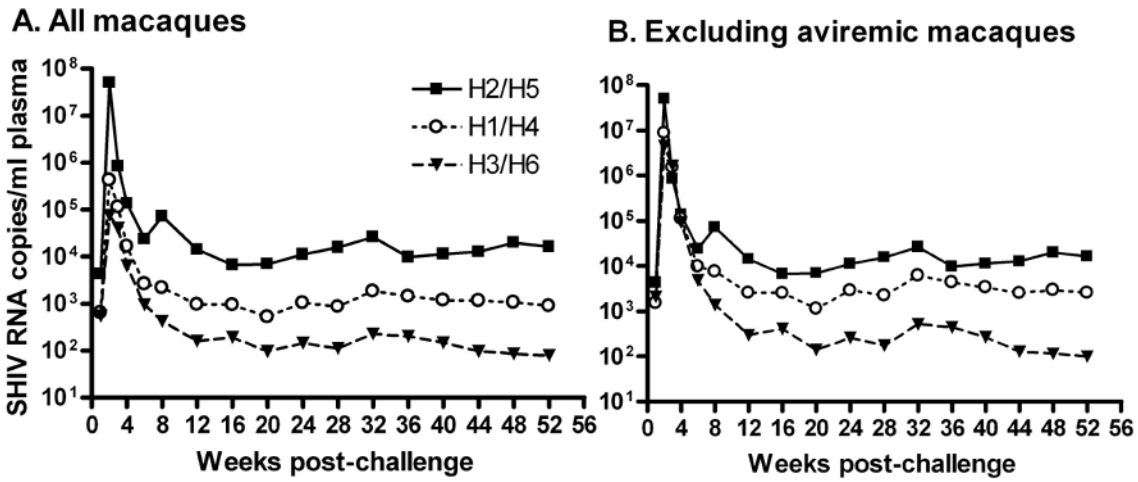

Figure 5. Viral loads of Mauritian cynomolgus macaques with H2/H5 and H3/H6 MHC class IB haplotypes are significantly correlated with susceptibility and resistance to $SHIV_{89.6P}$ infection respectively

Geometric mean viral loads are plotted for macaques grouped according to the indicated haplotypes.

Table 1

Immunization and challenge protocol in cynomolgus and rhesus macaques[a].

Week	Tat protein[b]	Ad-Tat[c]	Ad vector control	Adjuvant control
0	Tat protein (SC/ID)	Ad-Tat (IN)	AdΔE3 (IN)	Alum (SC)
2	Tat protein (SC/ID)			Alum (SC)
6	Tat protein (SC/ID)			Alum (SC)
11	Tat protein (SC/ID)			Alum (SC)
12		Ad-Tat (IT)	AdΔE3 (IT)	
15	Tat protein (SC/ID)			Alum (SC)
21	Tat protein (SC/ID)			Alum (SC)
24		Tat protein (SC)	Alum (SC)	
28	Tat protein (SC/ID)			Alum (SC)
32	Tat protein (SC/ID)			Alum (SC)
36	Tat +ISCOM (IM)	Tat protein (SC)	Alum (SC)	ISCOM (IM)
50	$SHIV_{89.6P}$ (IV)[d]	$SHIV_{89.6P}$ (IV)	$SHIV_{89.6P}$ (IV)	$SHIV_{89.6P}$ (IV)

[a] Cynomolgus and rhesus macaques in each immunization group are listed in Fig. 2 and 3, respectively.

[b] HIV_{IIIB} Tat protein: 10 μg given subcutaneously (SC) in alum + 6 μg given intradermally (ID) without adjuvant. Last immunization was 16 μg given with ISCOM intramuscularly (IM).

[c] Ad-recombinant dose: $5X10^8$ pfu each in PBS administered IN: intranasally; IT: intratracheally.

[d] Intravenous (IV) challenge with $SHIV_{89.6Pcyn243}$,15 MID_{50}, for cynomolgus; $SHIV_{89.6P}$, 30 MID_{50}, for rhesus.

Table 2

Pre-challenge Tat–specific cellular immune responses.

		ELISPOT			Proliferation		
Macaques	**Vaccination group**	**Peak[a] (SFC)**	**Responders[b] (%)**	**Frequency[c] (Mean %)**	**Peak[a] (SI)**	**Responders[b] (%)**	**Frequency[c] (%)**
Cynomolgus							
	Tat protein	26 (0 -77)	67	7	5.2 (<2 – 15.9)	78	12
	Adeno-Tat	51 (0 – 237)	78	14	4.7 (<2 – 12.4)	67	10
Rhesus							
	Tat protein	22 (0 - 93)	38	6	6.8 (<2 – 14.4)	88	34
	Adeno-Tat	109 (0 - 260)	88	22	4.7 (<2 – 13.2)	88	13

[a] Mean (range) of peak positive responses, weeks 2-50.

[b] Percent of macaques exhibiting a positive response, weeks 2-50.

[c] Mean of frequency of positive responses for each macaque over the 8 to 10 time points evaluated.

Table 3

Tat-specific T cell proliferation by MHC class IB haplotype in vaccinated macaques.

Macaque	Peak Stimulation indices			Pre- challenge	Post-challenge[a]
	Pre-challenge	Post-challenge[a]			
H3 Haplotype					
4014	11.4	11.4			
4015	15.9	19.3			
4017*	5.3	12.9			
4021	7.7	6.7	**H3 mean**	**10.2 ± 2.3**	**12.6 ± 2.6**
H6 Haplotype					
4004	4.7	5.2			
4008	0	3.0			
4009	5.9	151.4			
4016*	3.1	0			
4022*	12.4	0			
4023*	6.5	0	**H6 mean**	**5.4 ± 1.7**	**26.6 ± 25.0**
			H3/H6 mean	**7.3 ± 1.5**	**21.0 ± 14.6**
Non-H3/H6 Haplotypes					
4001	0	0			
4002	5.5	7.7			
4007	2.9	0			
4010	0	0			
4011	2.0	2.9			
4013	0	0	**Non-H3/H6 mean**	**1.7 ± 0.9**	**1.8 ± 1.3**
			H3/H6 vs Non-H3/H6[b]	**p = 0.011**	

Negative proliferative responses are recorded as 0. Aviremic macaques are marked by an asterisk.

[a] Responses weeks 1-8 post-challenge.

[b] H3/H6 macaques were combined for this analysis, as proliferative responses did not differ between groups.