Low-Dose Penile SIVmac251 Exposure of Rhesus Macaques Infected with Adenovirus Type 5 (Ad5) and Then Immunized with a Replication-Defective Ad5-Based SIV gag/pol/nef Vaccine Recapitulates the Results of the Phase IIb Step Trial of a Similar HIV-1 Vaccine

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The Step Trial showed that the MRKAd5 HIV-1 subtype B Gag/Pol/Nef vaccine did not protect men from HIV infection or reduce setpoint viral plasma RNA (vRNA) levels but, unexpectedly, it did modestly enhance susceptibility to HIV infection in adenovirus type 5 (Ad5)-seropositive, uncircumcised men. As part of the process to understand the results of the Step Trial, we designed a study to determine whether rhesus macaques chronically infected with a host-range mutant Ad5 (Ad5hr) and then immunized with a replication-defective Ad5 SIVmac239 Gag/Pol/Nef vaccine were more resistant or susceptible to SIV infection than unimmunized rhesus macaques challenged with a series of escalating dose penile exposures to SIVmac 251. The Ad5 SIV vaccine induced CD8+ T cell responses in 70% of the monkeys, which is similar to the proportion of humans that responded to the vaccine in the Step Trial. However, the vaccine did not protect vaccinated animals from penile SIV challenge. At the lowest SIV exposure dose (105 50% tissue culture infective doses), 2 of 9 Ad5-seropositive animals immunized with the Ad5 SIV vaccine became infected compared to 0 of 34 animals infected in the other animal groups (naïve animals, Ad5-seropositive animals immunized with the empty Ad5 vector, Ad5-seronegative animals immunized with the Ad5 SIV vaccine, and Ad5-seronegative animals immunized with the empty Ad5 vector). Penile exposure to more concentrated virus inocula produced similar rates of infection in all animal groups. Although setpoint viral loads were unaffected in Step vaccinees, the Ad5 SIV-immunized animals had significantly lower acute-phase plasma vRNA levels compared to unimmunized animals. Thus, the results of the nonhuman primate (NHP) study described here recapitulate the lack of protection against HIV acquisition seen in the Step Trial and suggest a greater risk of infection in the Ad5-seropositive animals immunized with the Ad5 SIV vaccine. Further studies are necessary to confirm the enhancement of virus acquisition and to discern associated mechanisms.
**MATERIALS AND METHODS**

**Animals.** All animals used in the present study were adult male RM (*Macaca mulatta*) housed at the California National Primate Research Center in accordance with the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care International Standards and with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (NIH). The Institutional Animal Use and Care Committee of the University of California, Davis, approved these experiments (protocol 11479). For blood collection, animals were anesthetized with 10 mg of ketamine hydrochloride (Park-Davis)/kg or 0.7 mg of tiletamine HCl and zolazepan (Telazol; Fort Dodge Animal Health, Fort Dodge, IA)/kg injected intramuscularly. All efforts were made to minimize suffering. Details of animal welfare and steps taken to ameliorate suffering were in accordance with the recommendations of the Weatherall report, “The Use of Nonhuman Primates in Research” (44a). Animals were housed in an air-conditioned facility with an ambient temperature of 21 to 25°C, a relative humidity of 40 to 60%, and a 12-h light/dark cycle. Animals were individually housed in suspended stainless steel wire-bottom cages and provided with a commercial primate diet. Fresh fruit was provided once daily, and water was freely available. Animals were assigned to experimental groups to distribute the TRIM5α genotype, MHC-I genotype, and organism of origin as evenly as possible (Table 1).

**Study design, Ad5hr inoculation and replication-incompetent Ad5 SIV vaccine.** Eighteen monkeys were infected with Ad5hr, fully competent for replication in monkey cells (19), by three sequential mucosal inoculations in order to induce natural immunity to the vector prior to immunization with the Ad5 SIVmac239 gag/pol/nef vaccine. At 20 weeks prior to immunization with the Ad5 SIVmac239 vaccine, Ad5hr was administered both orally and nasally at a dose of 1.5 × 10⁹ PFU (Fig. 1), and at 12 and 8 weeks prior to immunization with the Ad5 SIVmac239 vaccine, 1.5 × 10⁹ PFU of Ad5hr was administered intratracheally (Fig. 1). One of the eighteen animals was euthanized for medical reasons unrelated to the study prior to the third Ad5hr inoculation. Eight weeks after the third Ad5hr inoculation (Fig. 1), nine of the Ad5hr-infected macaques were immunized with 10¹⁰ particles of each individual rAd5 SIV gag/pol/nef vector Ad5 SIVmac239 Gag/Pol/Nef (4, 40). This group is designated as the Ad5 Vx-SIV group. The other eight Ad5hr-infected macaques were inoculated with 10¹⁰ particles of the empty replication-incompetent MRKAd5 vector, and this group is designated as the Ad5 Vx-empty group. In addition, nine macaques designated as the Vx-SIV group were never exposed to Ad5hr, but they were immunized with the Ad5 SIV vaccine, and another nine macaques, never exposed to Ad5hr, were inoculated with the empty replication-incompetent Ad5 vector and were designated the Vx-empty group. At 4 and 24 weeks after the primary immunization, the animals were boosted with the same vector construct and then 1 week later they were challenged by penile exposure to SIV (Fig. 1). The interval between the last immunization and when Step Trial participants were HIV exposed is unknown, but sexual activity was uninterrupted by the vaccine protocol, thus to model HIV exposure soon after the complete vaccine regimen was completed and when vaccine-induced immunity and the potential for protection were greatest, SIV exposures began 1 week after the last immunization. Thus, animals would be expected to have strong SIV-specific and Ad5-specific immunity at the time the SIV exposures were begun. Eight animals never exposed to Ad5hr, the Ad5 vector, or SIV antigens served as controls (naïve control group) (Fig. 1). The study endpoints were SIV infection and plasma vRNA levels after challenge.

**Penile SIVmac251 exposure/challenge.** A cell-free stock of SIVmac251 (UCD-6/04) was produced in *Staphylococcus enterotoxin* A-stimulated rhesus monkey peripheral blood mononuclear cells (PBMC) (42) and used for these studies. This SIVmac251 stock (UCD-6/04) contains ca. 10⁹ vRNA copies/ml and 10⁵ 50% tissue culture infection doses (TCID₅₀)/ml when titered on CEMX174 cells. On the day of challenge, serial 10-fold dilutions of the SIV stock were made in phosphate-buffered saline; a 2-ml portion of the virus inoculum was used for all of the penile exposures in the present study. For the penile SIVmac251 challenge inoculation, we modified our previously published procedure (27). Briefly, a small cup was fashioned from a closed end of a disposable 15-ml conical centrifuge tube, and 2 ml of virus inoculum was placed into the cup. The penis was extended, the foreskin was retracted, and the glans and shaft were inserted into the inoculum and held there for 5 min. Then, the penis was allowed to retract into the foreskin and the animal was placed into its cage in dorsal recumbency. An additional 250 μl of virus inoculum was then dropped into the space between the foreskin and the glans, and the animals were allowed to recover from anesthesia. Animals in the present study could be exposed 10 times to an inoculum of 10⁵ TCID₅₀, 10 times to an inoculum of 10⁶ TCID₅₀, and twice in 1 day to an inoculum of 10⁷ TCID₅₀ (Fig. 1). Although not all animals were challenged on the same day, the study was scheduled so that Ad5 Vx-SIV and Ad5 SIV-empty animals were challenged on the same days and Vx-SIV and SIV-empty animals were challenged on the same days. The SIV inoculation series for a particular animal was stopped when vRNA levels steadily increased in two consecutive plasma samples.

**Detection and quantitation of Ad5hr DNA in nasal secretions.** For several days following infection, nasal secretions were collected and stored at −70°C. A nested PCR assay (3) that amplifies a portion of the Ad5 fiber gene was used to detect Ad5 DNA. If a sample was positive in the nested PCR assay, and then a TaqMan PCR was used to measure the number of adenovirus DNA copies in 5 μl of sample, and all samples were tested in duplicate. For the TaqMan assay, the forward primer was 5′-TTG TAT GAT GAT GCC GCA GTG GTG TCA TAT CAC G-3′, the reverse primer was 5′-TTT CTA AAC TTG TTA TTC AGG CTG AAG TAC G-3′, and the probe was 5′-FAM-CCG GGT CTG GTG CAG TTT GCC CGC-3′ previously published (7). A standard curve made using serial 10-fold dilutions of the pCR-Ad5/Ad6 plasmid (nucleotides [nt] 18697 to 19197 of the Ad5hr exon gene cloned into TOPO2.1) was used to quantify copies in the samples. Briefly, 5-μl preboiled (95°) samples were added to a solution containing 12.5 μl of ABI Universal master mix, 2.0 μl of 10 μM Ad5/6 forward primer, 2.0 μl of 10 μM Ad5/6 reverse primer, 0.625 μl of 10 μM Ad5 probe, and 2.875 μl of water. The default amplification protocol for the ABI Sequence Detection System 7000 was used. Samples below the detection limit (100 copies) were reported as negative. The nested PCR assay was used as a screening assay on all samples (Fig. 2A), and the TaqMan PCR assay was used to estimate copy number in samples that were positive in the nested PCR assay (Fig. 2B).

**Assessment of Ad5hr antibody responses.** Ad5 neutralizing antibody titers were determined as previously described (41). Plasma, heat inactivated at 56°C, was plated into 96-well flat-bottom plates (50 μl of 4-fold dilutions) and incubated with 50 μl of Ad5-luciferase stock (Ad5-Apt-Luc; Crucell Holland B.V., Leiden, Netherlands) at ca. 500 PFU/cell for 1 h at 37°C in 5% CO₂. A549 cells (10⁴ in 100 μl in F-12 Kiga’s medium containing 10% fetal bovine serum, 50 μg of gentamicin/ml, and 2 mM glutamine) were added to each well. Positive controls consisting of virus plus cells with no plasma, and negative controls consisting of cells plus medium with no virus or plasma, were included in each plate. After incubation at 37°C in 5% CO₂ for 24 h, 100 μl of medium was removed from each well and replaced with 100 μl of BriteLite solution (BriteLite Plus 100 kit; Perkin-Elmer), mixed, and incubated for 2 min. Luciferase activity was detected by transferring 200 μl of each cell-substrate mixture to a 96-well black flat-bottom plate and measuring the luminescence within 15 min using a Victor² 1420 multilabel counter (Perkin-Elmer). Neutralizing titers were defined as the plasma dilutions resulting in 50% reduction in luciferase activity relative to that of preinfection samples diluted 1:20.

**SIV RNA isolation and cDNA synthesis.** Plasma samples were thawed at room temperature, and RNA was isolated from 0.5 ml of plasma using a QIAamp Ultrasens viral kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s protocol and eluted in 50 μl. The vRNA was reverse transcribed into cDNA using SuperScript III reagents (Invitrogen, Carlsbad,

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CA) with 2 μl of 50 μM dT30VN, 2 μl of 10 mM deoxynucleoside triphosphates, and 22 μl of viral RNA. This mixture was heated to 65°C for 5 min, followed by incubation on ice for 2 min. A master mix of the following was then added: 8 μl of 5 × first-strand buffer, 2 μl of 0.1 M dithiothreitol, 2 μl of RNase OUT recombinant RNase inhibitor (40 U/μl), and 2 μl of SuperScript III RT (200 U/μl), followed by incubation at 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min, followed in turn by the addition of 1 μl of Escherichia coli RNase H (3U/μl) and additional incubation at 37°C for 20 min. cDNA was stored at −20 to −80°C until amplification.

**Quantitative SIV RNA analysis.** A well-described reverse transcription-PCR was used to detect and quantify SIVgag RNA levels in plasma samples (14, 43). The copy number of SIVgag was calculated based on standard curves for a SIVgag plasmid spanning a concentration range from 0.1 to 108 copies. The limit of quantitation for the assay is 125 copies/ml based on plasmid spiking experiments with negative plasma. Although samples with 1 copy/ml were consistently detected in a well, 125 copies/ml were needed to place the point on the linear portion of the assay standard curve. Thus, a sample that was negative was graphed as

**TABLE 1 Animals, immunization groups, and outcome of challenge**

| Immunization group | Animal no. | Animal group | Animal origin\(^a\) | TRIM5α genotype\(^b\) | MHC-I allele(s)\(^c\) | Peak plasma vRNA\(^d\) |
|--------------------|------------|--------------|----------------------|----------------------|----------------------|----------------------|
| **Immunized**      |            |              |                      |                      |                      |                      |
| 33776              | Ad5 Vx-SIV | 1/8 Chinese  | TFP/TFP              |                      | A02, A11             | 5.77                 |
| 35380              | Ad5 Vx-SIV | Indian       | Q/TFP                |                      | A01                 | 5.9                  |
| 35584              | Ad5 Vx-SIV | 1/4 Chinese  | Q/TFP                |                      | B01                 | –                    |
| 35629              | Ad5 Vx-SIV | Indian       | Q/TFP                |                      | –                   | –                    |
| 36224              | Ad5 Vx-SIV | Indian       | TFP/TFP              |                      | A01                 | –                    |
| 36321              | Ad5 Vx-SIV | 1/8 Chinese  | Q/TFP                |                      | A01                 | 6.63                 |
| 36338              | Ad5 Vx-SIV | Indian       | TFP/TFP              |                      | B01                 | 6.8                  |
| 36658              | Ad5 Vx-SIV | 1/4 Chinese  | Q/TFP                |                      | A01                 | 6.84                 |
| 36982              | Ad5 Vx-SIV | Indian       | Q/TFP                |                      | A02                 | –                    |
| **Unimmunized**    |            |              |                      |                      |                      |                      |
| 35987              | Ad5 Vx-empty| Indian       | Q/CypA               |                      | –                   | 7.32                 |
| 36016              | Ad5 Vx-empty| Indian       | TFP/TFP              |                      | A01, B01            | 7.91                 |
| 36173              | Ad5 Vx-empty| Indian       | TFP/TFP              |                      | A01, A02            | –                    |
| 36380              | Ad5 Vx-empty| Indian       | TFP/TFP              |                      | A11, B08            | –                    |
| 36523              | Ad5 Vx-empty| Indian       | TFP/TFP              |                      | –                   | –                    |
| 36637              | Ad5 Vx-empty| Indian       | Q/Q                  |                      | A02                 | 6.61                 |
| 36961              | Ad5 Vx-empty| Indian       | Q/TFP                |                      | A08                 | 8.1                  |
| 36965              | Ad5 Vx-empty| Indian       | Q/TFP                |                      | B03, B04            | 7.43                 |
| **Immunized**      |            |              |                      |                      |                      |                      |
| 35919              | Vx-SIV     | Indian       | TFP/TFP              |                      | A01, A08            | 6.79                 |
| 35939              | Vx-SIV     | 1/4 Chinese  | Q/TFP                |                      | A02, B08            | 5.26                 |
| 36264              | Vx-SIV     | Indian       | Q/TFP                |                      | A02, B08            | –                    |
| 36272              | Vx-SIV     | Indian       | Q/Q                  |                      | B01                 | 5.9                  |
| 36303              | Vx-SIV     | 1/4 Chinese  | TFP/TFP              |                      | B01                 | 6.54                 |
| 36323              | Vx-SIV     | Indian       | TFP/TFP              |                      | –                   | 7.38                 |
| 36609              | Vx-SIV     | Indian       | TFP/CypA             |                      | –                   | 5.61                 |
| 36629              | Vx-SIV     | Indian       | Q/TFP                |                      | A02                 | 6.01                 |
| 37332              | Vx-SIV     | Indian       | TFP/TFP              |                      | A02                 | –                    |
| 34310              | Vx-empty   | Indian       | TFP/TFP              |                      | A02, B01, B17       | 6.93                 |
| **Unimmunized**    |            |              |                      |                      |                      |                      |
| 34976              | Vx-empty   | Indian       | Q/TFP                |                      | –                   | 7.44                 |
| 36270              | Vx-empty   | Indian       | Q/Q                  |                      | A02, B01            | 6.52                 |
| 36344              | Vx-empty   | Indian       | TFP/TFP              |                      | A08                 | –                    |
| 36411              | Vx-empty   | Indian       | Q/TFP                |                      | A01                 | 7.21                 |
| 36520              | Vx-empty   | Indian       | Q/TFP                |                      | B17                 | –                    |
| 36643              | Vx-empty   | Indian       | Q/TFP                |                      | A11                 | 6.44                 |
| 36784              | Vx-empty   | 1/8 Chinese  | Q/TFP                |                      | B08                 | 7.19                 |
| 36859              | Vx-empty   | 1/4 Chinese  | TFP/TFP              |                      | A02                 | –                    |
| **Unimmunized**    |            |              |                      |                      |                      |                      |
| 36254              | Naive control| Indian      | TFP/CypA             |                      | –                   | 7.44                 |
| 36503              | Naive control| Indian      | Q/TFP                |                      | –                   | –                    |
| 37302              | Naive control| Indian      | Q/TFP                |                      | –                   | –                    |
| 35581              | Naive control| Indian      | TFP/TFP              |                      | –                   | 5.64                 |
| 36199              | Naive control| Indian      | TFP/TFP              |                      | 7.93                 |
| 36368              | Naive control| Indian      | TFP/TFP              |                      | 7.92                 |
| 36589              | Naive control| Indian      | Q/CypA               |                      | 7.61                 |
| 37079              | Naive control| Indian      | 1/8 Chinese          |                      | 7.61                 |

\(^a\) The country of origin for the parental stock of the animal. The designation “Indian” indicates the animal’s parents were imported from India. For Indian × Chinese hybrid animals, the fraction of Chinese parentage is noted.

\(^b\) Trim5α alleles that are associated with effects on SIV replication (18). There are three alleles listed, and the allele on each chromosome is indicated: TFP, Q and CypA. The TFP and CypA alleles are associated with lower plasma vRNA levels in SIV-infected RM (18, 23).

\(^c\) MHC-I alleles A01, B17, and B08 are associated with lower plasma vRNA levels in SIV-infected RM (26, 34).

\(^d\) Expressed as the log10 vRNA copies/ml plasma. A dash indicates the animal was not infected.
having one copy/ml. The results were analyzed with SDS 7900 system software version 2.3 (Applied Biosystems). The results for each sample are reported as log_{10} vRNA copies per ml of plasma RNA.

**MHC-I haplotyping and TRIM 5α genotyping.** MHC-I haplotyping was performed by the Rhesus Macaque MHC Typing Core, University of Wisconsin Hospital and Clinics, as described previously (25). Trim5α polymorphisms were determined by amplifying and sequencing TRIM5α genes from genomic DNA using a described procedure (18). Based on the nucleotides at positions 339 to 349 of the B30.2/SPRY region, animals were classified into three categories: Trim5TFP, Trim5Q, or Trim5CypA (Table 1). The Trim5TFP and Trim5CypA alleles have been reported to modestly suppress SIVmac251 replication in infected animals (18, 23).

**Intracellular staining for cytokine production by SIV-specific T cells.** For intracellular staining of PBMC, cryopreserved samples were thawed and rested overnight at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium (Gibco/Invitrogen, Inc.) containing 10% fetal calf serum. The next day, the cells were adjusted to 10^6/ml and incubated with anti-CD28 and anti-CD49d antibodies (1 μg/ml, final concentration; BD Biosciences) as costimulatory molecules, in a total volume of 200 μl of RPMI 1640 –10% fetal bovine serum. Samples were stimulated with two pools of peptides spanning SIV gag (including a pool of peptides that span p27), three pools of peptides spanning SIV pol, and one pool of peptides spanning SIV nef. All peptides were 20mers that overlapped by 10 amino acid residues (ProImmune, Inc.) that were stored at 5 μg/ml in dimethyl sulfoxide (DMSO). Negative control cultures contained costimulatory molecules and DMSO, and a positive control culture was stimulated with staphylococcal enterotoxin B (0.2 μg/ml; Sigma-Aldrich). During stimulation, the cells were incubated for 6 h at 37°C in the presence of brefeldin A (Sigma-Aldrich). After stimulation, the cells were washed and then incubated with anti-CD3-Pacific Blue, anti-CD8-APC-Cy7, and anti-CD4-peridinin chlorophyll protein-Cy5.5 (Clone L200) for 20 min and

![FIG 1](http://jvi.asm.org) Overview of the experimental design. The designations used for each animal group are indicated in the left margin, and the dose of SIV used for each penile exposure is indicated by the colored hash marks.

![FIG 2](http://jvi.asm.org) Virus shedding after Ad5shr inoculation. (A) Number of animals with detectable Ad5shr DNA in nasal secretions after each Ad5shr inoculation based on the nested PCR assay. (B) Level of Ad5shr DNA in nasal secretions after the first Ad5shr inoculation, 20 weeks before the first immunization with the replication defective Ad5 SIV vaccine based on the TaqMan PCR assay.
then washed again and fixed with 1% paraformaldehyde. Fixed samples were permeabilized (0.5% saponin), incubated with anti-gamma interferon (anti-IFN-γ)-APC clone B27, anti-tumor necrosis factor alpha (TNF-α)-PE-Cy7 clone MBA11, and anti-interleukin-2 (anti-IL-2)-PE clone MQ1-17H12 for 20 min at room temperature, and then washed with permeabilizing buffer and fixed with 1% paraformaldehyde. All monoclonal antibodies were from Pharmingen/Becton Dickinson, San Diego, CA. The data were acquired using a FACSArray flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Treestar, Inc.) and a Macintosh G5 computer (Apple, Inc.). At least 100,000 events in the forward scatter/side scatter lymphocyte gate were acquired from all cultures. The background level of cytokine staining varied between the samples and the peptide pools. For reporting the single cytokine positive CD8+ T cell responses, a sample was considered positive if the frequency cytokine+ cells was >0.04 after subtracting the DMSO control. The cutoff value of 0.04% was based on the background responses in unimmunized animals.

**Statistical methods.** To define the penile exposure that resulted in the inoculation the previous week. The peak viral load was defined as the highest log_{10} vRNA level in any plasma sample collected over the 7-week postinfection observation period. All of the analyses described below were conducted separately for each of three dosage groups (10^3 TCID50; 10^3 TCID50 and 10^4 TCID50 combined; and 10^4 TCID50, 10^5 TCID50 and 10^6 TCID50 combined). Kaplan-Meier estimates of the survival function were generated for each arm or group of arms of interest, and a log-rank test was used to compare the survival functions between animal groups. Box plots of plasma vRNA levels in the infected animals were generated and a Wilcoxon rank-sum test (WRS) was used to test equality between animal groups. Finally, a discrete time survival model (15, 16) was used to model the relative probability of infection per penile SIV challenge of one group versus another. All P values are two-sided, and P values of <0.05 are considered significant.

**RESULTS**

**Virology of Ad5hr infection in RM.** Using procedures previously described (36), 18 male RM (Table 1, Fig. 1) were inoculated orally and intranasally with 1.5 × 10^6 infectious particles of Ad5hr404 (19) 20 weeks before immunization with the Ad5 SIVmac239 Gag/Pol/Nef vaccine or the Ad5 empty vector. At weeks −12 and −8, all 18 animals were reinoculated intraorally with the same dose of Ad5hr404 (Fig. 1). Using a sensitive nested PCR assay, Ad5hr DNA was detected in the nasal secretions of 17 of 18 RM in the first 48 h after the Ad5hr inoculation at week −20, but by week −12 no RM was shedding Ad5hr (Fig. 2A). After reinoculation at weeks −12 and −8, Ad5hr DNA was detected in the nasal secretions of only two or three animals. We also measured the levels of Ad5hr DNA present in nasal secretions using a less sensitive TaqMan nested PCR. Ad5hr DNA could be measured at low levels (35 Ad5 DNA copies/μl secretions) in one of six animals that were still positive by nested PCR.

**Ad5hr neutralizing antibody responses.** One week after Ad5hr inoculation (week −19), 8 of 18 animals had Ad5-specific serum neutralizing antibody responses with titers in the range of 128 to 2,048. All 18 animals were positive for Ad5hr neutralizing antibodies 2 weeks after the second Ad5hr infection (week −10) with titers in the range of 32 to >5,120 (Fig. 3). After the third Ad5hr inoculation, all macaques exhibited neutralizing antibody titers in the range of 128 to >5,120. The Step Trial included 1,500 participants with low (<200) Ad5 antibody titers at enrollment and, 1,500 participants with high Ad5 titers of >200 (2). Based on their Ad5-specific antibody titers, all but one of the Ad5hr-infected RM in the present study would fall into the cohort of Step Trial participants with high Ad5 antibody titers.

**Breadth of Ad5 SIV vaccine induced CD8+ T cell responses.** Of the 18 animals immunized with the Ad5 SIV vaccine (Ad5 Vx-SIV and Vx-SIV), 11 (61%) made CD8+ T cell responses to Gag, 10 (55%) developed CD8+ T cell responses to Pol, and 6 (33%) made CD8+ T cell responses to Nef at some point after the first vaccination (Table 2). Animals infected with Ad5hr before immunization with the Ad5 SIV vaccine (Ad5 Vx-SIV) made CD8+ T cell responses to only two of the three vaccine antigens, i.e., Gag and Pol (Table 2), while most of the Ad5-seronegative Vx-SIV animals responded to all three antigens. While the proportion of animals in the two groups responding to Gag was similar, Pol-specific responses were detected in only three of nine (33%) Ad5 Vx-SIV animals compared to seven of nine (77%) of the Vx-SIV animals (Table 2). Finally, Nef-specific CD8+ T cell responses were not detected in the Ad5 Vx-SIV animals but were common (6 of 9 [67%]) in the Vx-SIV animals (Table 2). The proportion of RM and humans making CD8+ T cell responses to the vaccine antigens after immunization with the MRKAd5 vaccine was similar, but the decreased breadth of immune responses attributed to preexisting Ad5 immunity seemed more marked in RM (Table 2).

**Strength and cytokine profile of Ad5 SIV vaccine-induced CD8+ T cell responses.** Four weeks after the first immunization, Gag-specific CD8+ T cell responses were detected in only 4 of 18 immunized macaques (Ad5 Vx-SIV and Vx-SIV), and the mean frequency of the Gag specific IFN-γ+ CD8+ T cells in responders was 0.095% of total CD8+ T cells (range, 0.05 to 0.14%; Fig. 4). Four weeks after the second immunization of 18 RM had Gag-specific CD8+ T cell responses, and the mean frequency of Gag-specific IFN-γ+ CD8+ T cells was 0.12% of total CD8+ T cells (range, 0.04 to 0.4%). By the day of the first penile SIV exposure (1 week after the third immunization), 7 of 18 vaccinated macaques had Gag-specific CD8+ T cell responses, with Gag-specific IFN-γ+ CD8+ T cells comprising...
TABLE 2 Summary of CD8+ T cell responses to the Ad5 SIV gag/pol/nef vaccine

| Animal no. | Group         | Gag  | Pol  | Nef  |
|------------|---------------|------|------|------|
| 33776      | Ad5 Vx-SIV    | −    | −    | −    |
| 35380      | Ad5 Vx-SIV    | −    | −    | −    |
| 35584      | Ad5 Vx-SIV    | +    | +    | +    |
| 35629      | Ad5 Vx-SIV    | + +   | −    | −    |
| 36224      | Ad5 Vx-SIV    | + +   | −    | −    |
| 36321      | Ad5 Vx-SIV    | −    | −    | −    |
| 36338      | Ad5 Vx-SIV    | −    | −    | −    |
| 36658      | Ad5 Vx-SIV    | + + + | −    | +    |
| 36982      | Ad5 Vx-SIV    | +    | −    | −    |
| Total (%)  | 5/9 (55)      | 3/9 (33) | 0/9 |
| % Step vaccinees  | 70 | 40 | 60 |

a Peptide pools covering Gag, Pol, and Nef were used to test PBMC collected at week 4 (n = 18), week 8 (n = 17), and week 24 (n = 9) after the first immunization.

b Gag p27 was tested at week 25 (n = 18).

c One cytokine was made upon antigen stimulation.

d Two cytokines were made upon antigen stimulation.

e Three cytokines were made upon antigen stimulation.

f Note that responses in the humans immunized with the HIV version of the vaccine were based on flow cytometry assays (38).

0.16% (range, 0.09 to 0.27%) of all CD8+ T cells in responding animals. The SIV Gag- and Pol-specific T cells in the Ad5 Vx-SIV animals were either IFN-γ alone or IFN-γ/IL-2+. In contrast, the Gag, Pol, and Nef CD8+ T cells in the Vx-SIV animals were predominantly positive for 2 or 3 cytokines (IFN-γ/IL-2+/TNF-α+) or IFN-γ/IL-2+/TNF-α−.

Effect of immunization on susceptibility to penile SIVmac251 exposure. Although the timing of HIV exposure relative to immunization in the Step Trial is not known, the animals in the present study were challenged with cell-free SIVmac251 applied to the foreskin and glans of the penis 1 week after the last immunization (Fig. 1). Animals were exposed in this manner to 103 TCID50 of SIVmac251 once a week for 10 weeks, then once a week for 10 weeks to 104 TCID50 of SIVmac251, and finally twice in 1 day to 105 TCID50 of SIVmac251. Exposures were stopped when there were two consecutive plasma samples with rising vRNA levels. At the end of the 22 SIVmac251 penile exposures, 6 of 8 (75%) of the naive control animals were SIV infected, and among the animals with preexisting Ad5 immunity, 5 of 8 (63%) of the Ad5 Vx-empty vector-immunized animals and 5 of 9 (56%) of Ad5 Vx-SIV-immunized animals were SIV infected (Fig. 5). In the animals that were not infected with Ad5 prior to immunization, 6 of 9 (67%) of the Vx-empty vector-immunized animals and 7 of 9 (78%) of the Vx-SIV-immunized animals were SIV infected (Fig. 5). Note that although one of the Vx-empty animals (animal 36344) had multiple episodes of transient viremia, the inoculation series continued uninterrupted, and a systemic infection never became established in this animal (Fig. 5).

For a more rigorous analysis of susceptibility to infection, Kaplan-Meier estimates of the survival function were generated for each animal group at each of the three challenge doses (Fig. 6). Although only 2 animals were infected after exposure to a dose of 103 TCID50, both of them were Ad5 Vx-SIV animals. Moreover, we used a log rank test to compare composites of the groups. Of particular interest were comparisons between the composite groups (i) immunized versus unimmunized animals with preexisting Ad5 immunity (Ad5 Vx-SIV versus Ad5 Vx-empty); (ii) immunized (Ad5 Vx-SIV + Vx-SIV) versus unimmunized animals (Ad5 Vx-empty + Vx-empty + control); and (iii) immunized animals with preexisting Ad5 immunity compared to all others. P values for those comparisons are presented in Table 3. At the lowest exposure dose (103 TCID50), a significant difference in risk of infection was detected between the immunized animals with preexisting Ad5 immunity (Ad5 Vx-SIV) compared to all of the other animal groups (P = 0.004 based on a log-rank test, Table 3). We also used a discrete time survival model (15, 16), assuming a leaky vaccine effect to test vaccine efficacy in one composite of the groups versus another. Using this model, among animals with preexisting Ad5 immunity, the estimated relative risk of infection for immunized versus unimmunized animals was > 1 either at the lowest exposure dose (103 TCID50) or when combining dose levels of 103 and 104 TCID50 (Table 3). A likelihood-ratio test based on the leaky model was conducted to compare the per-exposure infection probability between the composite groups at the lowest exposure dose: the relative risk of infection approached statistical significance in immunized animals compared to unimmunized animals (P = 0.056), and the relative risk was significantly higher in immunized animals with preexisting Ad5 immunity compared to all of the other animals (P = 0.010). Note that when infections at the low and middle exposure doses (103 and 104 TCID50) are combined, the relative risk of the Ad5 Vx-empty group was lower than the Vx-empty group (P = 0.05, based on a log-rank test) but not from the unimmunized control group (P = 0.31, based on a log-rank test).

The TFP and CypA Trim5α alleles are associated with lower plasma vRNA levels in RM infected with the SIVsm family of viruses (SIVsmE660 and SIVsmE543), but there is minimal effect on infections with the SIVmac family of viruses (18, 23). The TFP

![FIG 4 Frequency of vaccine-induced IFN-γ-secreting SIV Gag-specific CD8+ T cells in immunized animals. PBMC samples collected at 4 and 8 weeks after the first immunization were stimulated with two peptide pools covering the p55 Gag precursor, while the samples collected at 25 weeks were stimulated only with a gag p24 peptide pool.](http://jvi.asm.org/Downloaded from may1, 2019 by guest)
FIG 5 Plasma SIV RNA levels after penile exposure. (A) Naive control animals; (B) animals infected with Ad5hr then immunized with the Ad5 SIV vaccine; (C) animals infected with Ad5hr then immunized with the empty Ad5 vector; (D) animals immunized with the Ad5 SIV vaccine; (E) animals immunized with the empty Ad5 vector. The color of the arrows under the x-axis indicates the SIV dose used for each inoculation and, in animals that became infected, the color of the symbol or line indicates the dose of SIV that resulted in the systemic infection. The dotted black line in panel E indicates a period of transient viremia in one animal that never developed a typical systemic infection and thus was not classified as infected. The horizontal black line indicates the cutoff for accurately quantifying vRNA in plasma.
and CypA Trim5α alleles restrict SIV replication by binding to the virus capsid protein to prevent uncoating and block replication, but the sequence specificity of the Trim5α-capsid interaction explains why the effect is limited to SIVsm viruses (18). However, it was recently reported that monkeys homozygous for the restrictive Trim5α alleles resist becoming infected after penile inoculation with SIVsm E660 (46), and thus we sought to determine whether the susceptibility of the animals in the present study to penile SIVmac251 inoculation was related to their TRIM5α genotype. Both of the Ad5 Vx-SIV animals that became infected with exposure to 10^3 TCID_{50} of SIV were homozygous for the TFP allele (Table 1) that is associated with resistance to infection with after penile inoculation with SIVsm E660 (46), and thus we sought to determine whether the susceptibility of the animals in the present study to penile SIVmac251 inoculation was related to their TRIM5α genotype. Both of the Ad5 Vx-SIV animals that became infected with exposure to 10^3 TCID_{50} of SIVmac251 were homozygous for the TFP allele (Table 1) that is associated with resistance to infection with after penile SIVsm E660 inoculation. In fact, if group assignment is not considered, 11 of 18 TFP/TFP animals and 3 of 3 TFP/CypA animals became infected after penile SIV inoculation compared to 11 of 18 TFP/Q animals, 3 of 3 Q/Q animals, and 1 of 1 Q/CypA animals (Table 1). Thus, in the present study there was no difference in the rate of SIV acquisition between animals with the nonpermissive TFP and CypA alleles and animals with the permissive Q allele.

Effect of immunization on SIVmac251 replication after transmission. As shown in Fig. 5, there was no evidence that prior infection with Ad5 altered SIV replication in the Ad5 Vx-SIV group. Thus, the Ad5 Vx-SIV and Vx-SIV animals are hereafter grouped together and referred to as the “immunized animals,” while the Ad5 VX-empty and VX-empty animals and naive control animals are “unimmunized animals.” The peak plasma vRNA level in immunized animals was ~1 log_{10} lower compared to the unimmunized animals (Fig. 7B). In addition, at 6 weeks postinfection, the plasma vRNA level in immunized animals was at least 1 log_{10} lower than in unimmunized animals (P < 0.05, WRS test, data not shown). Finally, the vRNA area under the curve (AUC) through 7 weeks postinfection from immunized animals was 15% lower compared to the unimmunized animals (P < 0.01, WRS test, data not shown). Thus, the immunized animals that became infected had significantly lower plasma vRNA levels than unimmunized animals in the first few weeks after infection. Of note, the 2 Ad5 Vx-SIV animals that became infected at a dose of 10^3 TCID_{50} had relatively low plasma vRNA levels from 3 to 7 weeks postinfection compared to unimmunized animals (Fig. 8), suggesting that there was a vaccine effect after infection in these two animals despite the fact that they became infected at the lowest SIV dose.

DISCUSSION
Several versions of the Merck Ad5-based vaccine have been tested in nonhuman primates (NHP), but preclinical studies of this vaccine have never been conducted in Ad5-seropositive animals that were then SIV challenged by penile inoculation. The present study was designed to determine whether a repli-
TABLE 3 Relative risk estimates for animal groups based on the indicated comparisons analyzed under a discrete time survival model assuming a leaky vaccine effect

| SIV dose | \( P_o^a \) | \( P_1^b \) | RR\(^c \) | \( P^d \) |
|----------|-------------|-------------|---------|-------|
| Ad5 Vx-SIV (II) vs. Ad5 Vx-empty (I) |
| \( 10^3 \) TCID\(_{50}\) | 0 | 0.024 | \( \approx \) | 0.15 |
| \( \leq 10^4 \) TCID\(_{50}\) | 0.007 | 0.027 | 4.46 | 0.15 |
| \( \leq 10^5 \) TCID\(_{50}\) | 0.039 | 0.033 | 1.12 | 0.77 |
| Ad5 Vx-SIV + Vx-SIV (II) vs. Ad5 Vx-empty + Vx-empty + control |
| \( 10^3 \) TCID\(_{50}\) | 0 | 0.011 | \( \approx \) | 0.08 |
| \( \leq 10^4 \) TCID\(_{50}\) | 0.021 | 0.030 | 1.40 | 0.40 |
| \( \leq 10^5 \) TCID\(_{50}\) | 0.035 | 0.039 | 1.10 | 0.69 |
| Ad5 Vx-SIV (II) vs. Vx-SIV |
| \( 10^3 \) TCID\(_{50}\) | 0 | 0.024 | \( \approx \) | 0.004 |
| \( \leq 10^4 \) TCID\(_{50}\) | 0.024 | 0.027 | 1.14 | 0.80 |
| \( \leq 10^5 \) TCID\(_{50}\) | 0.037 | 0.033 | 0.88 | 0.71 |

\(^a\) Average per exposure infection probability for group I in the paired composite groups, over different challenge dose levels, under the leaky model assuming homogeneous per exposure infection probability within the group for a given challenge level and independence between the probability of infection and the number of prior challenges.

\(^b\) Average per exposure infection probability for group II in the paired composite groups, over different challenge dose levels, using the same model and assumptions as in footnote \( a \).

\(^c\) Relative risk (RR) ratio of averaged per exposure infection probability for group II versus that for group I using the same model and assumptions as in footnotes \( a \) and \( b \).

\(^d\) That is, the \( P \) value based on the log-rank test comparing the two composite groups.

Dedicated Ad5-based SIV gag/pol/neo vaccine that is very similar to the vaccine used in the Step Trial would alter susceptibility to infection after penile SIV exposure. HIV infection rates in the Step Trial were the same in Ad5-seronegative vaccine and placebo recipients, but infection rates were more than twice as high in Ad5-seropositive vaccinees compared to placebo recipients (2). As in the Step Trial, there was no evidence of protection from SIV infection in immunized animals, and there was an indication that the vaccine may have enhanced the susceptibility of animals that were Ad5 seropositive at the time they were immunized with the Ad5 SIV vaccine. As in the animals in the present study, the elevated risk of HIV infection seen in Ad5-seropositive men was absent in Ad5-seronegative men receiving the vaccine (2). Importantly, Ad5-seropositive animals immunized with the empty Ad5 vector were not susceptible to the low-dose SIV challenge, suggesting that SIV-specific immunity was responsible for the enhanced susceptibility. However, because this conclusion is based on only two of nine animals becoming infected at the lowest challenge dose, a larger study is needed to confirm the results and to define the mechanisms behind any enhanced infectivity.

The conclusion that the Merck Ad5 HIV-1 subtype B Gag/Pol/Nef vaccine may have enhanced HIV acquisition in the Step Trial was based on the finding that 49 of the 914 male vaccine recipients were infected with HIV versus 33 of the 922 male placebo recipients (2). Thus, there was a difference of 16 infections among the approximately 1,800 men (0.89%) in the two groups in the trial (2). The conclusion of the present study that an SIV vaccine enhanced SIV acquisition is based on 2 infected animals out of 18 immunized animals and 0 of 25 unimmunized animals infected, a difference of 2 infections among the 47 NHP (4.2%). Thus, results in humans and NHP suggest that an Ad5 vector-based AIDS vaccine marginally increased virus acquisition, making it less likely that the findings in these studies are statistical artifacts. Further studies are warranted to determine whether a similar risk exists in women and to characterize the mechanisms underlying increased susceptibility to HIV infection in people vaccinated with Ad5 vector-based HIV vaccines.

Despite the small number of animals in the present study, it is possible to propose some preliminary hypotheses to explain the host-virus interactions responsible for the study outcome. At the low and middle virus doses, the infection risk of the Ad5 Vx-empty group was lower than the Vx-empty group, thus there was no evidence of enhanced susceptibility to penile SIV exposure in the Ad5-seropositive NHP immunized with the empty Ad5 vector. These results lead us to hypothesize that vaccine-induced vector-specific immune responses did not contribute to altered susceptibility in the immunized NHP. Rather, it seems that vaccine-induced SIV-specific immune responses are responsible for the enhanced susceptibility in Ad5-seropositive, immunized NHP. Further, as with the Step Trial, this effect was not seen in the immunized animals that were Ad5 seronegative at immunization. Although the presence of SIV-specific CD4\(^+\) T cells in mucosal tissues was not examined here, the hypothesis that vaccine-elicited SIV-specific T cells contribute to enhanced susceptibility is consistent with the fact that HIV and SIV preferentially infect virus specific CD4\(^+\) T cells (1, 10, 28). The hypothesis that Ad5-specific immunity does not explain the results of the Step Trial is supported by the results of a recent case-control study that found no evidence that Ad5 seropositivity increases susceptibility to HIV infection (6).

Given the dual role of CD4\(^+\) T cells as orchestrators of the immune responses to HIV vaccines and preferred targets for HIV infection, the balance of CD4\(^+\) versus CD8\(^+\) T cell and B cell responses induced by candidate AIDS vaccines may be critical in determining vaccine efficacy (13). Unfortunately, a similar empty vector control group was not included in the Step Trial, so the role of Ad5-specific and HIV-specific T cells in determining the Step Trial results is not known.

There was no significant effect of vaccination on setpoint plasma vRNA levels in the Step Trial (2). With the caveat that such analysis can be vulnerable to bias, a post hoc analysis of 87 participants who became infected during the trial found that Shortly after any enhanced infectivity.

FIG 7 Effect of immunization on peak plasma SIV RNA levels after penile challenge. (A) Peak plasma vRNA levels of immunized animals that became infected were significantly lower compared to the unimmunized, Ad5 empty vector animals that became infected. (B) Peak plasma vRNA levels of SIV-immunized animals that became infected were significantly lower than all unimmunized animals (Ad5 empty vector animals and naive control animals combined) that became infected. WRS, Wilcoxon rank sum test.
immunized individuals with specific MHC-I alleles had lower viral loads than placebo recipients (12). Further, HIV-1 genome sequences from newly infected vaccine recipients were genetically more distant from the vaccine Gag/Nef protein sequences than the HIV-1 sequences from infected placebo recipients, providing evidence of selective pressure on HIV founder populations from vaccine-induced T cell responses in the Step Trial (39). Thus, the Step data suggest that the Ad5 HIV-1 subtype B vaccine had a limited effect on virus replication and evolution in some immunized individuals with specific MHC-I alleles. In contrast, we found that the RM immunized with the replication defective Ad5-based SIV gag/pol/nef vaccine had significantly lower plasma vRNA levels in acute infection (peak and 6 weeks postinfection) than did the unimmunized animals. The animals in the present study were not maintained after 7 weeks postinfection, so it is not possible to determine whether the vaccine effect extended into the set-point phase of infection, but it seems likely that control of replication would have steadily waned as cytotoxic-T-lymphocyte escape variants emerged. While lower set point plasma vRNA levels in Step Trial were limited to individuals with specific MHC-I alleles (12), in the present study the control of SIV replication in the acute phase of infection extended to all immunized animals without regard to MHC-I haplotype.

The seemingly conflicting findings of enhanced susceptibility to infection in some vaccinated animals but postinfection control of virus replication in all immunized animals suggests that either (i) the same vaccine elicited T cell responses that enhanced virus transmission also may have contributed to control of virus replication after infection or (ii) vaccine-elicited T cell responses enhanced virus transmission in some vaccinated animals but contributed to control of virus replication after infection in others. In fact, compared to unimmunized animals (Ad5 Vx-empty, Vx-empty, and control), the two Ad5 Vx-SIV animals that became infected at a dose of 10^4 TCID_{50} had relatively low plasma vRNA levels from 3 to 7 weeks postinfection (Fig. 8), providing some support for the first hypothesis, although further studies are needed to determine the relative importance of the two possibilities.

The Merck Ad5 HIV-1 subtype B vaccine elicited CD8\(^+\) T cell responses in 73% of the recipients, but there were no obvious differences between the breadth, magnitude, or polyfunctional profile of HIV-specific T-cell responses of the infected and noninfected Step Study vaccine recipients (30). However, it was recently shown that the number of HIV-Gag specific interferon-γ secreting T cells in the Step Trial vaccine recipients can be linked to polymorphisms located in specific HLA-B alleles that are associated with natural immune control of HIV replication (11), providing an immune mechanism that can explain the sieving of HIV-1 founder genotypes (39). In addition, it is clear that Ad5-specific antibodies blunted the vaccine-induced HIV-specific immune responses of Step Trial participants (30) and altered the quality of the HIV-specific T cell immunity induced by the Merck Ad5 HIV-1 subtype B vaccine (37). In the present NHP study, approximately 70% of immunized animals made CD8\(^+\) T cell responses to at least one vaccine antigen at some point after immunization. However, preexisting Ad5-specific immunity decreased the number of animals responding to SIV Pol and Nef peptides, and most Gag CD8\(^+\) T cell responses were comprised of monofunctional IFN-γ-secreting cells.

Ad5 vector-based vaccines are highly effective against a chimeric simian-human immunodeficiency virus 89.6P (SHIV89.6P) challenge (21, 22, 40), leading to the conclusion that NHP models using SHIVs are not adequate for preclinical evaluation of candidate HIV vaccines, especially those designed to elicit effector T cell responses (33). In fact, subsequent NHP studies showed that although Ad5-SIV vaccines are ineffective against a high-dose SIVmac239 challenge, the immunized RM that became infected controlled viral replication better than naive animals (4, 5, 29). The NHP model described here carefully mimicked the design of the Step Trial and demonstrates that NHP studies using challenge viruses and routes of exposure designed to mimic the details and complexity of HIV sexual transmission can reflect the results of human clinical trials of AIDS vaccines. NHP models of HIV transmission provide the opportunity to understand the complex interactions between host immune responses to the vaccine vector, the expressed antigen, and HIV susceptibility. The results of the
Step Trial and the current report suggest that some candidate AIDS vaccines have the potential to enhance HIV acquisition.

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