Vaccine protection against SIVmac239 acquisition

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The biological characteristics of HIV pose serious difficulties for the success of a preventive vaccine. Molecularly cloned SIVmac239 is difficult for antibodies to neutralize, and a variety of vaccine approaches have had great difficulty achieving protective immunity against it in rhesus monkey models. Here we report significant protection against i.v. acquisition of SIVmac239 using a long-lasting approach to vaccination. The vaccine regimen includes a replication-competent herpesvirus engineered to contain a near-full-length SIV genome that expresses all nine SIV gene products, assembles noninfectious SIV virion particles, and is capable of eliciting long-lasting effector-memory cellular immune responses to all nine SIV gene products. Vaccinated monkeys were significantly protected against acquisition of SIVmac239 following repeated marginal dose i.v. challenges over a 4-month period. Further work is needed to define the critical components necessary for eliciting this protective immunity, evaluate the breadth of the protection against a variety of strains, and explore how this approach may be extended to human use.

SIV | HIV | AIDS vaccine | DNA electroporation | recombinant herpesvirus

HIV has evolved a variety of immune evasion strategies that allow persistent viral replication despite host immune responses to the infection (1, 2). These immune evasion strategies predict great difficulty in development of an effective preventive vaccine and likely have been important factors in the repeated failures of vaccine efficacy trials in humans (1, 3–8). SIVmac239 has been widely used for preclinical vaccine/challenge experiments in monkeys in attempts to model vaccine approaches for their protective capacity. One advantage of this strain is that it is molecularly cloned and of defined sequence, essentially homogeneous. This avoids complications that can arise with the use of uncloned virus stocks that may contain mixtures of easy-to-neutralize as well as difficult-to-neutralize variants present in the population. The clonal nature of the virus stock helps create well-defined, well-controlled conditions ideally suited for laboratory experiments. SIVmac239 is also difficult for antibodies to neutralize. While most monkeys infected with SIVmac239 do make antibodies with neutralizing activity against it, the titers are relatively low, approximately corresponding to the lowest quartile of what is seen following primary infection of humans with HIV-1 (9).

We recently described a recombinant herpesvirus that contains a near-full-length genome of SIVmac239 (10). The herpesvirus used is the gamma-2 herpesvirus of rhesus monkeys known as rhesus monkey rhadinovirus (RRV) (11, 12). As with other herpesviruses, infection with RRV persists for life. The recombinant strain, rRRV-SIVnfl, was engineered to contain a 306-bp deletion in the SIV *pol* gene that removes the active site from the essential reverse transcriptase enzyme. The coding sequences for all nine SIV genes are otherwise intact in this recombinant strain. Infection of cells by rRRV-SIVnfl results in production not only of replication-competent herpesvirus virion particles, but also of SIV virion particles that are noninfectious (10). Notably, rRRV-SIVnfl is capable of eliciting cellular immune responses to all nine SIV gene products (10). Here we describe the use of rRRV-SIVnfl in a vaccine regimen that provides protection against i.v. acquisition of SIVmac239.

Results

In this vaccine trial, a mixture of five rRRV variants was administered to six rhesus monkeys (*SI Appendix*, Fig. S1). Three of these variants were rRRV-SIVnfl, each with a different promoter to drive expression of the SIVnfl genome: the CMV immediate early promoter/enhancer, SIV's own long terminal repeat promoter enhancer, and a hybrid early/late promoter from RRV (*SI Appendix*, Fig. S1) (10). We used this combination of the three because the extent to which one may be better than another in terms of the ability to express in vivo in monkeys, the ability to persist, and the magnitude, quality, and persistence of resultant immune responses is unknown. Two additional recombinant strains expressing the SIV envelope (Env) protein, rRRV-SIV*env*, were also used (*SI Appendix*, Fig. S1). One of these strains encodes SIVmac239 *env*, and the other encodes the closely related SIVmac316 *env* protein. Both strains contain a truncating stop codon at E767 in the cytoplasmic domain to maximize surface expression of the encoded protein (13, 14). The codon usage of these *env* expression cassettes was modified to reflect the codon usage of RRV glycoprotein to allow adequate expression in the monkeys (15).

For the trial, 1 mL of a mixture of the five strains was administered i.v. to each of six Indian-origin rhesus macaques. Each rRRV vector strain was present at 1.0×10^{10} vector genomes per inoculum. A second dose of the same rRRV constructs was administered i.v. and orally at week 25. Following vaccine administration, all six monkeys seroconverted to the SIV envelope glycoproteins gp120 and gp140 (Fig. 1 *A* and *B*). These antibodies persisted for the entire 46-wk follow-up period (Fig. 1*C*). These anti-SIV antibodies had neutralizing activity against SIVmac316

Significance

Given the extreme difficulty in developing an effective vaccine against HIV, monkey models are being used in an attempt to discover novel approaches that may result in protective immunity. The difficult-to-neutralize cloned virus strain SIVmac239 has proven notoriously refractory to a variety of vaccine approaches. Here we describe significant vaccine protection against SIVmac239 acquisition following intravenous challenge. Only live attenuated strains of SIV have previously provided such protection against SIVmac239 acquisition.

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The authors declare no conflict of interest.

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(Fig. 1*D*) but did not detectably neutralize SIVmac239 (*SI Appendix*, Fig. S2). In the six monkeys, 50% neutralization titers (ID_{50}) to SIVmac316 at week 46 ranged from 1:220 to 1:3,130 (Fig. 1*D*). These titers are 9.5- to 130-fold lower than the titer present in serum from a monkey infected with SIVmac239Δ*nef* for 19 wk (Fig. 1*D*).

We performed intracellular cytokine staining (ICS) assays to monitor SIV-specific T cell responses in all six animals throughout the vaccine phase. These assays included pools of peptides spanning all nine SIV proteins. At week 4 after rRRV vaccination, low to modest levels of SIV-specific $CD8^+$ T cell responses against all nine SIV proteins were detected, although not necessarily in the same animal (Fig. 2*A*). Most of these responses contracted to below detection limits in the ensuing weeks. A similar pattern was observed in the frequency of vaccine-induced SIV epitope-specific $CD8^+$ T cells, as revealed by staining with peptide/MHC I tetramers to defined epitopes (Fig. 2 *B* and *C*). $CD4^+$ T cell responses against Gag, Pol, and Vpx were also detected in some of the animals, albeit at very low frequencies (*SI Appendix*, Fig. S3).

Beginning at week 46, immune responses were boosted by repeated immunizations with an SIVnfl-expressing recombinant (r)DNA plasmid delivered by i.m. electroporation (EP rDNA). The rDNA-SIVnfl vector used the CMV promoter to drive expression. The SIVmac239 Env protein was truncated at E767, and the *nef* gene contained a 6-bp deletion corresponding to Nef amino acids 239–240 to abrogate down-regulation of MHC-I (16). The *tat* gene also encoded a L35Q substitution to inactivate the immunodominant Mamu-A*01–restricted $Tat_{28–35}$SL8 epitope (17). Because subdominant $CD8^+$ T cell responses can be actively suppressed by dominant $CD8^+$ T cell responses in the context of rDNA immunization (18), the rationale for the Tat L35Q change in the rDNA-SIVnfl vector was to broaden the repertoire of vaccine-induced SIV-specific $CD8^+$ T cells in the *Mamu-A*01*$^+$ vaccinees in the present study. A total of four EP rDNA immunizations were delivered at 3-wk intervals.

At 1 day after the third and fourth EP rDNA boosters, the vaccinated macaques were treated with 3 mg/kg body weight of an mAb that blocks the immune checkpoint (IC) receptor cytotoxic T lymphocyte antigen-4 (CTLA-4). CTLA-4 is up-regulated on T cells shortly after activation and suppresses immune responses by interfering with CD28 signaling, a prerequisite for proper T cell activation (19). Consequently, blocking CTLA-4 during antigen exposure can amplify the ensuing effector immune response. The EP rDNA immunizations resulted in repeated boosting of the anti-SIV antibody levels (Fig. 1*C*). Neutralizing activity (ID_{50}) to SIVmac316 increased from the preboost range of 1:220–1:3,130 to 1:5,000–1:24,500 following the fourth EP DNA boost (Fig. 1*D*; $P = 0.03$). The median of this range was only slightly less than the ID_{50} titer of 1:29,600 present in serum from a monkey infected with SIVmac239Δ*nef* for 19 wk (Fig. 1*D*). Again, neutralizing activity against SIVmac239 was not detected (*SI Appendix*, Fig. S2).

The EP rDNA booster vaccinations also increased the frequency of $CD8^+$ T cell responses against all nine SIV proteins (Fig. 2). This effect was particularly pronounced for responses targeting Gag and Nef epitopes. Indeed, Gag-specific $CD8^+$ T cells composed 51% of peripheral $CD8^+$ T cells in monkey rh2445 at 2 wk after the third EP DNA immunization. Despite some contraction after the fourth EP rDNA dose, the median total frequency of SIV-specific $CD8^+$ T cell responses in these animals was 5% at study week 67, that is, 2 wk before the first SIVmac239 challenge. A memory phenotype analysis conducted at this time point revealed that ≥87% of vaccine-induced MHC-I tetramer-positive $CD8^+$ T cells displayed an effector memory signature ($CD28^-CCR7^-$; *SI Appendix*, Fig. S4). Vaccine-induced SIV-specific $CD4^+$ T cell responses also expanded following the EP rDNA booster immunizations (*SI Appendix*, Fig. S3).

Beginning at week 69 following the initial rRRV administrations, 14 wk after the final EP rDNA boost, we proceeded with a series of i.v. marginal dose challenges with SIVmac239. Six MHC-I-matched unvaccinated rhesus monkeys served as controls. The dose used for each challenge contained an estimated ~0.3–0.5 infectious unit per rhesus monkey by the i.v. route. Challenges were performed every 3 wk until the sixth challenge was completed in accordance with the protocol. Viral loads through the 13th day after each challenge were used to determine which monkeys were rechallenged on the 21st day. Only animals that remained aviremic after an SIV challenge were reexposed to SIV; those with positive viral loads continued to be bled to determine the viral load kinetics after infection.

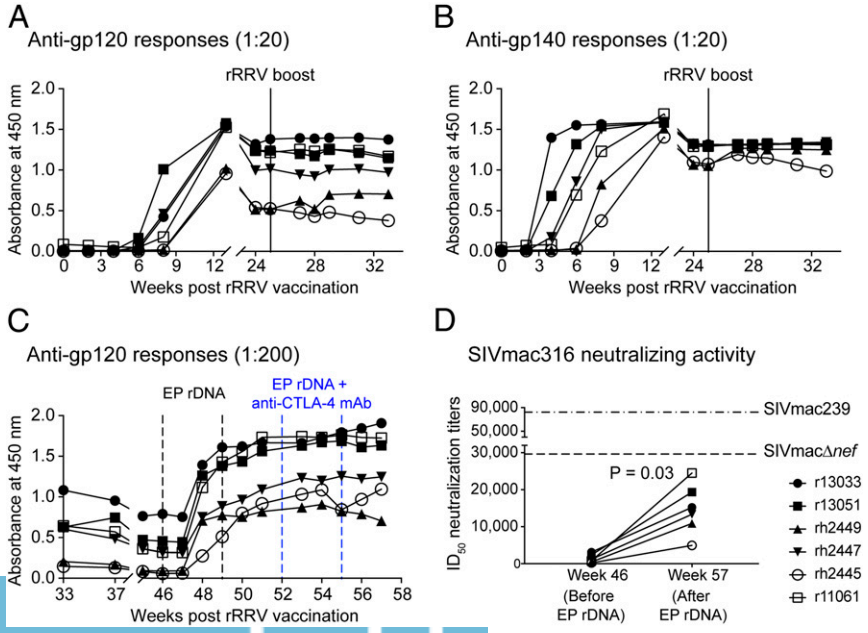

Fig. 1. (*A* and *B*) Antibody responses following rRRV immunization and boosting by EP rDNA. Antibody responses to SIV gp120 SU (*A*) and gp140 (*B*) proteins were measured by ELISA over the first 33 wk following rRRV immunization. Serum samples diluted 1:20 were used for these assays. (*C*) Antibody responses to SIV gp120 SU protein pre- and post-EP rDNA boost measured by ELISA. Serum samples diluted 1:200 were used for these assays. (*D*) Neutralizing activity (ID_{50} titers) against SIVmac316 before (week 46) and after (week 57) the EP rDNA booster immunizations. The ID_{50} titer of an animal infected with SIVmac239Δ*nef* for 19 wk is shown as a horizontal dashed line. The ID_{50} titer of an animal chronically infected with SIVmac239 is shown as a horizontal dashed-dotted line. The *P* value was determined by the Wilcoxon matched-pairs signed-rank test.

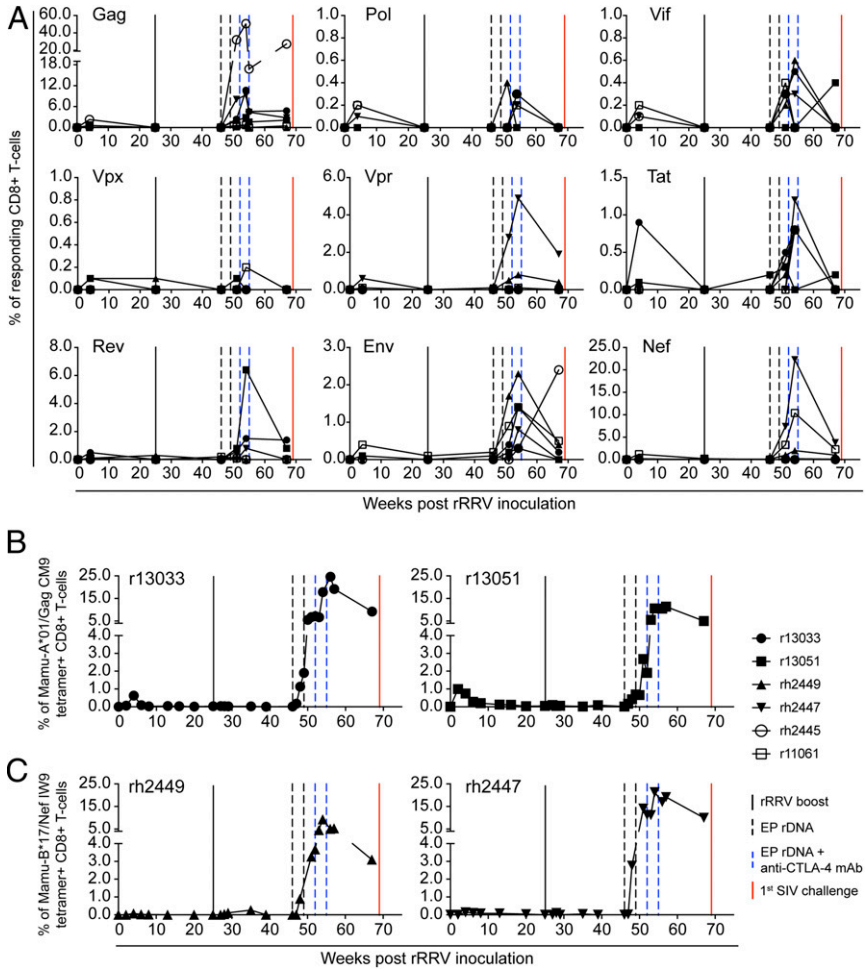

Fig. 2. Ontogeny of SIV-specific CD8$^+$ T cell responses in rRRV/EP rDNA-vaccinated monkeys. (*A*) Vaccine-induced CD8$^+$ T cell responses against the entire SIV proteome were quantified by ICS assays using pools of peptides corresponding to each of the nine SIVmac239 gene products. Each panel depicts the frequency of CD8$^+$ T cell responses in PBMCs against one SIV protein. The percentages of responding CD8$^+$ T cells against each protein were calculated by adding the background-subtracted frequencies of positive responses producing any combination of IFN-γ, TNF-α, and CD107a. (*B* and *C*) Frequencies of vaccine-induced, SIV epitope-specific CD8$^+$ T cell responses in *Mamu-A*01*$^+$ (*B*) and *Mamu-B*17*$^+$ (*C*) macaques. The panels show the percentages of Mamu-A*01/Gag CM9 tetramer$^+$ (*B*) and Mamu-B*17/Nef IW9 tetramer$^+$ (*C*) CD8$^+$ T cells in PBMCs during the vaccine phase. Each symbol corresponds to one rRRV/EP rDNA-vaccinated animal.

Notably, four of six vaccinees remained uninfected after six SIVmac239 challenges (Fig. 3*A*). In comparison, five of the six control animals became infected by the sixth SIV challenge (Fig. 3*A*). This difference in the rate of SIV acquisition between vaccinees and control animals was statistically significant (P = 0.05, log-rank test). The rRRV/EP rDNA vaccine regimen also resulted in a 79% reduction in the per-exposure probability of infection compared with the contemporaneous control group (P = 0.04, Fisher's exact test). Since we had historical controls that received the same dose of the same challenge stock with the same regimen through six challenges that could be included in the analysis, we also compared the rate of acquisition between vaccinees and contemporaneous (n = 6) plus historical (n = 10) controls (Fig. 3*B*). This approach strengthened the conclusion that vaccinees were significantly protected against SIV infection (P = 0.003, log-rank test; P = 0.002, Fisher's exact test). Of note, there was no significant difference in the per-exposure probability of infection between the contemporaneous controls and the historical controls (P = 0.33, Fisher's exact test).

The two SIV-infected vaccinees exhibited modestly reduced viral loads compared with the control group (Fig. 4). The difference was statistically significant when median peak viral loads were compared between vaccinees and contemporaneous plus historical controls (P = 0.01). As for the four vaccinees that resisted SIV infection, their vaccine-induced SIV-specific cellular and humoral immune responses were still readily detectable at the end of the challenge phase, albeit at somewhat decreased levels compared with prechallenge time points (*SI Appendix*, Fig. S5).

Discussion

Vaccine-mediated protection against acquisition of SIVmac239 infection is exceedingly difficult to achieve. To our knowledge, only live-attenuated SIV vaccines have conferred protective immunity against SIVmac239 acquisition in Indian-origin rhesus macaques. Considering the stringency of this SIV challenge model, our finding that the rRRV/EP rDNA vaccine regimen reduced the per-exposure probability of infection by 79% is not only encouraging, but also perhaps instructive. There may be a number of distinct advantages to the use of recombinant herpesviruses as vaccine vectors. Herpesviruses have large DNA genomes and can accommodate large amounts of genetic information in a stable fashion. We have pushed the limits of this here by using rRRV-SIVnfl vectors, which harbor >9,000 bp of inserted sequences and express all but 102 amino acids, >95% of the SIV proteome (10). There are eight distinct human herpesviruses from which to choose (20). These eight human herpesviruses

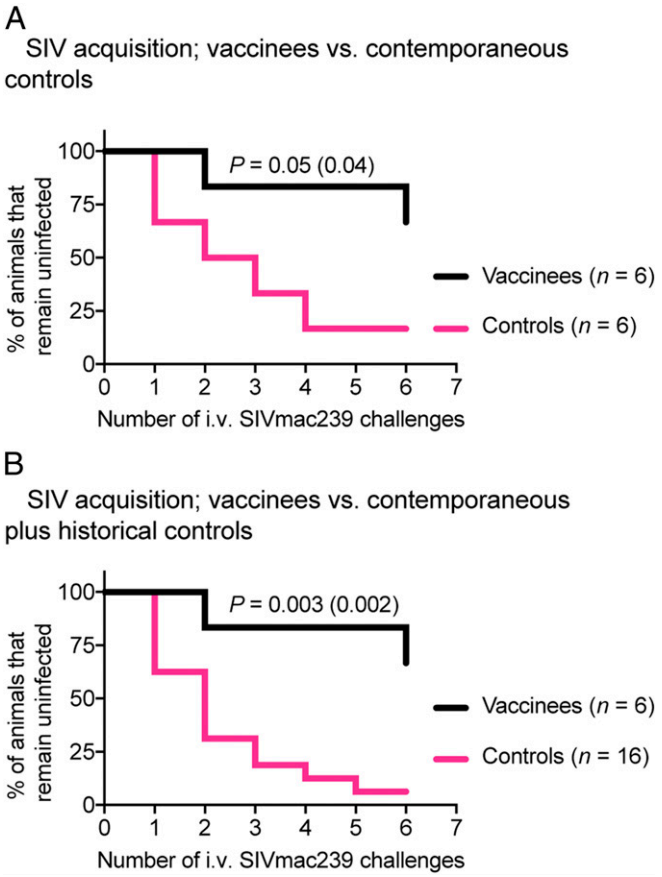

Fig. 3. SIVmac239 acquisition in vaccinated and control monkeys. Vaccinated and control monkeys were subjected to repeated (every 3 wk) i.v. challenges with a marginal dose of SIVmac239. Viral loads were determined between each SIV exposure. Once an animal became infected, it was no longer challenged. (*A*) Kaplan–Meier analysis of SIV acquisition in vaccinees ($n = 6$) vs. contemporaneous control animals ($n = 6$). (*B*) Kaplan–Meier analysis of SIV acquisition in vaccinees ($n = 6$) vs. contemporaneous ($n = 6$) plus historical ($n = 10$) control animals. *P* values outside parentheses were determined using the log-rank test. *P* values enclosed in parentheses were determined by the Fisher exact test based on the per-exposure probability of acquiring SIV infection through six SIVmac239 challenges.

have distinct biological properties, differ in the cell types for primary replication, differ in the sites of persistence, and even differ somewhat in the composition of genes that they carry (20). There is no way of knowing a priori whether one virus will perform better or worse than another in the context of a recombinant vaccine for HIV/AIDS. Finally, and perhaps most importantly, herpesviruses persist for life, and immune responses to them persist in an up, on, active fashion (21). Given the properties of HIV, it seems unlikely that immunologic memory will be sufficient to provide long-term vaccine protection against it (22).

The EP rDNA booster vaccinations resulted in robust expansions of both cellular and humoral immune responses against SIV. It is difficult to estimate the extent to which the EP rDNA boosters contributed to the efficacy of the rRRV/EP rDNA vaccine regimen; nonetheless, the impressive immunogenicity seen in this study highlights some key advantages of this vaccine platform. Because DNA does not elicit antivector immunity, it can be used to repeatedly boost immune responses irrespective of the vector used as a prime. rDNA vaccines are also stable and can be coupled with various adjuvants (23), including IC inhibitors, as demonstrated here. Despite the clinical success of mAbs against CTLA-4 and programmed death-1 as cancer immunotherapies (24), these drugs remain largely unexplored as prophylactic vaccine adjuvants. Although the extent to which anti–CTLA-4 therapy contributed to the overall immunogenicity and efficacy of the rRRV/EP rDNA vaccine regimen is unclear, certain aspects of the biology of CTLA-4 make it an attractive target for enhancing vaccine-induced immune responses against HIV. For example, CTLA-4 regulates T follicular helper cell responses and germinal center reactions (25, 26). Consequently, blocking CTLA-4 during HIV vaccination might improve anti-Env humoral immune responses. Furthermore, since CTLA-4 contributes to the maintenance of peripheral tolerance, anti–CTLA-4 therapy during vaccination may release host regulatory controls that normally limit the activation of B cells capable of producing neutralizing antibodies (27, 28). However, because systemic IC blockade can be associated with immune adverse effects (29), vaccine strategies will need to be optimized to safely harness the potent immunostimulatory effects of IC modulation.

Is it reasonable to think that a recombinant herpesvirus could be used in humans? Well, a replication-competent, attenuated strain of the zoster herpesvirus has been injected into millions of people as the licensed vaccine for chicken pox in children and its recurrence as shingles in older adults (30, 31). In addition, a replication-competent strain of CMV was given to human volunteers decades ago but was not advanced to larger-scale testing (32, 33). If a recombinant herpesvirus turns out to outperform a vast array of other vaccine approaches in preclinical monkey testing, it is reasonable to think that attempts could be made to advance to small-scale testing in humans. In the case of the studies described here, the human equivalent would be human herpesvirus-8. It is likely that one or more herpesviral genes would need to be deleted from any such recombinant to help avoid any long-term adverse consequences in some percentage of the recipients. Further preclinical studies are needed for the system that we describe here.

Are both rRRV and EP rDNA-SIVnfl vaccinations needed for the protective effects that were observed? Are there simple ways of maximizing protection against heterologous strains? How long do the protective effects last? Would EP rDNA boosts be needed at periodic intervals to maintain the protective state? Will this approach protect against vaginal and rectal challenges? Of note, neither the levels of vaccine-induced Env-binding antibodies nor the titers of anti-SIVmac316 neutralizing antibodies before SIVmac239 challenge correlated with protection from infection. Similarly, the total magnitude of vaccine-elicited SIV-specific $CD8^+$ T cell responses before the first SIVmac239 challenge was also not predictive. Future studies will need to focus on identifying the key components of the vaccine regimen, on the immune correlates of protection, and on the mechanisms of protective immunity afforded by the rRRV/EP rDNA vaccine regimen. In any event, the results described here are sufficiently promising to justify further efforts along these lines.

Materials and Methods

Research Animals. Twelve rhesus macaques (*Macaca mulatta*) of Indian origin were used in this study. All animals were housed at the Wisconsin National Primate Research Center (WNPRC) and were cared for under a protocol approved by the University of Wisconsin Graduate School's Animal Care and Use Committee and in accordance with the guidelines of the Weatherall Report (34). Vaccinations were performed under anesthesia (ketamine administered at 5–12 mg/kg depending on the animal), and all efforts were made to minimize suffering. Euthanasia was performed whenever an animal experienced conditions deemed distressful by a veterinarian at the WNPRC. All euthanasias were performed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and consisted of an i.v. overdose (≥50 mg/kg or to effect) of sodium pentobarbital or equivalent, as approved by a clinical veterinarian, preceded by ketamine (at least 15 mg/kg body weight) given via the i.m. route. The MHC I genotype, sex, and age of each monkey at the beginning of the SIV challenge phase are shown in *SI Appendix*, Table S1.

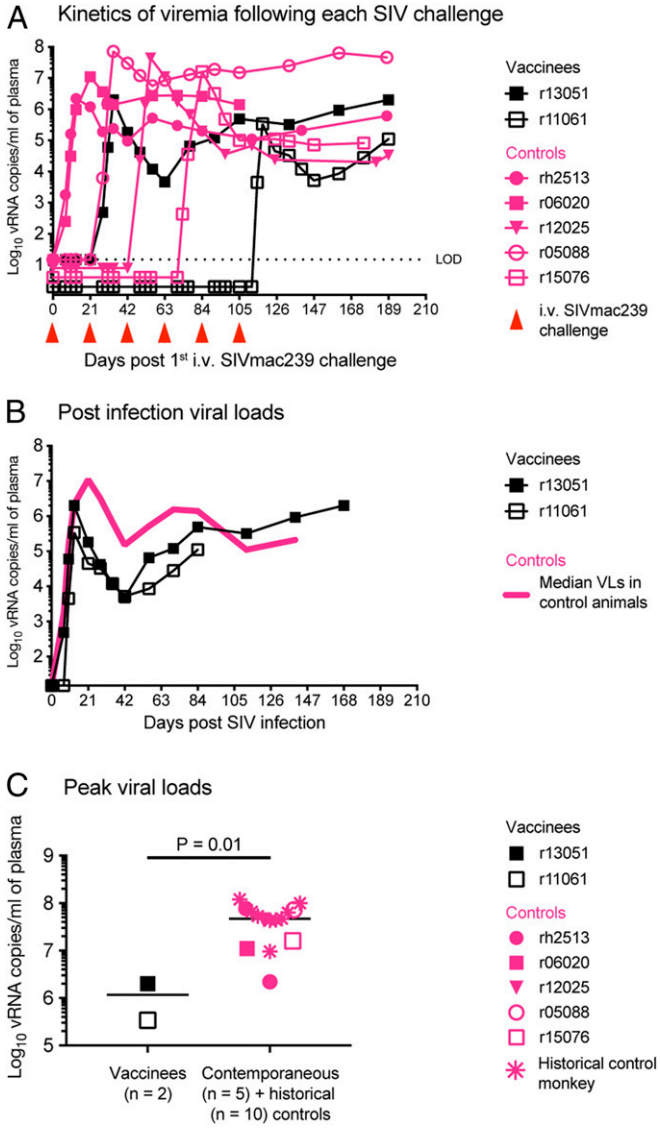

Fig. 4. Outcome of repeated i.v. SIVmac239 challenges in vaccinated and control rhesus macaques. (*A*) Viral load kinetics in vaccinees and control animals following each i.v. marginal-dose challenge with SIVmac239. A total of six challenges were performed every 3 wk. The four vaccinees and one control monkey that remained aviremic after this six-challenge series are not displayed. LOD, limit of detection. (*B*) Individual viral loads from the two SIV-infected vaccinees were plotted alongside the median viral loads from the five contemporaneous control animals at corresponding time points. (*C*) Comparison of peak viral loads between the two SIV-infected vaccinees and control macaques. The latter animals include contemporaneous ($n = 5$) and historical ($n = 10$) macaques, all of which were challenged with the same i.v. SIVmac239 regimen (same dose of the same stock) used here. *P* values were determined using the Mann–Whitney *U* test. The lines represent median values.

Cell Culture. Cells from a rhesus monkey fibroblast cell line (RF, NEPRC) were cultured and maintained as described previously (10).

Generation of Recombinant RRVs. Recombinant (r)RRV stocks containing a near-full-length SIVmac239 (SIVnfl) genome were generated as described previously (10, 11, 35). The SIVnfl genome harbored a 520-bp deletion in the 5′ LTR region, a 306-bp deletion in the *pol* gene corresponding to the active site of reverse transcriptase, and a 414-bp deletion in the 3′ LTR region. SIVnfl also encoded a V5 tag in the C terminus of Nef. In total, three rRRV/SIVnfl constructs were generated using the CMV immediate-early enhancer and promoter, an intact 5′ SIVmac239 LTR, or a combination of the RRV ORF26 (p26) and polyadenylated nuclear (PAN) RNA promoters to drive expression of the SIVnfl insert (10). In addition, two rRRVs encoding SIVmac239*env* or SIVmac316*env* were generated. These *env* inserts had an RRV gH-like codon usage to allow adequate expression in vivo (15). Moreover, to increase the surface expression of Env, both SIVmac239 and SIVmac316 *env* inserts encoded a truncated Env protein due to a E767stop mutation (13, 14).

Vaccinations. For vaccination, 1 mL of PBS containing 1.0×10^{10} genome copies (GCs) of each of the five rRRV constructs described above (5.0×10^{10} GCs total) was administered i.v. to the six vaccinees. All animals seroconverted to RRV following the rRRV vaccination. A second rRRV vaccination was performed 25 wk later, when the aforementioned rRRV inoculum (same dose in 1 mL of PBS) was given simultaneously via the i.v. and oral routes. This second rRRV vaccination did not appear to boost SIV-specific immune responses. A series of four rDNA immunizations, given at 3-wk intervals, began 46 wk after the first rRRV inoculation. The DNA vector used for these immunizations was the pCMVkan plasmid (36). It encoded the same SIVnfl insert delivered by the rRRV/SIVnfl vectors, except for three modifications. First, the *nef* gene contained a 6-bp deletion corresponding to Nef amino acids 239–240 to abrogate down-regulation of MHC I (16). Second, the *tat* gene encoded a L35Q substitution to inactivate the immunodominant Mamu-A*01–restricted $Tat_{28-35}SL8$ epitope (17). Because subdominant $CD8^+$ T cell responses can be actively suppressed by dominant $CD8^+$ T cell responses in the context of rDNA immunization (18), the rationale for the Tat L35Q change in the pCMVkan-encoded SIVnfl insert was to broaden the repertoire of vaccine-induced SIV-specific $CD8^+$ T cells in the *Mamu-A*01*$^+$ vaccinees in the present study. The third modification was the aforementioned Env E767stop substitution to increase surface expression of Env. Four milligrams of pCMVkan-expressing SIVnfl were delivered per occasion using the TriGrid in vivo electroporation (EP) system (Ichor Medical Systems). Four sites in the left and right thighs and shoulders were used for each EP rDNA immunization, with each site receiving 1.0 mg of pCMVkan-expressing SIVnfl per occasion. This amount of rDNA was delivered in a final volume of 0.5 mL of PBS. At 1 d after the third and fourth EP rDNA immunizations, the vaccinated animals received 3 mg/kg of the anti–CTLA-4 mAb ipilimumab (37) (Bristol-Myers Squibb) via the i.v. route.

ELISA. Anti-Env antibodies were measured by ELISA as described previously (10).

SIVmac316 and SIVmac239 Neutralization Assays. SIV neutralization was measured by the TZM-bl assay, as described previously (10, 38).

MHC-I Tetramer Staining of $CD8^+$ T Cells. Fluorochrome-labeled Mamu-A*01 (MBL International) and Mamu-B*17 (NIH Tetramer Core Facility) tetramers were used to quantify SIV-specific $CD8^+$ T cells in peripheral blood mononuclear cells (PBMCs) according to a recently published protocol (39). Samples were acquired using FACSDiva version 6 (BD Biosciences) on a special order research product BD LSR II flow cytometer (BD Biosciences) equipped with a 50-mW, 405-nm violet, 100-mW 488-nm blue, and 30-mW 635-nm red laser. FlowJo 9.9 was used to analyze flow cytometry data, as described previously (39). All tetramer frequencies mentioned in this manuscript correspond to percentages of live $CD14^-CD16^-CD20^-CD3^+CD8^+tetramer^+$ lymphocytes.

ICS Assay. The antigen stimuli consisted of 16 pools of SIVmac239 peptides (15mers overlapping by 11 aa) corresponding to Gag amino acids 1–263 (1) and 253–510 (2); Pol amino acids 1–354 (3), 344–700 (4), and 690–1060 (5); the amino acids corresponding to the entire Vif (6), Vpx (7), Vpr (8), Tat (9), and Rev (10) ORFs; Env amino acids 1–175 (11), 161–355 (12), 340–531 (13), 516–707 (14), and 692–879 (15); and the amino acids spanning the entire Nef ORF (16). The final concentration of each 15mer in the ICS tubes was 1.0–10.0 μM, depending on the peptide pool. Because of low cell numbers in the ICS assay performed at week 4 post-rRRV inoculation, two pools of Env gp120 or gp41 peptides were created by combining pools 11–13 and pools 14 and 15, respectively. The ICS assay was set up and the data were analyzed as described previously (10).

SIVmac239 Challenges. Vaccine efficacy was assessed by subjecting vaccinated and control macaques to repeated (every 3 wk) i.v. challenges with SIVmac239. The challenge inoculum consisted of 1x $MmID_{50}$ of the SIVmac239 10/1/1990 stock, which corresponds to 0.3 pg of Gag p27. Plasma samples obtained on days 7, 10, and 13 after each SIV exposure were screened for the presence SIV RNA. Only the macaques that remained aviremic on all three days were rechallenged in the next cycle. Conversely, animals that had positive viral loads at any of these time points were considered infected and thus were not

subjected to additional SIV challenges. SIV viral loads were measured using 0.5 mL of EDTA-anticoagulated rhesus macaque plasma based on a modification of a previously published protocol (40). The limit of reliable quantitation on an input volume of 0.5 mL of plasma was 15 vRNA copies/mL.

Statistical Analyses. The titers of vaccine-induced Env-binding antibodies measured before and after the EP rDNA booster immunizations were compared using the Wilcoxon matched-pairs signed-rank test. The Kaplan–Meier method and log-rank test were used to determine whether the rRRV/EP rDNA vaccine regimen affected the acquisition of SIV infection. For this analysis, the time to productive infection was analyzed using the Kaplan–Meier method, and the differences between vaccinees and control animals were evaluated using the log-rank test. As mentioned above, only the animals that remained aviremic on days 7, 10, and 13 following each SIV exposure were rechallenged on day 21. Monkeys that showed a positive viral load at any of these time points were not rechallenged. Based on this setup, the per-exposure probabilities of infection in vaccinees and control animals were calculated by dividing the number of monkeys that acquired SIV infection following six i.v. SIVmac239 challenges by the total number of challenges performed during this period. This resulted in per-exposure probabilities of infection of 6.2% for the vaccinated animals ($n = 6$), 29.4% for the contemporaneous control animals ($n = 6$), and 40.5% when both contemporaneous and historical control animals ($n = 16$) were considered. The historical monkeys used in this analysis were subjected to the same i.v. SIVmac239 challenge regimen (same dose and virus stock) used here as part of previous experiments conducted by our group. The aforementioned infection rates were used to determine the extent to which the rRRV/EP rDNA vaccine regimen reduced the probability of acquiring infection per exposure, which ranged from 79% to 85%, depending on whether or not the historical control animals were considered. The total number of animals that remained infected or uninfected after six i.v. SIVmac239 challenges was compared between the vaccinated and control animals using the Fisher exact test. This analysis was performed with contemporaneous controls alone or combined with historical controls. All significance tests were two-tailed and calculated using Prism 7 (GraphPad Software).

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