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Immunization of Macaques with Single-Cycle Simian Immunodeficiency Virus (SIV) Stimulates Diverse Virus-Specific Immune Responses and Reduces Viral Loads after Challenge with $SIV_{mac}239$

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Genetically engineered simian immunodeficiency viruses (SIV) that is limited to a single cycle of infection was evaluated as a nonreplicating AIDS vaccine approach for rhesus macaques. Four Mamu-A*01$^+$ macaques were inoculated intravenously with three concentrated doses of single-cycle SIV (scSIV). Each dose consisted of a mixture of approximately equivalent amounts of scSIV strains expressing the $SIV_{mac}239$ and $SIV_{mac}316$ envelope glycoproteins with mutations in *nef* that prevent major histocompatibility complex (MHC) class I downregulation. Viral loads in plasma peaked between 10^4 and 10^5 RNA copies/ml on day 4 after the first inoculation and then steadily declined to undetectable levels over the next 4 weeks. SIV Gag-specific T-cell responses were detected in peripheral blood by MHC class I tetramer staining (peak, 0.07 to 0.2% CD8$^+$ T cells at week 2) and gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays (peak, 50 to 250 spot forming cells/10^6 peripheral blood mononuclear cell at week 3). Following the second and third inoculations at weeks 8 and 33, respectively, viral loads in plasma peaked between 10^2 and 10^4 RNA copies/ml on day 2 and were cleared over a 1-week period. T-cell-proliferative responses and antibodies to SIV were also observed after the second inoculation. Six weeks after the third dose, each animal was challenged intravenously with $SIV_{mac}239$. All four animals became infected. However, three of the four scSIV-immunized animals exhibited 1 to 3 log reductions in acute-phase plasma viral loads relative to two Mamu-A*01$^+$ control animals. Additionally, two of these animals were able to contain their viral loads below 2,000 RNA copies/ml as late as 35 weeks into the chronic phase of infection. Given the extraordinary difficulty in protecting against $SIV_{mac}239$, these results are encouraging and support further evaluation of lentiviruses that are limited to a single cycle of infection as a preclinical AIDS vaccine approach.

The development of a safe and effective AIDS vaccine is proving to be a particularly elusive challenge. A number of vaccine approaches based on prime and boost regimens with recombinant DNA and/or viral vectors designed to stimulate potent cellular immune responses to human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) antigens are currently being evaluated. Initial optimism for these vaccines was driven by animal studies demonstrating promising levels of protection against certain challenge viruses, most notably the simian-human immunodeficiency virus chimera SHIV 89.6P (6, 10, 50, 56). There is now increasing evidence that the efficacy of these vaccine approaches may depend upon the challenge virus and that they are unlikely to be as effective against naturally transmitted isolates of HIV-1 (19, 55). When two of the most promising recombinant vaccine approaches were evaluated for protection against $SIV_{mac}239$, which more closely resembles naturally transmitted isolates of HIV-1 in terms of resistance to neutralizing antibodies, coreceptor usage, and the nature of disease course, the results of protection were much less impressive (25, 55). The ability of nonpersisting recombinant vaccines to stimulate lasting protective immunity against the extraordinary diversity of circulating HIV-1 field isolates is also questionable. Thus, while recombinant vectors pose few vaccine-associated risks, there is little reason for optimism with respect to their potential to afford reliable protection beyond idealized laboratory conditions (14, 20).

Live attenuated strains of SIV have provided considerably more robust protection in nonhuman primate trials (13, 62, 63). However, depending on the particular mutation or combination of mutations used to attenuate the virus, a certain percentage of immunized animals may eventually develop AIDS as a result of vaccination (8, 9, 64). When this occurs, it appears to be the result of the accumulation of genetic changes during continuous rounds of replication in vivo that ultimately restores a pathogenic phenotype to the virus (2). Hence, there

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are justifiable safety concerns that will likely preclude the use of live, attenuated strains of HIV-1 in humans for the foreseeable future.

In an effort to develop an AIDS vaccine approach with a more promising safety/efficacy profile, we have devised a genetic system for producing strains of SIV, and potentially HIV-1, that are limited to a single cycle of infection (17). Single-cycle lentiviruses have a number of theoretical advantages over other vaccine approaches. Lentiviruses that are limited to one round of infection should stimulate a full spectrum of virus-specific immune responses comparable to the breadth of antibody and cellular immune responses elicited by live, attenuated viruses including antibodies to the native, oligomeric envelope glycoprotein and $CD8^+$ and $CD4^+$ T-cell responses to multiple viral epitopes. Additionally, in the absence of any vector-derived gene products, complications associated with vector-specific immunity should be alleviated. Single-cycle lentiviruses should also be considerably safer than live attenuated vaccine strains, since the virus can no longer regain a pathogenic phenotype through continuous rounds of replication.

Our approach for producing single-cycle SIV (scSIV) is based on Gag-Pol-complementation of an SIV genome that is deficient for Pol expression as a result of a combination of mutations in the *gag-pol* frameshift site and in *pol* (17). Gag and Gag-Pol are normally made from the same mRNA transcript at a molar ratio of approximately 20:1. This ratio is achieved by ribosomal frameshifting between the *gag* and *pol* reading frames (11). Ribosomal frameshifting physically occurs during protein translation within a conserved 7-nucleotide "slippery sequence" in the *gag-pol* mRNA (28). By introducing mutations into this 7-nucleotide element that prevent the translation of Pol and by providing Gag-Pol in *trans* from a second construct that also contains the same mutations in the slippery sequence, we have developed a unique two-plasmid system for producing Gag-Pol-complemented virus by cotransfection into monolayer cells (17). The principal advantage of this approach is that if recombination between the two constructs occurs, the resulting virus will remain defective for replication, since neither the viral genome nor the Gag-Pol expression construct retains a functional frameshift site. To minimize the potential for recovery of replication-competent viruses as a result of nucleotide reversion of changes within the frameshift site, additional downstream deletions were introduced into the viral *pol* gene. Cells infected with scSIV produced by this approach express all of the viral gene products except for Pol and release immature virus particles that are unable to complete subsequent rounds of infection (17).

In the present study, we evaluated scSIV as a potential nonreplicating AIDS vaccine strategy by immunizing four rhesus macaques with concentrated doses of scSIV and then challenging them with $SIV_{mac}239$ to assess protection. Each of the scSIV-immunized animals made antibody and T-cell responses to multiple viral antigens. After challenge, three of the animals exhibited reductions in their acute- and chronic-phase viral loads relative to unvaccinated, control animals. Given the extraordinary difficulty in protecting against $SIV_{mac}239$, these preliminary results are encouraging and support further evaluation of single-cycle lentiviruses as an AIDS vaccine approach using nonhuman primate models.

MATERIALS AND METHODS

Single-cycle SIV constructs. Six different full-length genomic constructs were used for the production of scSIV. These included p239FS$^-$ΔIN-EGFP, p239FS$^-$ΔIN-Nef, p239FS$^-$ΔIN-Nef^{sff}, p316FS$^-$ΔIN-EGFP, p316FS$^-$ΔIN-Nef, and p316FS$^-$ΔIN-Nef^{sff}. Each construct contained three nucleotide changes in the *gag-pol* frameshift site (FS$^-$; TTTTTTA→CTTCCTA, changes are underlined) to inactivate Pol expression (54) and a 105-bp deletion (nucleotides 4529 to 4634) (47) in the integrase coding region of *pol* (ΔIN) to prevent the recovery of replication-competent virus by nucleotide reversion (17). Envelope sequences from the parental $SIV_{mac}239$ genome were exchanged with *env* sequences from $SIV_{mac}316$ to generate constructs for the production of macrophage-tropic viruses (41). In p239FS$^-$ΔIN-Nef^{sff} and p316FS$^-$ΔIN-Nef^{sff}, a premature stop codon followed by two single-nucleotide deletions were introduced into the *nef* gene (Nef^{sff}; GGC→TGA, 9788 to 9790, Δ9791, Δ9797) (47) to eliminate residues essential for major histocompatibility complex (MHC) class I downregulation (59). Since this region of *nef* overlaps the 3′ long terminal repeat (LTR), these changes were also introduced into the 5′ LTR to prevent restoration of *nef* through LTR-LTR recombination. In p239FS$^-$ΔIN-EGFP and p316FS$^-$ΔIN-EGFP, the *nef* gene was replaced with the gene for enhanced green fluorescent protein (EGFP) (3).

The expression construct pGPfusion was created to provide Gag-Pol in *trans* (17). This construct was derived from pRS102 (kindly provided by Tahir Rizvi) in which the cytomegalovirus immediate-early promoter drives Gag-Pol expression and the Mason-Pfizer monkey virus *cis*-transport element facilitates Rev-independent nuclear export of *gag-pol* mRNA transcripts (49). The *gag-pol* frameshift site of pGPfusion was disrupted with the same three nucleotide changes that were introduced into the SIV genome plus an additional insertion to place *gag* and *pol* in the same reading frame (TTTTTTA→CTTCCTCA, changes are underlined). Therefore, pGPfusion expresses Gag-Pol but not Gag.

Preparation of single-cycle SIV. Single-cycle SIV was prepared by cotransfection of 293T cells with a full-length, frameshift site mutant provirus (FS$^-$ΔIN) and the Gag-Pol expression construct pGPfusion. 293T cells were seeded at 3×10^6 cells per dish in 100-mm tissue culture dishes the day before transfection. On the day of transfection, the medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS], L-glutamine, penicillin, and streptomycin) was replaced and each plate was transfected with 5 μg of each construct by the method of calcium phosphate precipitation using reagents provided in the ProFection kit (Promega, Madison, WI). The next day, the medium was replaced with 3 ml of fresh DMEM supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin, and culture supernatants containing scSIV were harvested 24 h later. Viral supernatants were then concentrated approximately 20-fold by repeated low-speed centrifugation at $2{,}000 \times g$ in YM-50 ultrafiltration units (Millipore, Bedford, MA) with a 50-kDa pore size before being divided into aliquots and frozen at −80°C. To produce scSIV for animal experiments, p239FS$^-$ΔIN-Nef^{sff} and p316FS$^-$ΔIN-Nef^{sff} were cotransfected into 293T cells with pGPfusion. The day after transfection, cells were washed twice with serum-free medium and the medium was replaced with fresh DMEM containing 10% rhesus serum (Equitech-Bio, Kerrville, TX) to prevent anaphylactic reactions to FBS antigens. The following day, culture supernatants were pooled and concentrated by ultrafiltration in YM-50 units as described above.

Infectivity assays. The relative infectivities of different scSIV strains were compared by infecting T2-SEAP cells. Virus samples were diluted to 2.5 μg/ml SIV p27 in R10 medium and used to infect 1×10^6 T2-SEAP cells in a volume of 100 μl (250 ng p27) for 2 h. Cultures were then expanded to a volume of 2 ml and maintained at 37°C and 5% CO_2. Four days after infection, the cells were analyzed for SIV Gag and EGFP expression using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). For intracellular Gag staining, cells were fixed and permeabilized with Fix and Perm reagents (Caltag Laboratories, Burlingame, CA) according to the manufacturer's instructions. Permeabilized cells were then stained with hybridoma supernatant containing the SIV Gag-specific monoclonal antibody 2F12 (23) at a 1:5 dilution followed by a phycoerythrin-conjugated donkey anti-mouse $F(ab')_2$ fragment (Jackson ImmunoResearch, West Grove, PA) at a 1:100 dilution. For EGFP analysis, cells were washed and fixed in 2% paraformaldehyde–phosphate-buffered saline. The percentage of Gag^+ and $EGFP^+$ cells was determined by gating on the live cell population and collecting 100,000 to 200,000 events for each sample. The production of secreted-alkaline phosphatase (SEAP) in the culture supernatant was also determined as an additional measure of infectivity. T2-SEAP cells (provided by Welkin Johnson, New England Primate Research Center) were derived from the $CD4^+$ human T2 cell line by the introduction of a stable Tat-inducible SEAP reporter construct (38). Infection of these cells with SIV therefore results in the dose-dependent release of SEAP into the culture supernatant. SEAP activity was

measured using the Phosphalight chemiluminescent detection kit (Tropix, Bedford, MA) according to the manufacturer's instructions and a 96-well format lumaplate reader (Wallac, Turku, Finland).

Animals and housing. Rhesus macaques (*Macaca mulatta*) were housed at the New England Primate Research Center in an animal biosafety level 3 containment facility in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. Research was conducted according to the principles described in the *Guide for the Care and Use of Laboratory Animals* (43) and was approved by the Harvard Medical School Animal Care and Use Committee. Animals positive for the MHC class I allele *Mamu-A*01* were identified by sequence-specific PCR (31) and selected for this study. All animals were also tested and found to be free of simian retrovirus type D, SIV, simian T lymphotrophic virus type 1, and herpes B virus prior to assignment.

Single-cycle SIV inoculations. Rhesus macaques were inoculated by intravenous (i.v.) injection with scSIV. Three doses of concentrated scSIV were given at weeks 0, 8, and 33. Each dose consisted of an approximately equal mixture of T-cell-tropic (scSIV239-Nef^{sff}) and macrophage-tropic (scSIV316-Nef^{sff}) viruses. The first two doses were from the same scSIV stocks and contained a total of 7.8 μg p27 equivalents (4.4 μg scSIV239-Nef^{sff} and 3.4 μg scSIV316-Nef^{sff}). The third dose contained a total of 11.6 μg p27 equivalents (5.2 μg scSIV239-Nef^{sff} and 6.4 μg scSIV316-Nef^{sff}). Inoculations were performed under ketamine-HCl anesthesia (15 mg/kg intramuscularly) by injecting 2- to 3-ml volumes of concentrated virus through a 22-gauge catheter placed aseptically in the saphenous vein.

MHC class I tetramer staining. Peripheral blood was analyzed by four-color flow cytometry for SIV-specific CD8$^+$ T cells. Whole blood (100 μl) was stained for 25 min at 37°C with CD3-fluorescein isothiocyanate (Becton Dickinson, San Jose, CA) and CD8-PerCP (Becton Dickinson) monoclonal antibodies and allophycocyanin-conjugated Mamu-A*01 $Gag_{181-189}$ tetramers (generously provided by John Altman, Emory University). Samples were then treated for 10 min with fluorescence-activate cell sorter lysing solution (Becton Dickinson) to remove erythrocytes. Cells were washed and fixed in 2% paraformaldehyde–phosphate-buffered saline. Data was collected on a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using the FlowJo software package (Tree Star, San Carlos, CA).

IFN-γ ELISPOT assays. Gamma interferon (IFN-γ)-producing T-cell responses were enumerated using an enzyme-linked immunospot (ELISPOT) assay for detection of macaque IFN-γ (Mabtech, Mariemont, OH). Peripheral blood mononuclear cells (PBMC) were stimulated in duplicate wells at 3×10^5, 1×10^5, and 3×10^4 cells per well with peptide pools (15-mers overlapping by 11 amino acids at 2.5 μg/ml each) representing the complete amino acid sequences of $SIV_{mac}239$ proteins. PBMC were incubated overnight in multiscreen plates (Millipore) coated with an IFN-γ capture antibody, and spots representing IFN-γ-producing T cells were detected in an enzyme-linked, colorimetric assay for bound IFN-γ. Spots were counted using an automated ELISPOT plate reader (Zellnet Consulting, New York, NY). To determine the frequency of virus-specific IFN-γ-producing T cells per million PBMC, the number of background spots in medium control wells was subtracted from the number of spots in peptide-stimulated wells.

Proliferation assays. T-cell-proliferative responses were monitored as an indicator of the development of CD4$^+$ T helper cell responses by conventional [^{3}H]thymidine incorporation assays. PBMC were stimulated in quadruplicate wells of a 96-well plate (1×10^5 cells per well) with aldrithiol-2-inactivated SIV particles (50 ng p27/ml) and mock-treated control supernatants (7). As a positive control, PBMCs were also stimulated with 5 μg/ml concanavalin A. T-cell proliferation was measured by the incorporation of [^{3}H]thymidine (1 μCi/well) over an 18-hour period 5 days after antigen stimulation. Stimulation indices were calculated as the ratio of the mean [^{3}H]thymidine incorporation in response to antigen to the mean incorporation for control wells.

Analysis of SIV-specific antibody responses. Binding antibodies to SIV proteins were analyzed by whole-virus enzyme-linked immunosorbent assay and Western blot analysis. Commercially available SIV Western blot strips (ZeptoMetrix, Buffalo, NY) were probed with plasma samples at a dilution of 1:100 and developed according to the manufacturer's instructions. Neutralizing antibody titers to $SIV_{mac}239$ and a lab-adapted strain of $SIV_{mac}251$ were determined by the ability to block infection of CEMx174SIV-SEAP cells harboring a Tat-inducible SEAP reporter construct as described previously (38). Serial twofold dilutions of plasma were incubated with 2 ng p27 equivalents of virus at 100 μl per well in 96-well plates for 1 h before the addition of 4×10^4 CEMx174SIV-SEAP cells (100 μl) for a final volume of 200 μl. After 48 to 72 h, 18 μl of supernatant was removed and SEAP activity was determined using the Phospha-Light SEAP detection kit (Applied Biosystems, Foster City, CA) and a lumaplate reader. Control wells for mock-infected cells and virus treated with pooled SIV-negative plasma were used to determine background and maximal SEAP production, respectively. The percent neutralization was calculated from the mean SEAP counts at each plasma dilution divided by the maximal SEAP counts after subtracting background activity. Fifty percent neutralization titers were calculated as the reciprocal of the plasma dilution at which infectivity was reduced by 50%.

Viral loads and CD4$^+$ T-cell counts. Viral loads in plasma were determined using a quantitative real-time reverse transcriptase PCR assay (35). This assay has a nominal threshold of detection of 25 copy equivalents/ml and an interassay coefficient of variation of <25%. CD4$^+$ T-lymphocyte counts were calculated from the total lymphocyte population determined by complete blood count analysis and the percentage of CD4$^+$ T cells determined by flow cytometry (63).

RESULTS

Modifications to single-cycle SIV that may enhance immunogenicity. To enhance the potential immunogenicity of scSIV for evaluation as a nonreplicating vaccine approach in animals, modifications were made in the *env* and *nef* genes. These genetic changes included the exchange of *env* sequences to generate scSIV strains differing in T-cell- versus macrophage-tropism and the introduction of mutations in *nef* to prevent MHC class I downregulation. Each of these modifications was introduced into a full-length proviral construct with mutations in the *gag-pol* frameshift site and a backup deletion in the integrase (IN) coding region of *pol* (scSIV FS$^-$ΔIN) (17).

Macrophages express costimulatory molecules characteristic of professional antigen-presenting cells and appear to be more resistant to the cytopathic effects of virus infection than CD4$^+$ T cells (21, 27, 45). Thus, virus-infected macrophages may be particularly effective antigen-presenting cells. To facilitate the infection of macrophages by scSIV, macrophage-tropic strains were created by exchanging *env* sequences from a T-cell-tropic scSIV genome based on $SIV_{mac}239$ (p239FS$^-$ΔIN) with *env* sequences from the macrophage-tropic strain $SIV_{mac}316$ (41). These *env* sequences include 8 amino acid differences, 6 in gp120 and 2 in gp41, which enhance the efficiency of macrophage infection approximately 100-fold relative to $SIV_{mac}239$ (41).

Nef interferes with cytotoxic T-lymphocyte (CTL) recognition of HIV-1- and SIV-infected cells by downregulating MHC class I molecules from the cell surface (12, 34, 53). This mechanism of immune evasion may also interfere with the induction of virus-specific CTL responses by reducing the density of peptide-MHC class I complexes on the surface of infected antigen-presenting cells. Specific residues of SIV Nef that contribute to MHC class I downregulation have been defined, and it is now possible to inactivate this activity while retaining other functional activities of the molecule (59). In a recent study, mutations in *nef* that selectively knockout MHC class I downregulation resulted in stronger virus-specific CD8$^+$ T-cell responses in infected animals (58). We therefore introduced a series of mutations in *nef* that eliminate residues near the C terminus of the full-length molecule that are essential for MHC class I downregulation in an effort to enhance the stimulation of virus-specific CTL responses in scSIV-immunized animals. This combination of mutations included a premature stop codon followed by two single-nucleotide deletions to prevent read-through of downstream *nef* sequences. The resulting *nef* mutant expresses a protein (Nef^{sff}) that is missing 25 residues from the C terminus that are required for MHC class I downregulation.

The infectivity of six different scSIV strains differing in *env*

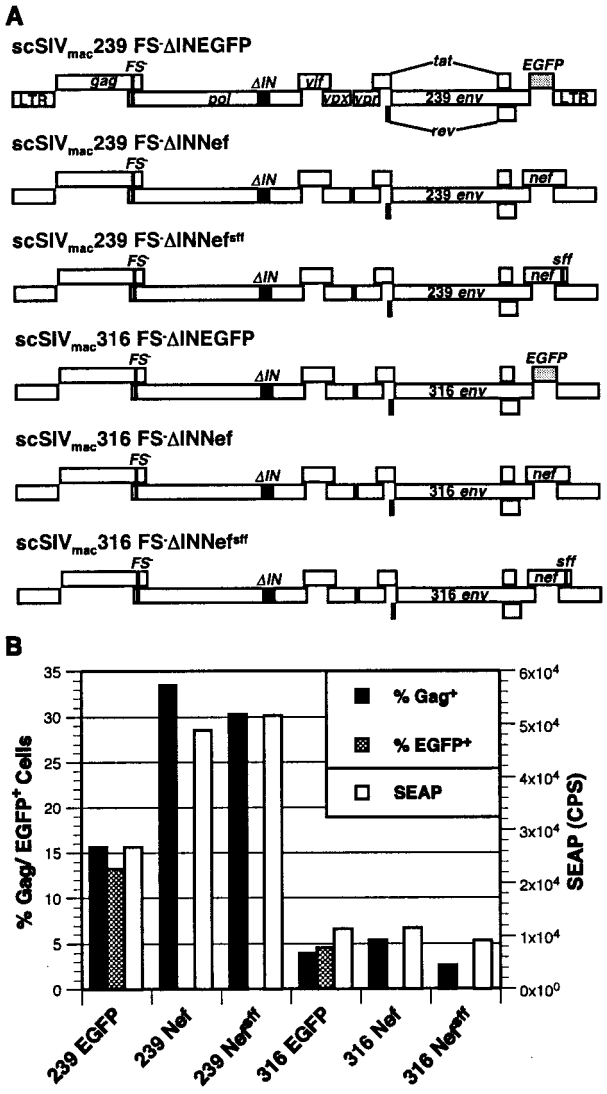

FIG. 1. Comparison of the relative infectivity of six different strains of scSIV on an immortalized human CD4$^+$ T-cell line. (A) Six different strains of scSIV were generated with differences in the *env* and *nef* genes. These included strains expressing the SIV$_{mac}$239 (239) and the SIV$_{mac}$316 (316) envelope glycoproteins together with wild-type *nef* (Nef), a *nef* mutant deficient for MHC class I downregulation (Nef^{sff}), or the gene for EGFP at the *nef* locus (3). (B) T2-SEAP cells (1×10^6) were infected with 250 ng p27 equivalents of each virus. Four days after infection, the infectivity of each strain was determined by flow cytometry and by SEAP production in the culture supernatant. Fixed cells were analyzed for both Gag and EGFP expression. Intracellular Gag staining was performed on permeabilized cells using the SIV Gag-specific monoclonal antibody 2F12 (23) and a phycoerythrin-conjugated donkey anti-mouse F(ab′)$_2$ fragment. SEAP activity was measured by chemiluminescent detection using a 96-well format lumaplate reader.

and *nef* was compared by infecting T2-SEAP cells with equivalent amounts of concentrated virus as determined by p27 antigen capture enzyme-linked immunosorbent assay. Four days after infection, infectivity was measured by both SEAP production in the culture supernatant (38) and intracellular staining for the SIV Gag protein (Fig. 1B). Both assays gave a similar pattern of infectivity on this immortalized human CD4$^+$ T-cell line. The T-cell-tropic 239 strains were three- to sixfold more infectious than the macrophage-tropic 316 strains per ng of p27 in each of these assays (Fig. 1B). A roughly twofold enhancement in the infectivity of the Nef-expressing 239 strains was observed relative to the *nef* deletion strain expressing EGFP. However, infectivity enhancement was not observed for the Nef-expressing 316 strains. Importantly, the infectivities of the scSIV strains expressing the wild-type Nef protein and the Nef^{sff} mutant were similar for both envelope variants (Fig. 1B). Thus, the elimination of 25 residues at the C terminus of Nef that participate in MHC class I downregulation did not appear to affect the infectivity of scSIV, at least in this particular cell line.

Single-cycle SIV inoculations. Four Mamu-A*01$^+$ rhesus macaques were inoculated intravenously with three concentrated doses of scSIV at weeks 0, 8, and 33. Each dose consisted of an approximately equivalent mixture of scSIV strains expressing the 239 and 316 envelope glycoproteins. In addition to nucleotide substitutions in the *gag-pol* frameshift site, each strain also contained a backup deletion in the IN coding region of *pol* and mutations in *nef* to prevent MHC class I downregulation (FS$^-$ΔIN Nef^{sff}). The first two doses were identical and consisted of 4.4 and 3.4 μg p27 of the same cryopreserved stocks of scSIV$_{mac}$239Nef^{sff} and scSIV$_{mac}$316Nef^{sff} for a total of 7.8 μg p27 equivalents. This corresponded to approximately 7×10^6 single-cycle infectious units as determined by titration on CEMx174 cells. Subsequent analysis of viral RNA in the inoculum indicated that each dose contained a total of 1.6×10^{10} RNA copy equivalents or approximately 0.8×10^{10} scSIV particles. Based on these measurements, the particle infectivity (0.8×10^{10} virions = 7×10^6 IU) ratio was approximately 1,000:1. For the third dose, new stocks of scSIV$_{mac}$239Nef^{sff} and scSIV$_{mac}$316Nef^{sff} were prepared. On week 33, each animal was inoculated with 5.2 and 6.4 μg p27, respectively, of these stocks for a total of 11.6 μg p27 equivalents.

Transient peaks of virus production in plasma were detectable after each dose of scSIV. After the first inoculation, viral loads in plasma in each of the four animals peaked between 10^4 and 10^5 RNA copies/ml on or before day 4. These viral load measurements then declined to undetectable levels over the following 4 weeks (Fig. 2). After the second and third doses, viral loads peaked between 10^2 and 10^4 RNA copies/ml on day 2 and were cleared below the threshold of detection in less than 2 weeks (Fig. 2). In contrast to the first inoculation, we captured both the up slopes and down slopes of plasma viremia following the second and third inoculations (Fig. 2). Therefore, these viral load measurements cannot be explained simply by the presence of residual virus inoculum. Since the half-life of SIV in plasma is extremely short, approximately 3 to 4 min (26, 66, 67), these viral load measurements almost certainly reflect the cellular production of noninfectious virus and the turnover of scSIV-infected cells. Repeated attempts to recover replication-competent virus from PBMC by coculture with CEMx174 cells following each dose of scSIV were also unsuccessful. These results are thus consistent with the predicted phenotype of a virus that is limited to a single cycle of infection and a single round of virus production in vivo.

SIV-specific CD8$^+$ T-cell responses. SIV-specific CD8$^+$ T-cell responses were monitored in the peripheral blood of scSIV-immunized animals by MHC class I tetramer staining.

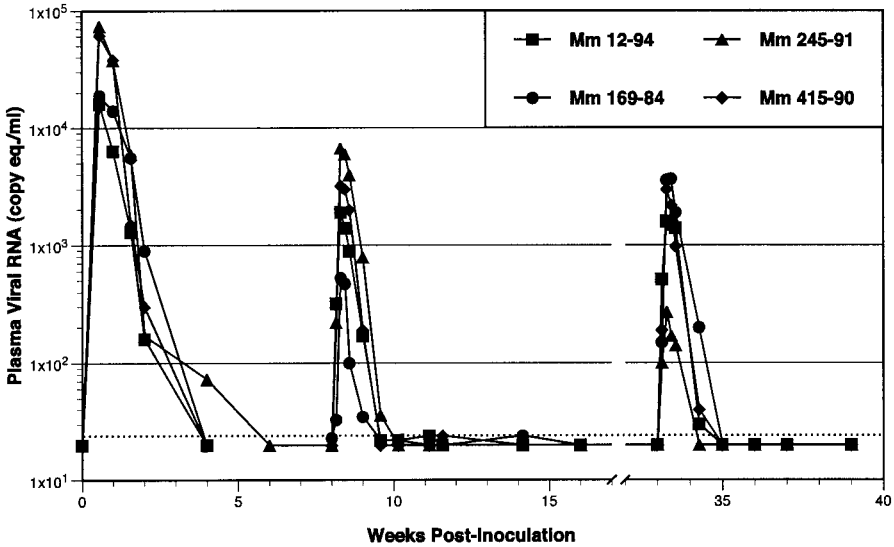

FIG. 2. Viral RNA loads in plasma after intravenous inoculation of four macaques with single-cycle SIV. Each animal was inoculated intravenously with three doses of concentrated scSIV at weeks 0, 8, and 33. The first two doses used identical amounts of the same cryopreserved stocks and contained a total of 7.8 μg p27 equivalents of scSIV (a mixture of 4.4 μg p27 of $scSIV_{mac}239Nef^{sff}$ and 3.4 μg p27 of $scSIV_{mac}316Nef^{sff}$). The third dose contained a total of 11.6 μg p27 equivalents of scSIV (5.2 μg p27 of $scSIV_{mac}239Nef^{sff}$ and 6.4 μg p27 of $scSIV_{mac}316Nef^{sff}$) from separate stocks. Viral RNA loads in plasma were determined using a quantitative reverse transcription-PCR assay (35) with a nominal sensitivity of 25 copy equivalents (eq.)/ml (dotted line) and an interassay coefficient of variation of <25%.

Whole blood was directly stained with Mamu-A*01-$Gag_{181-189}$ tetramers together with CD3- and CD8-specific antibodies to determine the frequency of $CD8^+$ T cells specific for the SIV Gag epitope $C_{181}TPYDINQM_{189}$. SIV-specific $CD8^+$ T-cell responses were detectable in all four animals after a single dose of scSIV. $Gag_{181-189}$-specific responses peaked 2 weeks after the first inoculation at 0.05% to 0.2% of the $CD8^+$ T-cell population and declined to baseline levels in three of the four animals by week 8 (Fig. 3). After boosting with scSIV on week 8, virus-specific $CD8^+$ T-cell responses were again detectable, this time after just 1 week. These responses also declined more gradually, consistent with the kinetics of secondary T-cell responses (Fig. 3). Thus, MHC class I tetramer staining clearly demonstrated the stimulation of both primary and secondary $CD8^+$ T-cell responses in all four scSIV-immunized animals. Overall, the rank order of $Gag_{181-189}$-specific responses was

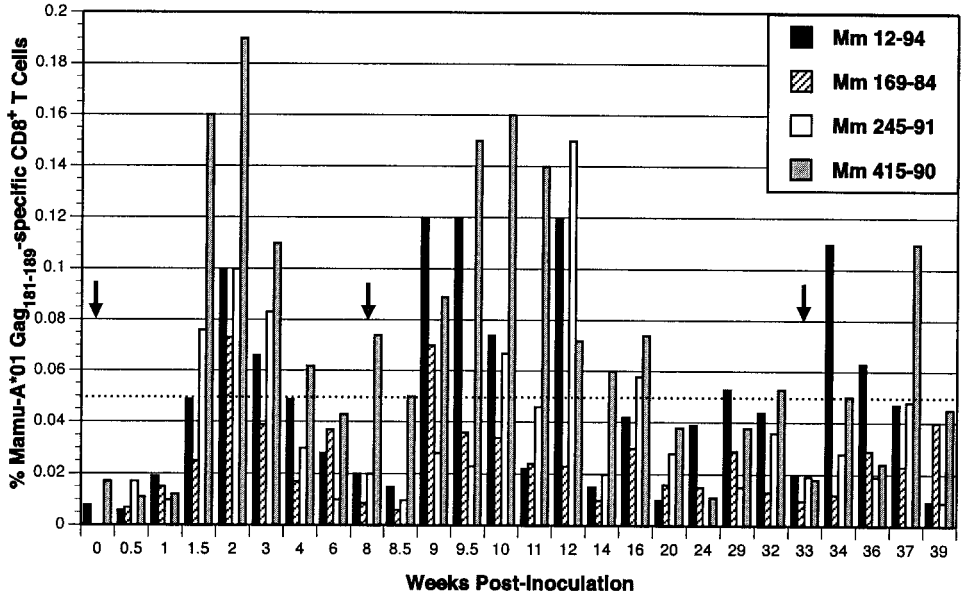

FIG. 3. SIV Gag-specific $CD8^+$ T-cell responses in four Mamu-A^*01^+ rhesus macaques immunized with scSIV. Whole blood was stained with monoclonal antibodies to CD3 and CD8 together with Mamu-A*01-$Gag_{181-189}$ tetramers and analyzed by flow cytometry. After gating on the $CD3^+$ $CD8^+$ lymphocyte population, the percentage of tetramer-positive cells was determined at each time point. The arrows at weeks 0, 8, and 33 indicate inoculations with scSIV. Values greater than 0.05% (dotted line) are considered positive.

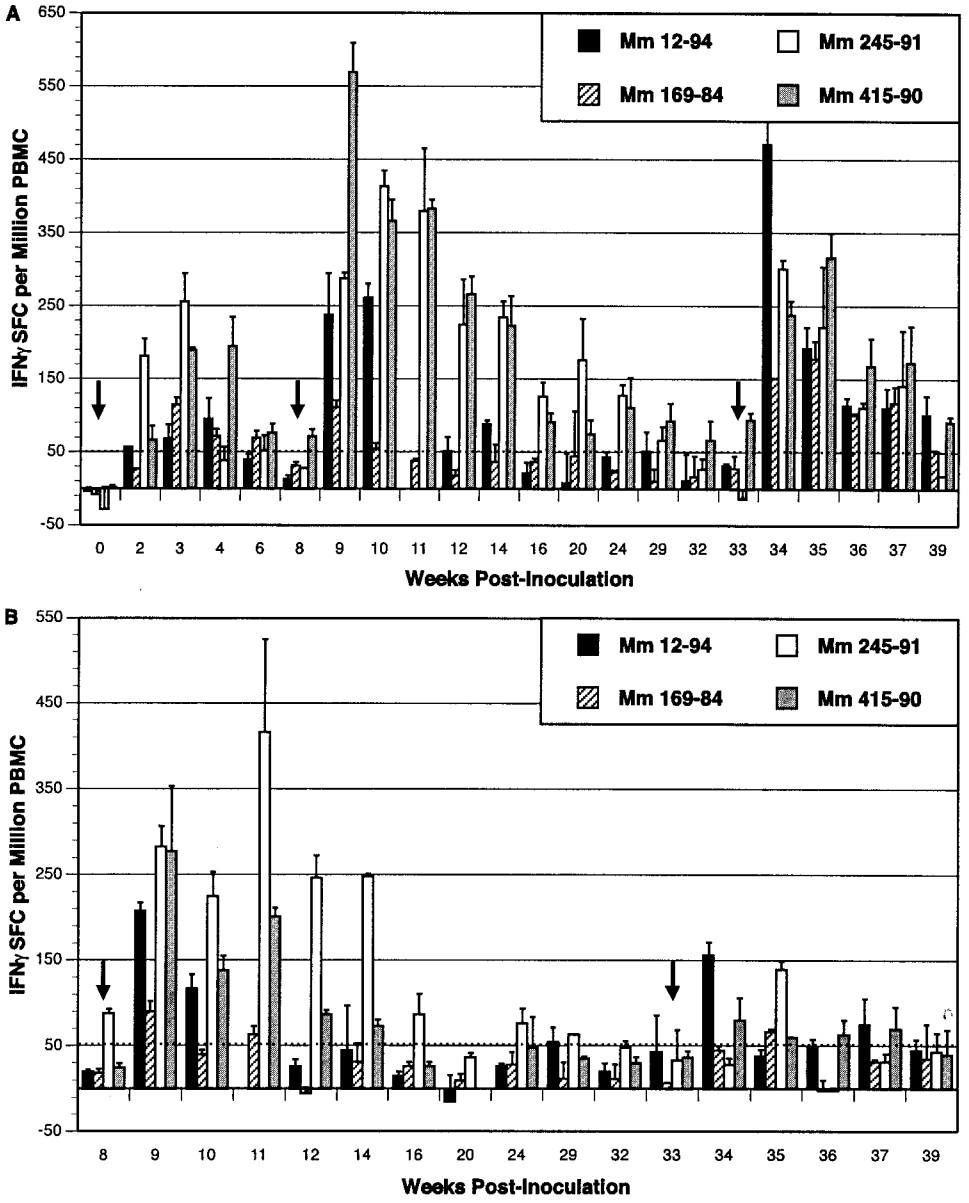

FIG. 4. SIV-specific IFN-γ T-cell responses in four scSIV-immunized macaques. PBMC were stimulated overnight with pools of overlapping Gag (A) and Env (B) peptides. IFN-γ-producing cells were enumerated in ELISPOT assays. The average numbers of SFC per million PBMC and the standard deviations (error bars) were determined from duplicate wells plated at 3×10^5 PBMC/well. Responses greater than 50 SFC/10^6 PBMC (dotted line) are considered positive. Arrows indicate scSIV inoculations at weeks 0, 8, and 33.

similar after each inoculation with the strongest responses in animals Mm 415-90 and Mm 12-94 (Mm 415-90 > Mm 12-94 > Mm 245-91 > Mm 169-84). However, there was no clear relationship between the magnitude of these responses and the transient viral loads in plasma (Fig. 2 and 3).

IFN-γ ELISPOT responses. Virus-specific T-cell responses were also monitored by IFN-γ ELISPOT assays in which PBMC were stimulated with pools of overlapping SIV peptides. SIV Gag-specific T-cell responses, ranging from 70 to 270 spot-forming cells (SFC) per million PBMC, peaked 3 weeks after the first dose of scSIV (Fig. 4A). This increase was statistically significant ($P = 0.04$, paired t test) as demonstrated by analysis of the mean IFN-γ T-cell responses at week 3 versus week 0 (baseline). One week after the second dose, these responses were boosted to 100 to 570 SFC per million PBMC ($P = 0.05$, paired t test compared to baseline) and exhibited a more gradual course of decline typical of secondary T-cell responses (Fig. 4A). After the third scSIV inoculation, there was again a significant increase in the mean frequency of Gag-specific IFN-γ T cells relative to the baseline ($P = 0.02$), and with the exception of the week 39 time point for Mm 245-91, these responses consistently remained above baseline levels until the time of challenge (Fig. 4A).

Similar IFN-γ T-cell responses to Env were observed after the second scSIV inoculation (Fig. 4B). Comparison of mean responses at week 8 (baseline) versus week 9 revealed a sig-

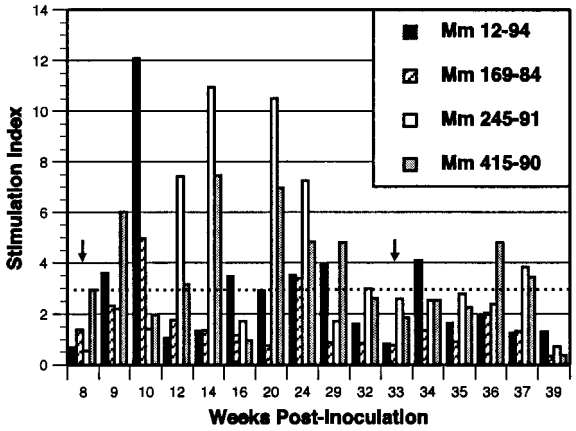

FIG. 5. SIV-specific T-cell proliferative responses in four scSIV-immunized macaques. PBMC were stimulated in quadruplicate wells at 1×10^5 cells/well with AT-2-inactivated $SIV_{mac}239$ (7) and matched microvesicle control supernatants prepared from uninfected cells. Five days after stimulation, virus-specific proliferation was determined in an 18-hour [^{3}H]thymidine incorporation assay. Stimulation indices were calculated by dividing the average counts per minute for virus-stimulated wells by the average counts per minute for control wells. Stimulation indices greater than 3 were considered positive (dotted line). Arrows indicate scSIV inoculations at weeks 8 and 33.

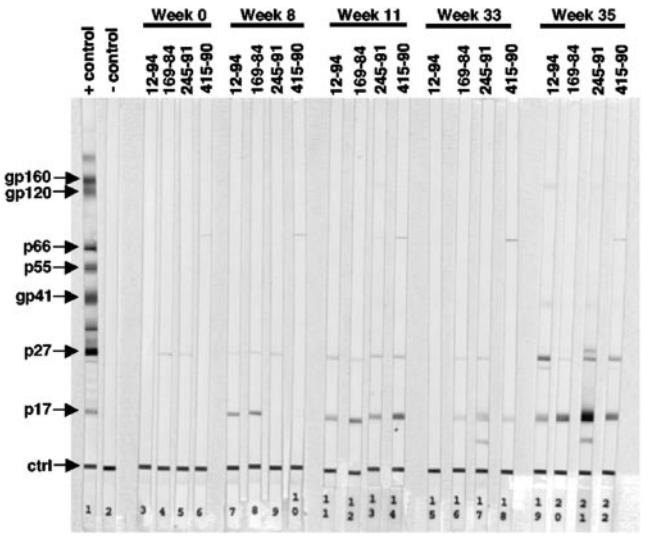

FIG. 6. Virus-specific antibody responses in scSIV-immunized animals. SIV Western blot strips were probed with plasma samples collected at weeks 0, 8, 11, 33, and 35 at a 1/100 dilution according to the manufacturer's instructions. The positions of major viral proteins are indicated on the left side of the blot. The serum control band (ctrl) represents an internal positive control for the reactivity of each strip. +, positive; −, negative.

nificant increase ($P = 0.02$, paired t test). However, after the third dose of scSIV, Env-specific recall responses were weak to undetectable and were not significantly different than baseline levels ($P = 0.4$, paired t test). Additional IFN-γ ELISPOT responses to Tat, Rev, and Nef were also observed in certain animals, although these responses were generally lower and were not sustained (data not shown). Thus, scSIV immunization is capable of eliciting T-cell responses to multiple viral antigens.

SIV-specific T-cell proliferative responses. Proliferative responses to whole virus were monitored in scSIV-immunized animals as an indicator of the development of $CD4^+$ T helper cell activity. Beginning at week 8, PBMC were stimulated with aldrithiol-2 (AT-2)-inactivated $SIV_{mac}239$ (7) and virus-specific proliferation was measured by [^{3}H]thymidine incorporation relative to PBMC stimulated with virus-free control supernatant. All four scSIV-immunized animals had T-cell proliferative responses with stimulation indices greater than 3 at two or more time points after the second dose of scSIV (Fig. 5). Furthermore, levels of proliferation 1 week after the second dose were significantly higher than baseline levels at the time of the second dose ($P = 0.03$, paired t test). Interestingly, the two animals that made the strongest IFN-γ T-cell responses, Mm 245-91 and Mm 415-90, also made the most consistent virus-specific proliferative responses. The strength of these responses was comparable to proliferative responses in animals immunized with the live, attenuated strains of SIV (22). However, for reasons that are currently unclear, the third dose of scSIV at week 33 did not significantly boost mean SIV-specific proliferative responses ($P = 0.3$, paired t test versus baseline levels at week 8).

SIV-specific antibody responses. Antibody responses to SIV were monitored in each of the scSIV-immunized animals by Western blot and by virus neutralization assays. The Western blot strips shown in Fig. 6 illustrate the seroconversion of each animal with successive doses of scSIV. Binding antibody responses were detectable in plasma from two of the animals 8 weeks after the first dose and were present in all four animals at week 11, 3 weeks after the second dose. Virus-specific antibody responses were further boosted after the third inoculation, as indicated by the increase in intensity of the p17 and p27 bands from week 33 to week 35 (Fig. 6). However, relative to the reactivity typically observed with serum from wild-type SIV-infected animals, the intensity of staining was low, suggesting that overall antibody titers were not as high as in animals persistently infected with replication-competent viruses. Indeed, only faint recognition of the gp120 and gp41 envelope bands was detectable in two animals (Mm 12-94 and Mm 245-91) at week 35 (Fig. 6). Furthermore, the decrease in antibody reactivity between the second and third inoculations, weeks 11 to 33 (Fig. 6), suggests that virus-specific antibody titers may wane after each inoculation. These observations are perhaps not surprising given the transient nature of scSIV infection and the limited amount of virus production in immunized animals.

Interestingly, the pattern of antibody reactivity in the scSIV-immunized animals also appeared to differ from the pattern that is usually observed in animals infected with wild-type virus. The p27 capsid protein is typically the most intense band on Western blots probed with serum from persistently infected animals, as demonstrated by the positive control strip (Fig. 6). In contrast, the p17 matrix protein was better recognized in each of our animals following scSIV inoculation (Fig. 6). Since virus particles released from scSIV-infected cells are Pol deficient and unable to complete proteolytic maturation, the preferential recognition of the p17 matrix band may reflect differences in the specificity of antibodies elicited by progeny virus in

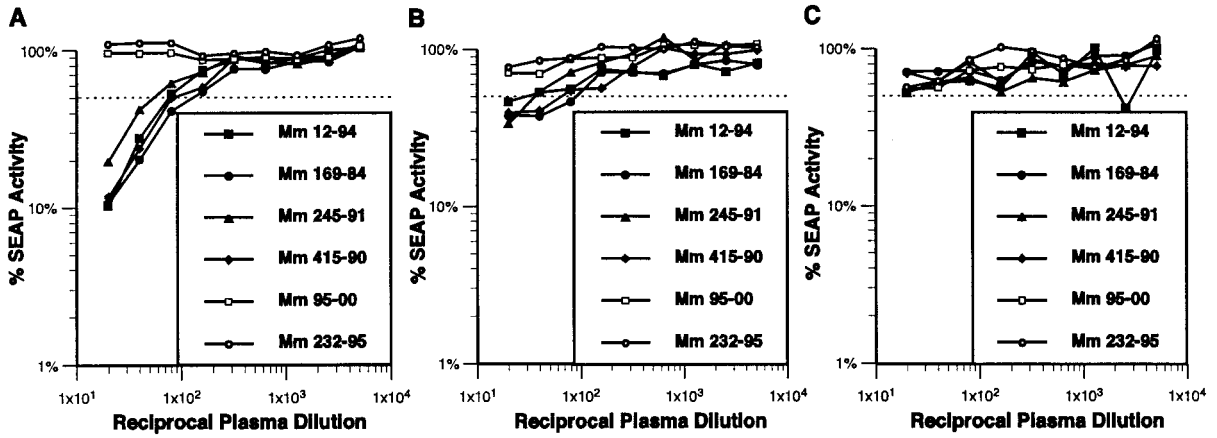

FIG. 7. Neutralizing antibody responses at the time of challenge. Serial twofold dilutions of plasma were tested for the ability to block the infection of CEMx174SIV-SEAP cells (38) with $SIV_{mac}251_{LA}$ (A), $SIV_{mac}316$ (B), and $SIV_{mac}239$ (C). Serial twofold dilutions of plasma were incubated with $SIV_{mac}251_{LA}$ (0.25 ng p27), $SIV_{mac}239$ (1.0 ng p27), and $SIV_{mac}316$ (5.0 ng p27) for 1 hour before the addition of 4×10^4 target cells. SEAP activity was measured in culture supernatants collected from duplicate wells 3 days after infection with $SIV_{mac}251_{LA}$ and $SIV_{mac}239$ and from quadruplicate wells 5 days after infection with $SIV_{mac}316$. The dashed lines indicate 50% neutralization.

which the predominant form of Gag is the uncleaved polyprotein precursor.

Plasma samples from each of the scSIV-immunized animals were able to neutralize $SIV_{mac}316$ and a lab-adapted strain of SIV ($SIV_{mac}251_{LA}$) but not $SIV_{mac}239$. Figure 7 shows the neutralization curves for each of the scSIV-immunized animals and two control animals at the time of challenge. Neutralizing antibody titers to $SIV_{mac}251_{LA}$ were first observed in three of the animals after the second dose of scSIV (Table 1). These neutralizing antibody responses were observed at week 11 prior to the development of Env-specific antibodies detectable by Western blotting (Table 1 and Fig. 6), an observation that most likely reflects the greater sensitivity of $SIV_{mac}251_{LA}$ neutralization by this assay. After the third dose, all four animals had neutralizing antibodies to $SIV_{mac}316$ and $SIV_{mac}251_{LA}$ until the day of challenge (Table 1). Similar to binding antibody responses that were detectable by Western blotting, these neutralizing antibody titers were generally low ($\leq$320). In contrast, we were unable to detect any neutralization of $SIV_{mac}239$ (Table 1). The lack of detectable neutralizing antibodies to $SIV_{mac}239$ probably reflects, at least in part, the inherent resistance of the $SIV_{mac}239$ envelope glycoprotein to neutralizing antibody responses (29). Indeed, neutralizing antibody titers to $SIV_{mac}239$ are often weak or undetectable in animals persistently infected with this virus (29, 48). Thus, given the transient nature of infection and limited expression of the viral envelope glycoprotein, it is not surprising that scSIV immunization failed to elicit detectable neutralizing antibody responses to $SIV_{mac}239$.

Intravenous challenge with $SIV_{mac}239$. Six weeks after the last dose of scSIV, each of the four scSIV-immunized animals and two additional Mamu-A*01$^+$, naive control animals were challenged intravenously with 10 animal infectious doses of an $SIV_{mac}239$ challenge stock that has been used previously by our laboratory (18) and by other laboratories (25). All six animals became infected. However, three of the four scSIV-immunized animals exhibited 1 to 3 log reductions in their acute-phase viral loads in plasma at week 2 postchallenge relative to the control animals (Fig. 8A). Furthermore, these three animals continued to control virus replication well into the chronic phase of infection. By week 35 postchallenge, viral loads for two of the scSIV-immunized animals remained below

TABLE 1. Neutralizing antibody titers in response to scSIV immunization

Wk (sample)	50% Neutralization titer for strain and macaque no.[a]:											
	Mm 12-94[b]			Mm 169-84[b]			Mm 245-91[b]			Mm 415-90[b]		
	239	316	251_{LA}	239	316	251_{LA}	239	316	251_{LA}	239	316	251_{LA}
0 (1st dose)	—	NT	—	—	NT	—	—	NT	—	—	NT	—
8 (2nd dose)	—	NT	—	—	NT	—	—	NT	—	—	NT	—
11	—	NT	—	—	NT	160	—	NT	20	—	NT	20
33 (3rd dose)	—	NT	—	—	NT	—	—	NT	—	—	NT	—
35	—	60	240	—	40	320	—	80	160	—	60	320
39 (challenge)	—	30	80	—	80	120	—	40	60	—	60	80

[a] Neutralizing antibody titers indicate the reciprocal of the plasma dilution that reduced SIV infection of CEMx174SIV-SEAP cells containing a Tat-inducible SEAP reporter construct by 50%. Antibody neutralization was tested against $SIV_{mac}239$ (239), $SIV_{mac}316$ (316), and a lab-adapted strain of $SIV_{mac}251$ (251_{LA}). Samples that were negative for virus neutralization at the lowest dilution tested (<1:20) are indicate by a dash (—). Time points that were not tested for neutralization of $SIV_{mac}316$ are indicated by NT. Two different pools of positive-control plasma from SIV-infected rhesus macaques gave titers of 3,840 and 10,240 against lab-adapted $SIV_{mac}251$, 2,560 against $SIV_{mac}316$, and 20 and 70 against $SIV_{mac}239$. Negative-control pools from uninfected animals were unable to neutralize either $SIV_{mac}251$ or $SIV_{mac}239$.

[b] Mm 12-94, Mm 169-84, Mm 245-91, and Mm 415-90 were immunized with $scSIV_{mac}239Nef^{sff}$ and $scSIV_{mac}316Nef^{sff}$ at weeks 0, 8, and 33.

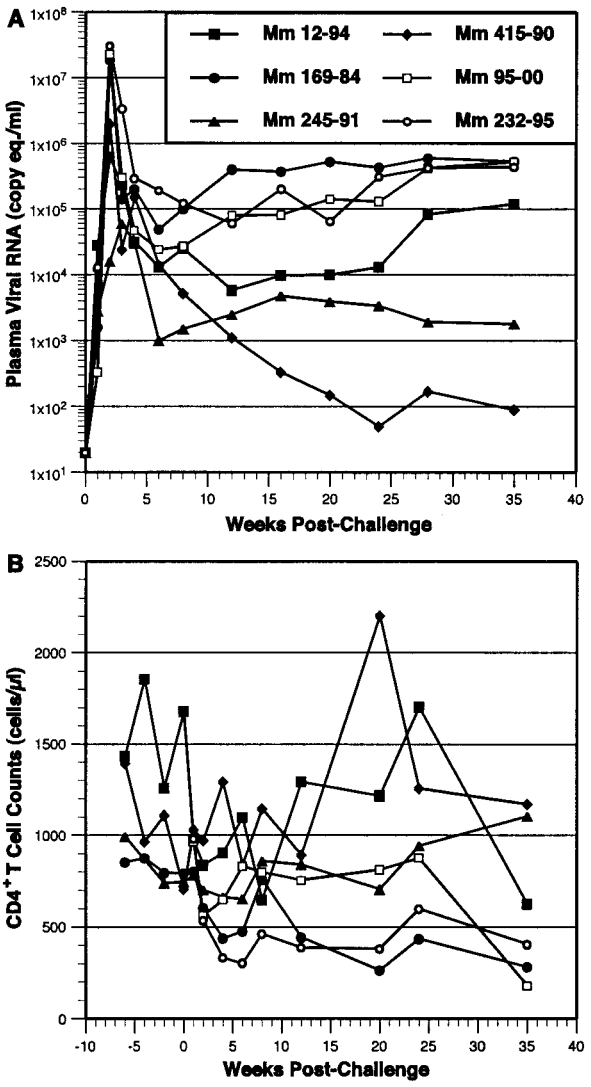

FIG. 8. Viral loads in plasma and $CD4^+$ T-cell counts after an intravenous $SIV_{mac}239$ challenge. Four scSIV-immunized animals and two unvaccinated control animals were challenged intravenously with 10 animal infectious doses (1.5 pg p27) of $SIV_{mac}239$. (A) Viral RNA loads in plasma were determined using a quantitative reverse transcription-PCR assay (35) with a nominal sensitivity of 25 copy equivalents (eq.)/ml and an interassay coefficient of variation of <25%. (B) $CD4^+$ T-cell counts in peripheral blood were monitored by flow cytometry and complete blood count analysis. All six macaques were Mamu-A*01^+. The solid symbols represent the scSIV-immunized animals, and the open symbols represent the unvaccinated control animals.

2,000 RNA copies/ml, 2 to 4 logs lower than set point viral loads in the control animals (Fig. 8A). These differences were also reflected in total $CD4^+$ T-cell counts. $CD4^+$ T-cell counts did not appear to drop as sharply after challenge in the scSIV-immunized animals and were somewhat better maintained during the chronic phase of infection than those of the unvaccinated control animals (Fig. 8B). The only animal that showed no evidence of protection, Mm 169-84 (Fig. 8), consistently made the weakest immune responses in each of the previous assays for virus-specific T-cell activity. The fact that Mm 169-84 was at least 19 years old at the time the study began and could be considered geriatric may have contributed to the weaker immune responses and the inability to control virus replication by this animal.

TABLE 2. Plasma viral RNA peaks following each inoculation

Animal	Peak plasma viral loads at[a]:			
	Dose:			Challenge[e]
	1[b]	2[c]	3[d]	
Mm 245-91	74,000	6,700	270	16,000
Mm 12-94	16,000	1,900	1,600	620,000
Mm 415-90	62,000	3,200	3,000	2,000,000
Mm 169-84	19,000	530	3,600	19,000,000
Avg	43,000	3,100	2,100	5,400,000

[a] Viral loads are expressed as RNA copy equivalents/ml of plasma.
[b] Plasma viremia 4 days after i.v. inoculuation with 4.4 μg p27 of $scSIV_{mac}239Nef^{sff}$ and 3.4 μg p27 of $scSIV_{mac}316Nef^{sff}$.
[c] Plasma viremia 2 days after i.v. inoculuation with 4.4 μg p27 of $scSIV_{mac}239Nef^{sff}$ and 3.4 μg p27 of $scSIV_{mac}316Nef^{sff}$.
[d] Plasma viremia 2 days after i.v. inoculuation with 5.2 μg p27 of $scSIV_{mac}239Nef^{sff}$ and 6.4 μg p27 of $scSIV_{mac}316Nef^{sff}$.
[e] Acute-phase viral loads 2 weeks after an i.v. challenge with 10 animal infectious doses of $SIV_{mac}239$.

We also observed progressive reductions in the transient peaks of plasma viremia following each dose of scSIV. With the exception of Mm 169-84, peak viral loads were successively lower after each scSIV inoculation (Table 2). The average change in $\log_{10}$ viral load per dose of scSIV was −0.68 (95% confidence interval of −1.28 to −0.09), indicating significantly lower viral loads with each inoculation ($P = 0.04$, one-sample t test). Furthermore, the rank order of peak viral loads after challenge corresponded to the rank order of peak viral loads after the third dose of scSIV (Table 2). These observations suggest that the ability to contain scSIV infection may improve after repeated inoculation and may ultimately be predictive of the ability to control pathogenic SIV infection after challenge.

Differences between the viral loads of the scSIV-immunized animals and the two unvaccinated control animals were not great enough to make statistically significant conclusions about protection from the limited number of animals in this initial pilot study. However, when these results were analyzed in comparison to historical control data from 17 additional unvaccinated, Mamu-A*01^+ rhesus macaques infected with $SIV_{mac}239$ (42), the outcome was statistically significant. After log transformation of the data, an independent sample t test was used to compare plasma viral load measurements between scSIV-immunized and control animals. Peak viral loads at week 2 postchallenge were on average 1.16 log lower ($P = 0.002$) in the scSIV-immunized animals than in the unvaccinated control animals. Likewise, set point viral loads, defined as weeks 12 to 16, were on average 1.37 log lower ($P = 0.05$) in the scSIV-immunized animals. Since the combination of Mamu-A*01 and -B*17 is associated with the ability to spontaneously control SIV infection (44) and one of the immunized animals (Mm 245-91) and five of the historical control animals were Mamu-B*17^+, a linear regression analysis was performed to determine if the outcome of challenge was independent of Mamu-B*17 status. This analysis revealed that, independent of Mamu-B*17, the scSIV-immunized animals still had signifi-

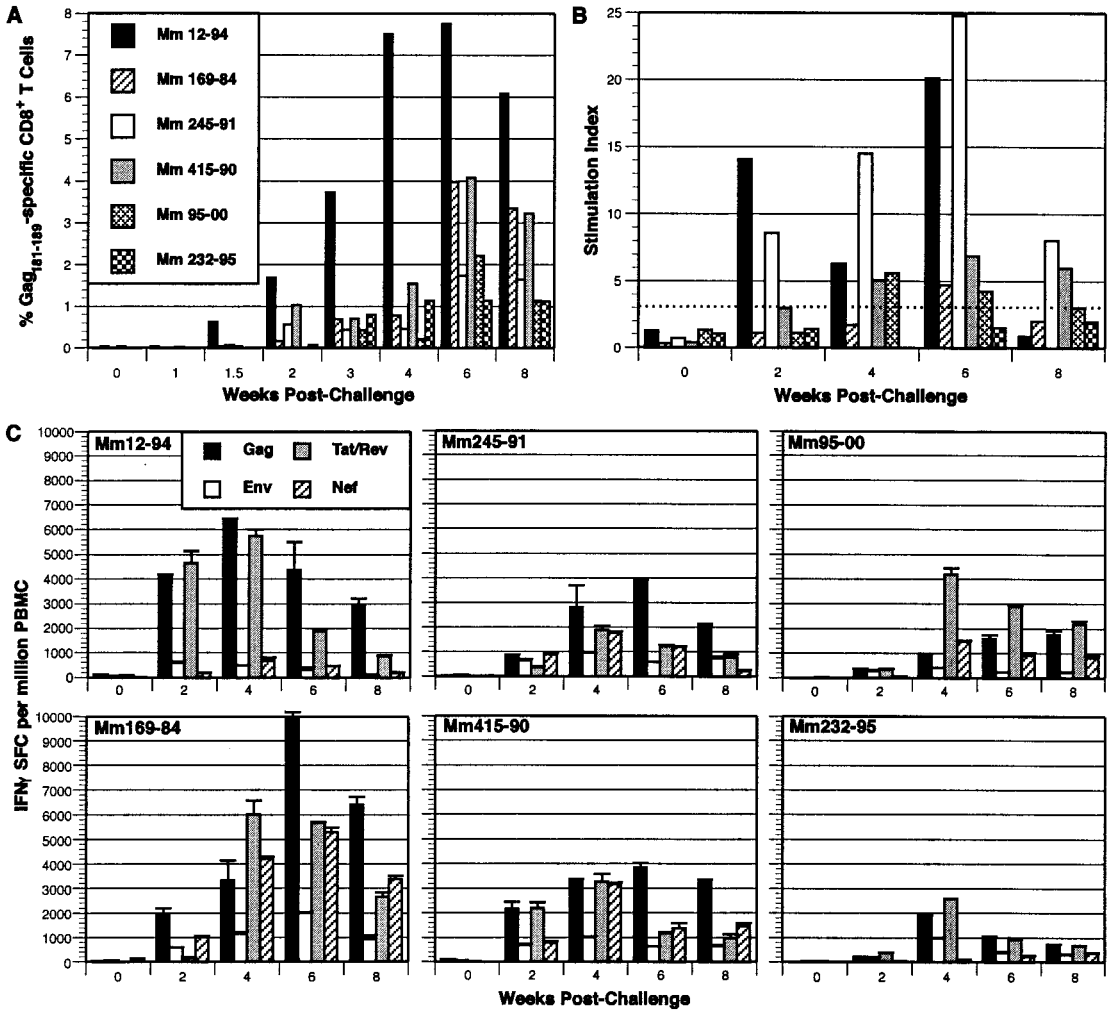

FIG. 9. SIV-specific T-cell responses postchallenge. (A) SIV $Gag_{181-189}$-specific $CD8^+$ T-cell responses were monitored by staining whole blood with monoclonal antibodies to CD3 and CD8 together with Mamu-A*01-$Gag_{181-189}$ tetramers. The percentage of tetramer-positive $CD8^+$ T cells was determined by flow cytometry after gating on the $CD3^+$ $CD8^+$ lymphocyte population. (B) SIV-specific T-cell proliferative responses were monitored by stimulating PBMC with AT-2-inactivated $SIV_{mac}239$ (7) and matched microvesicle control supernatants. Five days after stimulation, virus-specific proliferation was determined in an 18-hour [^{3}H]thymidine incorporation assay. Stimulation indices greater than 3 were considered positive (dotted line). (C) IFN-γ-producing T-cell responses to the SIV Gag, Env, Tat/Rev, and Nef proteins. PBMC were stimulated with pools of overlapping peptides, and cells expressing IFN-γ were enumerated in ELISPOT assays. The average numbers of SFC per million PBMC and standard deviations (error bars) were determined from duplicate wells plated at 3×10^4, 1×10^5, or 3×10^5 PBMC/well.

cantly lower peak and set point viral loads relative to the control animals, 1.16 log lower at peak ($P = 0.002$) and 1.51 log lower at set point ($P = 0.003$). Thus, despite the limited number of animals in the present study, statistical analyses using historical control data from earlier studies that used the same $SIV_{mac}239$ challenge stock suggest that the reduced acute- and chronic-phase viral loads in our scSIV-immunized does indeed reflect a limited degree of protection.

SIV-specific T-cell and antibody responses postchallenge. Virus-specific T cell responses were monitored for 8 weeks after challenge by MHC class I tetramer staining, IFN-γ ELISPOT assays, and proliferation assays. No clear associations with the ability to contain plasma viral loads were observed. However, SIV-specific T-cell responses were generally stronger and appeared earlier in the scSIV-immunized animals.

$Gag_{181-189}$-specific $CD8^+$ T cells were first detectable in the blood of each of the scSIV-immunized animals by week 2 postchallenge and peaked between 1.75% and 7.75% of the total $CD8^+$ T-cell population (Fig. 9A). In contrast, $Gag_{181-189}$-specific responses did not appear until week 3 and peaked at 1.14% and 2.23% of the $CD8^+$ T-cell population in each of the control animals. Statistical comparison of the timing of first detection of these responses confirmed that the $Gag_{181-189}$-specific $CD8^+$ T-cell responses in the immunized animals appeared significantly earlier than in the control animals ($P = 0.004$, independent sample t test). However, there was not a significant difference in peak $Gag_{181-189}$-specific $CD8^+$ T-cell frequencies in inoculated versus control animals ($P = 0.2$, independent sample t test).

The strength of IFN-γ T-cell responses in the scSIV-immunized animals appeared to reflect the overall magnitude of

TABLE 3. Postchallenge neutralizing antibody titers

Wk	50% Neutralization titer for strain and macaque no.[a]:																	
	Mm 12-94[b]			Mm 169-84[b]			Mm 245-91[b]			Mm 415-90[b]			Mm 95-00[b]			Mm 232-95[b]		
	239	316	251_{LA}	239	316	251_{LA}	239	316	251_{LA}	239	316	251_{LA}	239	316	251_{LA}	239	316	251_{LA}
0	—	30	80	—	80	120	—	40	60	—	60	80	—	—	—	—	—	—
2	—	480	1,920	—	—	160	—	120	240	—	80	240	—	—	—	—	—	—
4	—	NT	7,680	—	NT	5,120	—	NT	5,120	—	NT	7,680	—	NT	160	—	NT	160
20	—	NT	10,240	—	NT	7,680	—	NT	15,360	20	NT	1,920	60	NT	5,120	20	NT	3,840

[a] Neutralizing antibody titers indicate the reciprocal of the plasma dilution that reduced SIV infection of CEMx174 cells containing a Tat-inducible SEAP reporter construct by 50%. Antibody neutralization was tested against $SIV_{mac}239$ (239), $SIV_{mac}316$ (316), and a lab-adapted strain of $SIV_{mac}251$ (251_{LA}). Samples that were negative for virus neutralization at the lowest dilution tested (<1:20) are indicate by a dash (—). Time points that were not tested for neutralization of $SIV_{mac}316$ are indicated by NT.

[b] Mm 12-94, Mm 169-84, Mm 245-91, and Mm 415-90 were immunized with three doses of $scSIV_{mac}239Nef^{sff}$ and $scSIV_{mac}316Nef^{sff}$. Mm 95-00 and Mm 232-95 are unvaccinated, Mamu-A*01$^+$ control animals. All six animals were challenged intravenously with $SIV_{mac}239$ 6 weeks after the third scSIV immunization.

antigen presentation, since the highest responses were observed in the animals with the highest viral loads (Fig. 9C). These responses were predominantly directed against Gag and Tat (Fig. 9C), consistent with the presentation of immunodominant epitopes from each of these viral antigens by Mamu-A*01 (4, 5, 39). Similar to CD8$^+$ T-cell responses, early IFN-γ T-cell responses at week 2 postchallenge were higher in the scSIV-immunized than in the control animals, but peak responses between the two groups were not statistically significant (Gag $P = 0.4$, Env $P = 0.4$, Tat/Rev $P = 0.8$, and Nef $P = 0.9$, independent sample t tests).

T-cell proliferative responses to whole, inactivated SIV were also detected in each of the scSIV-immunized animals after challenge and may reflect improved maintenance of CD4$^+$ T-cell helper cell activity (Fig. 9B). These responses were particularly robust for animals Mm 12-94 and Mm 245-91. Whether virus-specific CD4$^+$ T-cell responses were a significant factor in reducing viral loads in the scSIV-immunized animals or simply reflect reduced CD4$^+$ T-cell turnover due to the preservation of these cells by other immune effector mechanisms is not clear. However, in either case, the ability to detect these responses provides further evidence of a positive outcome after challenge in the scSIV-immunized animals.

Neutralizing antibody titers to $SIV_{mac}251_{LA}$ were significantly higher in the scSIV-immunized animals than in the control animals at weeks 2 and 4 postchallenge ($P = 0.01$ and $P < 0.001$, respectively, independent sample t test). Three of the four scSIV-immunized animals also showed an early increase in neutralizing antibody titers to $SIV_{mac}316$ by week 2 postchallenge (Table 3). Thus, scSIV immunization appears to have established virus-specific memory B cells capable of responding more rapidly to the challenge virus. However, by week 20 postchallenge, there was no significant difference in neutralizing antibody titers to $SIV_{mac}251_{LA}$ between the two groups ($P = 0.5$, independent sample t test). Furthermore, no neutralization of $SIV_{mac}239$ was detected in any of the animals until week 20 postchallenge. Even then, neutralizing antibody titers were low and present in only three of the animals (Table 3). Similar to virus-specific T-cell responses, there was no clear association between neutralizing antibody titers and the ability to contain virus replication. Thus, while scSIV immunization may have established memory B-cell responses that led to rapid increases in neutralizing antibody titers against $SIV_{mac}316$ and $SIV_{mac}251_{LA}$ after challenge, it is unclear to what extent, if any, these antibodies contributed to the ability to contain $SIV_{mac}239$ infection.

DISCUSSION

A variety of AIDS vaccine candidates based on prime and boost regimens with different recombinant vector systems are now in clinical trials. While these vaccine approaches are relatively safe and stimulate potent cellular immune responses, a number of factors suggest that they are unlikely to provide reliable protection against HIV-1 infection in at-risk human populations (14, 20). Thus, there is an urgent need to pursue innovative vaccine concepts. We have devised a unique strategy for producing strains of SIV, and potentially HIV-1, that are limited to a single cycle of infection. These strains express all of the viral gene products except Pol and are specifically designed to prevent the emergence of replication-competent virus by recombination or nucleotide reversion (17). To evaluate such single-cycle viruses for their potential as nonreplicating vaccines, we immunized four rhesus macaques with three concentrated doses of single-cycle SIV and then challenged them with $SIV_{mac}239$ to assess protection.

Consistent with the phenotype of a virus that is limited to a single round of infection and virus production, transient peaks of plasma viremia were detected after each scSIV inoculation. These plasma viral load measurements cannot be explained by the presence of residual virus inoculum, since we observed increases in viral RNA loads during the first 2 days after the second and third inoculations. Since the half-life of free virus in the blood is relatively short compared to the half-life of infected cells, our viral load measurements almost certainly represent the production of noninfectious virus by scSIV-infected cells. Previous estimates for the half-life of free virus particles based on the infusion of SIV and HIV-1 directly into the blood of naive macaques range from 3.3 to 15.1 min (26, 66, 67). In contrast, estimates for the half-lives of HIV-1-infected T-cell populations stand at 0.7 day for productively infected CD4$^+$ T cells (36) and 6 to 44 months for latently infected resting memory CD4$^+$ T cells (46, 57). Thus, the peak heights and clearance rates of plasma viremia after scSIV inoculation directly reflect the number and turnover rate of productively infected cells.

Progressive reductions in the peaks of plasma viremia and increased rates of clearance were observed after each inocu-

lation with equivalent or higher doses of scSIV. These changes were most striking for the first two inoculations. After the first dose of scSIV, viral loads peaked between 10^4 and 10^5 RNA copies/ml and were cleared below the threshold of detection in approximately 4 weeks. After the second dose, viral loads peaked between 10^2 and 10^4 RNA copies/ml and were cleared within 2 weeks. Since identical doses of the same frozen stocks were used for the first and second inoculations, adaptive immune responses elicited by the first inoculation are likely to be the most important factor responsible for these differences. Indeed, virus-specific T-cell responses were detected by MHC class I tetramer staining and IFN-γ ELISPOT assays within 3 weeks after the first dose of scSIV and were later boosted by subsequent inoculations. Immunization with scSIV also elicited virus-specific antibodies. However, antibody responses were generally weak and were unable to neutralize $SIV_{mac}239$ at the highest concentrations tested (1:20), despite an identical match in envelope glycoprotein sequences with one of the two scSIV vaccine strains.

$Gag_{181-189}$-specific T-cell frequencies ranging from 0.05% to 0.2% of the $CD8^+$ T-cell population in peripheral blood were detected by MHC class I tetramer staining after just a single dose of scSIV. Perhaps not surprisingly, these responses were lower than responses in animals infected with wild-type SIV. Mamu-$A*01^+$ rhesus macaques infected with $SIV_{mac}239$ typically make $Gag_{181-189}$-specific $CD8^+$ T-cell responses that range from 0.5% to 10% of the total $CD8^+$ T-cell population after the resolution of acute-phase viral loads in excess of 10^7 copies of viral RNA per ml of plasma (33, 42). Nevertheless, despite a transient peak of virus production that was approximately 3 logs lower than acute viremia in wild-type SIV-infected animals, each of the scSIV-immunized animals made virus-specific $CD8^+$ T-cell responses that were clearly detectable by MHC class I tetramer staining.

The detection of T-cell proliferation in response to the stimulation of PBMC with chemically inactivated preparations of SIV suggests that scSIV immunization also elicited virus-specific $CD4^+$ T helper cell responses. Previous studies have shown that proliferative responses to viral antigens in wild-type SIV-infected animals and in HIV-infected individuals are usually weak or undetectable (15, 22, 60). Strong proliferative responses are generally only observed in individuals that are able to spontaneously control their viral loads or have contained virus replication as a result of antiretroviral drug therapy (51, 52). This is presumably due to the rapid turnover of activated $CD4^+$ T cells by apoptosis and the cytopathic effects of virus infection (16, 24, 40, 61). Our ability to detect robust proliferative responses, at least transiently, in scSIV-immunized animals comparable in magnitude to the responses of animals immunized with live attenuated strains of SIV (22) may reflect the uncoupling of $CD4^+$ T-cell activation from the destructive effects of ongoing virus replication. Since $CD4^+$ T helper cells are important for the maintenance of effective CTL and antibody surveillance against other viral pathogens (37, 65), these responses may ultimately be important for achieving reliable, long-term protective immunity against HIV-1.

IFN-γ-producing T-cell responses were also observed against multiple viral antigens. These responses were primarily directed against the structural antigens Gag and Env but also included lesser responses to the accessory proteins Tat, Rev, and Nef (data not shown). Since these assays did not differentiate between $CD4^+$ and $CD8^+$ T-cell responses, we do not know the relative contribution of each these cell types to IFN-γ production. Nevertheless, it is interesting to speculate whether these responses may have been skewed toward greater $CD4^+$ T-cell frequencies relative to wild-type SIV infection. In scSIV-immunized animals, endogenous processing and presentation of viral antigens by MHC class I molecules to $CD8^+$ T cells should be limited to the first round of infection. However, noninfectious virus particles released from scSIV-infected cells may still stimulate $CD4^+$ T cells through exogenous pathways of antigen processing and presentation. Thus, scSIV infection may favor exogenous antigen presentation by MHC class II molecules enhancing virus-specific $CD4^+$ T-cell responses.

With the exception of IFN-γ T-cell responses to Gag, the third dose of scSIV at week 33 did not significantly boost SIV-specific T-cell responses. Mamu-$A*01$-$Gag_{181-189}$ tetramer staining, Env-specific IFN-γ ELISPOT assays, and proliferation assays all revealed only marginally elevated T-cell responses following the third scSIV inoculation. This was true despite a roughly 50% increase in the dose of the virus inoculum and a peak of virus production only slightly lower than the peak of viremia after the second inoculation. The explanation for this phenomenon is currently unclear. It is possible that sufficient expansion of the SIV-specific T-cell population had already occurred by the time of the third inoculation that the particular dose of scSIV administered was not high enough to drive further T-cell activation or proliferation. Alternatively, reduced T-cell activation following the third dose of scSIV may reflect a state of functional inactivation or anergy brought about by repeated scSIV inoculation. This latter possibility is intriguing in light of recent studies suggesting that a $CD4^+$ $CD25^+$ population of regulatory T cells present in individuals persistently infected with HIV-1 may suppress virus-specific T-cell proliferation and cytokine production in response to viral antigens (1, 30).

Following an intravenous challenge with $SIV_{mac}239$, three of the four scSIV-immunized animals exhibited 1 to 3 log reductions in their acute-phase viral loads relative to two unvaccinated control animals. These animals were also better able to control virus replication well into the chronic phase of infection. This was particularly true for two of the animals that maintained set point viral loads 2 to 4 logs lower than the control animals as late as 35 weeks postchallenge. The only animal that showed no evidence of protection was at least 19 years old at the time the study began and made the weakest virus-specific T-cell responses, possibly reflecting an age-related deficit in the ability to respond to scSIV immunization.

While the three animals with reduced viral loads generally had the strongest SIV-specific T cell responses, none of the immune responses measured before or after challenge correlated with the ability to contain $SIV_{mac}239$ infection in an obvious way. This is perhaps similar to the situation with live attenuated strains of SIV. Although live attenuated viruses afford the most reliable protection achieved thus far with animal models, the immune correlates responsible for this protection remain to be identified (13, 14, 62, 63). Interestingly, the rank order of acute-phase viral loads 2 weeks postchallenge in the scSIV-immunized animals directly corresponded to the

rank order of viral loads after the third dose of scSIV (Table 2). With the exception of a single animal, we also observed progressive reductions in the transient peaks of viremia following each dose of scSIV (Table 2). Thus, the peaks of plasma viremia following each dose of scSIV may be predictive of the outcome of challenge with wild-type virus. If this relationship is borne out in future investigations, scSIV may be useful as a surrogate to assess protection in lieu of challenge with pathogenic, replication-competent viruses. These observations also suggest that the degree of protective immunity may improve incrementally with repeated doses of scSIV.

Previous studies have demonstrated reduced acute-phase viral loads after challenging animals that were vaccinated according to prime and boost regimens with $SIV_{mac}239$ (25, 55). Immunization of macaques with another strain of scSIV pseudotyped with vesicular stomatitis virus glycoprotein G also resulted in reduced primary viremia after challenge with $SIV_{mac}239$ (32). However, by 30 weeks postchallenge, the viral loads of the vaccinated animals in these studies were indistinguishable from those of the control animals (25, 32, 55). Thus, the ability of two of the scSIV-immunized animals in our study to maintain viral loads below 2,000 RNA copies/ml for more than 35 weeks after challenge is particularly intriguing. These trends are further supported by statistical analyses using historical control data suggesting that the reductions in both the acute- and chronic-phase viral loads were significant. Given the difficulty in achieving vaccine protection against $SIV_{mac}239$, these observations are encouraging and suggest that continued studies are warranted to try to improve upon the protection afforded by this approach. Even if single-cycle lentiviruses are ultimately not practical for use in clinical trials, studies using scSIV may be helpful for defining the factors necessary for protective immunity.

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