Pathogenicity and Mucosal Transmissibility of the R5-Tropic Simian/Human Immunodeficiency Virus SHIV$_{AD8}$ in Rhesus Macaques: Implications for Use in Vaccine Studies

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There is an urgent need to develop new pathogenic R5 simian/human immunodeficiency viruses (SHIVs) for the evaluation of candidate anti-HIV vaccines in nonhuman primates. Here, we characterize swarm SHIV$_{AD8}$ stocks, prepared from three infected rhesus macaques with documented immunodeficiency at the time of euthanasia, for their capacity to establish durable infections in macaques following inoculation by the intravenous (i.v.) or intrarectal (i.r.) route. All three viral stocks (SHIV$_{AD8-CE8J}$, SHIV$_{AD8-CK15}$, and SHIV$_{AD8-CL98}$) exhibited robust replication *in vivo* and caused marked depletion of CD4$^+$ T cells affecting both memory and naïve CD4$^+$ T lymphocyte subsets following administration by either route. Eleven of 22 macaques inoculated with the new SHIV$_{AD8}$ stocks were euthanized with clinical symptoms of immunodeficiency and evidence of opportunistic infections (*Pneumocystis*, *Candida*, and *Mycobacterium*). A single but unique founder virus, also present in the SHIV$_{AD8-CE8J}$ swarm stock, was transmitted to two animals following a single i.r. inoculation of approximately 3 50% animal infectious doses, which is close to the threshold required to establish infection in all exposed animals. Because the three new SHIV$_{AD8}$ viruses are mucosally transmissible, exhibited tier 2 sensitivity to anti-HIV-1 neutralizing antibodies, deplete CD4$^+$ T lymphocytes *in vivo*, and induce AIDS in macaques, they are eminently suitable as challenge viruses in vaccine experiments.

Nonhuman primate surrogate models of human immunodeficiency virus (HIV)/AIDS have been used for the past 25 years in the quest to develop an effective prophylactic vaccine. In many of these studies, two genetically distinct simian immunodeficiency virus (SIV) lineages (SIVmac239/SIVmac251 and SIVsmE660) have been utilized as challenge viruses in experiments that have focused almost exclusively on immunogens eliciting antivirus cell-mediated immune responses. SIV/HIV-1 chimeric viruses (simian/human immunodeficiency viruses [SHIVs]), which usually carry the HIV-1 *env* gene inserted into an SIVmac239 genetic background, have also been used to challenge vaccinated macaques. These SHIVs were originally constructed to assess vaccine-directed anti-HIV humoral immune responses. The early pathogenic SHIVs invariably expressed a CXCR4-utilizing (X4) gp120 envelope glycoprotein in infected macaques and induced rapid and complete elimination of CD4$^+$ T lymphocyte subsets and death from immunodeficiency within a few months of virus inoculation (19, 20, 41). Furthermore, most vaccine regimens consistently suppressed the replication of SHIV challenge viruses (1, 2, 43). Because the rapid disease phenotype observed and the uniformly successful vaccine outcomes were discordant with the results obtained following challenge with commonly used (R5) pathogenic SIVs and were inconsistent with the invariable presence of R5-tropic HIV-1 strains in infected individuals following seroconversion (10), attention shifted to the construction of R5-tropic SHIVs.

The effort to develop R5 SHIVs, in fact, received high priority from a HIV Vaccine Summit convened in 2008 at Bethesda, MD, following the announced failure of the phase 2b STEP HIV-1 vaccine trial (9). Although several clade B and clade C R5-tropic SHIVs have been constructed (5, 13, 17, 29, 36), only one,

SHIV$_{SF162}$ (16), has been widely used as a challenge virus in vaccine experiments. Even though SHIV$_{SF162}$ generates high levels of peak plasma viremia during acute infection, it expresses an HIV-1 envelope glycoprotein with an extremely low tier 1 neutralization phenotype (44), making it exquisitely sensitive to most anti-HIV-1 neutralizing antibodies (NAbs).

We previously reported the construction of the R5 SHIV$_{AD8}$ and its replicative adaptation to macaques following serial passage in rhesus monkeys (34), but a challenge virus inoculum suitable for vaccine experiments had not been prepared and characterized. In this earlier work, animals were inoculated by transfusing whole blood from SHIV$_{AD8}$-infected monkeys or with small virus stocks prepared from peripheral blood mononuclear cells (PBMC) and lymphoid tissues collected from macaques with high virus loads. Our goal in the present study was to characterize three new SHIV$_{AD8}$ stocks prepared from animals that had developed documented immunodeficiency for use in passive-transfer and vaccine experiments. We report that each of these "swarm" virus stocks exhibits tier 2 sensitivity to NAbs and is efficiently transmitted to macaques by intravenous (i.v.) and mucosal routes. Infected animals experience variable

levels of set point viremia, sustain depletion of both memory and naïve CD4$^+$ T cell subsets, and develop clinical disease.

MATERIALS AND METHODS

Preparation of SHIV$_{AD8}$ virus stocks. PBMC recovered at the time of euthanasia from three animals infected with R5-SHIV$_{AD8}$ derivatives were cocultured with concanavalin A (ConA)-stimulated PBMC from uninfected rhesus monkeys (Rh PBMC), as previously described (34). Supernatant media were assayed for progeny virus production by measuring ^{32}P-reverse transcriptase (RT) activity (51). SHIV$_{AD8}$ stocks prepared from each macaque were designated SHIV$_{AD8-CE8J}$, SHIV$_{AD8-CK15}$, and SHIV$_{AD8-CL98}$, corresponding to their animal identifications (IDs). The infectious titers of SHIVAD8$_{-CE8J}$, SHIV$_{AD8-CK15}$, and SHIV$_{AD8-CL98}$ were 1.83 × 10^4 50% tissue culture infective doses (TCID$_{50}$)/ml, 4.09 × 10^4 TCID$_{50}$/ml, and 3.49 × 10^5 TCID$_{50}$/ml, respectively, as determined in Rh PBMC. The replication kinetics of these viral stocks were evaluated by spinoculating (1,200 × g for 1 h) ConA-stimulated PBMC with these virus stocks, following normalization for ^{32}P-RT activity (33). Virus replication was assessed by ^{32}P-RT assay of the culture supernatant.

Animals. Rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (30) and were housed in a biosafety level 2 facility. Phlebotomies, euthanasia, and tissue sample collections were performed as previously described (8). Bronchoalveolar lavage (BAL) fluid lymphocytes were prepared from uninfected and infected animals using a pediatric bronchoscope (Olympus BF3C40; Olympus America, Inc., Melville, NY) as previously described (18). All animals were negative for major histocompatibility complex (MHC) class I alleles *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17*.

SHIV$_{AD8}$ inoculation and quantitation of plasma viral RNA. One milliliter of SHIV$_{AD8-CE8J}$, SHIV$_{AD8-CK15}$, or SHIV$_{AD8-CL98}$ (titers as indicated above) was inoculated i.v. into two macaques. For intrarectal (i.r.) inoculation, 100 TCID$_{50}$ to 10,000 TCID$_{50}$ of each virus stock was administered atraumatically in a volume of 1 ml. Macaques that remained uninfected were reinoculated with the initial i.r. challenge dose; animals still uninfected following the two initial inoculations were challenged with a 10-fold-higher dose intrarectally (Table 1).

Plasma viral-RNA levels were determined by real-time PCR (7900 HT Sequence Detection System; Applied Biosystems, Foster City, CA), using reverse-transcribed viral RNA from plasma samples of SHIV$_{AD8}$-infected macaques and primer pairs corresponding to the SIVmac239 *gag* gene, as previously described (8). The cDNA was amplified employing the following thermal-cycling conditions: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min with primer pairs corresponding to SIVmac239 gag gene sequences (forward, nucleotides [nt] 1890 to 1909, and reverse, nt 2010 to 1991) and probe (nt 1963 to 1990). Plasma from infected macaques, previously quantitated by the branched-DNA method (6), served as standards for the RT-PCR assay.

Lymphocyte-immunophenotyping and intracellular-cytokine assays. EDTA-treated blood samples and BAL fluid lymphocytes were stained for flow cytometric analysis as described previously (12, 31, 32), using combinations of the following fluorochrome-conjugated monoclonal antibodies (MAbs): CD20 fluorescein isothiocyanate (FITC), CD3 phycoerythrin (PE), CD4 peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5), CD8 allophycocyanin (APC), CD95 (APC), and CD28 (FITC). All antibodies were obtained from BD Biosciences (San Diego, CA). Flow-cytometric acquisitions were done with a FACSCalibur flow cytometer (Becton, Dickinson, Franklin Lakes, NJ).

For intracellular cytokine assays, immune stimulation using SIVmac239 Gag peptides (New England Peptide, Gardner, MA), 15 amino acids in length, was performed on frozen or freshly prepared lymphocytes as described previously (33).

Measurement of anti-gp120 antibodies. An enzyme-linked immunosorbent assay (ELISA) was designed to detect antibodies generated against HIV *env* with specific gp120 protein as an antigen. Ninety-six-well

TABLE 1 Number of challenges needed to cause infection

Animal	No. of challenges needed to cause infection at (TCID$_{50}$/ml)[a]:		
	100	1,000	10,000
SHIVAD8-CE8J infected			
CL5E	●		
DA24	○○	●	
DBJE		●	
DBZL		●	
CZH		●	
DBX9		●	
SHIVAD8-CL98 infected			
DA55	○○	●	
DB18	○○	○●	
CL5B		○○	●
DB0F		○○	●
SHIVAD8-CK15 infected			
DC1W		●	
DC87		●	
DBRL		●	
DC65		●	
BIH		○	●
ENF		○	●

[a] ○, challenge that did not result in detectable SHIVAD8 infection; ●, challenge that did result in detectable SHIVAD8 infection.

microtiter plates were coated at 4°C overnight with antigen (Advanced Biosciences, Kensington, MD) at 2 μg/ml in phosphate-buffered saline (PBS) solution using 50 μl/well on a rocking platform. The plates were rinsed four times with wash buffer (PBS-0.05% Tween 20), followed by addition of 200 μl/well of blocking buffer (1% bovine serum albumin [BSA], 0.05% Tween 20 in PBS) and incubation for 2 h at room temperature. Plasma samples were diluted in blocking buffer at 1:100 dilution, and 100 μl was added per well. The plates were incubated for 1 h at room temperature, followed by four washes with washing buffer. Secondary-antibody conjugated with horseradish peroxidase (goat anti-rhesus IgG; Southern Biotech, Birmingham, AL) was diluted at 1:8,000 in blocking buffer, and 100 μl was added to each well, followed by incubation for 1 h at room temperature. The plates were washed four times and then developed by addition of 100 μl/well of an *o*-phenylenediamine (SigmaFast tablets; Sigma, St. Louis, MO) for 30 min at room temperature. The reaction was stopped by adding 100 μl of 1 M H$_2$SO$_4$. Replicate samples were read at 492-nm absorbance with a microplate reader. The background reactivities were subtracted for each time point using prechallenge plasma controls. The results are presented as the optical density (OD) (absorbance value) read at 492 nm using a Multiscan FC microplate reader (Thermo Scientific, Waltham, MA).

Neutralization assays. Neutralizing activity in plasma samples of SHIVAD8-infected animals was determined as previously described (34). The assay was performed with a 1:20 dilution of plasma samples in TZM-bl target cells using an infectious Env-pseudovirus of SHIV$_{AD8-CK15}$ generated in 293-T/17 cells. Any sample resulting in a 50% reduction of luciferase activity compared to that obtained with an uninfected control sample was considered positive for NAbs. All experiments were performed in quadruplicate and repeated twice.

The neutralization phenotypes (tiers) of SHIV$_{AD8}$ virus stocks were determined by TZM-bl assay (27) using plasma samples from a cohort study, which exhibited a wide range of neutralizing activities against subtype B HIV-1 isolates, as previously described (7).

In addition to the phenotype determination, the neutralization sensitivity of SHIV$_{AD8}$ virus stocks was measured against available monoclonal

antibodies ([4], [38], [48], [50], [52], [53]) using the TZM-bl luciferase reporter gene assay as described previously ([27], [44], [45]). DEAE-dextran was added to a final concentration of 20 μg/ml to enhance viral entry and to obtain a baseline infection level of ~200,000 relative light units. Indinavir was added to the medium to a final concentration of 1 μM to limit infection of target cells to a single round of viral replication. Neutralization curves were fitted by nonlinear regression using a 5-parameter hill slope equation as described previously ([44]). The antibody concentrations required to inhibit infection by 50% or 80% are reported as 50% or 80% inhibitory concentrations (IC_{50} or IC_{80}), respectively.

Viral-RNA extraction and cDNA synthesis. From each plasma specimen and inoculum stock, 20,000 viral-RNA copies were extracted using the QIAamp Viral RNA Minikit (Qiagen, Valencia, CA). Reverse transcription of RNA to single-stranded cDNA was performed using Super-Script III reverse transcription according to the manufacturer's recommendations (Invitrogen). In brief, a cDNA reaction mixture of $1\times$ RT buffer, 0.5 mM each deoxynucleoside triphosphate, 5 mM dithiothreitol, 2 U/ml RNaseOut (RNase inhibitor), 10 U/ml of Super-Script III reverse transcriptase, and 0.25 mM antisense primer SIVRR1 (5′-CAC TAG CTT ACT TCT AAA ATG GCA GC-3′) was incubated at 50°C for 60 min and at 55°C for 60 min and then heat inactivated at 70°C for 15 min, followed by treatment with 2 U of RNase H at 37°C for 20 min. The newly synthesized cDNA was used immediately or frozen at −80°C.

SGA of SHIV env. A 3.5-kb fragment that included the entire env gene was sequenced from two animals (DBJE and DBX9) at peak viremia and the inoculum stock using a limiting-dilution PCR so that only one amplifiable molecule was present in each reaction. Single-genome amplification (SGA) was performed by serially diluting cDNA distributed among independent PCRs to identify a dilution at which amplification occurred in <30% of the total number of reactions. PCR amplification was performed with $1\times$ PCR buffer, 2 mM $MgSO_4$, 0.2 mM each deoxynucleoside triphosphate, 0.2 μM each primer, and 0.025 U/μl of Platinum Taq High Fidelity polymerase (Invitrogen, Carlsbad, CA) in a 20-μl reaction mixture. First-round PCR was performed with sense primer SIVIntF1 (5′-GAA GGG GAG GAA TAG GGG ATA TGA C-3′) and antisense primer SIVRR1 (5′-CAC TAG CTT ACT TCT AAA ATG GCA GC-3′) under the following conditions: 1 cycle of 94°C for 2 min and 35 cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 5 min, followed by a final extension of 68°C for 10 min. Next, 1 μl of the first-round PCR product was added to a second-round PCR that included the sense primer SIVEnvF1 (5′-CCT CCC CCT CCA GGA CTA GC-3′) and the antisense primer SIVEnvR1 (5′-TGT AAT AAA TCC CTT CCA GTC CCC CC-3′) performed under the same conditions used for first-round PCR but with a total of 45 cycles. Correctly sized amplicons were identified by agarose gel electrophoresis and directly sequenced with second-round PCR primers and 6 HIV-specific primers using BigDye Terminator technology (Applied Biosystems, Foster City, CA). To confirm PCR amplification from a single template, chromatograms were manually examined for multiple peaks, indicative of the presence of amplicons resulting from PCR-generated recombination events, Taq polymerase errors, or multiple variant templates. Sequences containing two or more ambiguous sites were excluded from analysis.

Sequence analysis. Sequences were aligned using ClustalW and hand edited using MacClade 4.08 to improve alignment quality. Phylogenetic trees were constructed using the neighbor-joining method. In both animals, sequences segregated into distinct homogeneous lineages with at most 2 changes from the consensus sequence of each lineage. Each low-diversity lineage was inferred to be a unique founder lineage.

Nucleotide sequence accession numbers. All sequences were deposited in GenBank with accession numbers JQ775968 to JQ776025.

RESULTS

Properties of new $SHIV_{AD8}$ swarm stocks prepared from animals with documented immunodeficiency. We previously re-ported the generation of the pathogenic R5-tropic $SHIV_{AD8}$ following serial passaging in macaques ([34]). Independent $SHIV_{AD8}$ stocks were prepared from PBMC, collected at the time of euthanasia, from three monkeys (CE8J, CK15, and CL98) that had developed symptomatic AIDS. The stocks prepared from the macaques were designated $SHIV_{AD8-CE8J}$, $SHIV_{AD8-CK15}$, and $SHIV_{AD8-CL98}$, corresponding to the IDs of the source animals. RT-PCR analyses revealed that each "swarm" stock possessed distinctive env gene sequences reflecting its replication in individual infected monkeys (Fig. 1). All three virus stocks replicated to high levels in ConA-stimulated rhesus macaque PBMC (see Fig. S1 in the supplemental material).

The replication properties of these $SHIV_{AD8}$ stocks in vivo were assessed following inoculation by parenteral and mucosal routes. In an initial experiment, pairs of macaques were inoculated intravenously with high doses (2×10^4 to 3×10^5 $TCID_{50}$) of each $SHIV_{AD8}$ stock. Individual monkeys generated peak plasma viral loads ranging from 1.2×10^5 to 1.9×10^7 viral-RNA copies/ml between days 10 and 14 postinfection (p.i.) (Fig. 2A). Levels of set point viremia ranged from 10^3 to 10^6 viral-RNA copies/ml plasma, consistent with values previously reported for "intermediate" $SHIV_{AD8}$ derivatives ([34]). All of the macaques sustained a gradual loss of circulating $CD4^+$ T cells, even in the case of one animal (A3E051) with modest levels (~1×10^3 RNA copies/ml) of plasma viremia (Fig. 2A and B). The depletion of circulating $CD4^+$ T lymphocytes affected both memory and naïve subsets (Fig. 2C and D). At the time of euthanasia, all six of the infected animals (DB93, DB1A, A3E050, CK5H, A3E051, and CJ7D) had sustained significant depletions of circulating memory $CD4^+$ T lymphocytes (144, 55, 9, 3, 4, and 56 circulating memory $CD4^+$ T cells/μl, respectively). As observed previously ([34]), these $SHIV_{AD8}$-infected macaques also experienced a decline in circulating naïve $CD4^+$ T cells during the chronic phase of infection and then a profound depletion of this T cell subset heralding the onset of clinical AIDS (Fig. 2D). This loss of naïve $CD4^+$ T lymphocytes had also previously been shown not to be associated with altered coreceptor utilization by the contemporaneously circulating $SHIV_{AD8}$ ([34]).

The specific targeting of memory $CD4^+$ T cells at an effector site by the new $SHIV_{AD8}$ stocks is best illustrated by the marked depletion of $CD4^+$ T lymphocytes in BAL specimens during acute infection (Fig. 2E). In four of these monkeys (DB93, DB1A, CJ7D, and A3E051), the levels of BAL fluid $CD4^+$ T lymphocytes stabilized or partially recovered, whereas in the remaining two (A3E050 and CK5H), this memory $CD4^+$ T cell subset was markedly depleted (Fig. 2E).

In summary, 4 of 6 animals inoculated intravenously with three new swarm $SHIV_{AD8}$ virus stocks exhibited a normal-progressor (NP) clinical course characterized by durably maintained set point virus loads and the gradual depletion of circulating $CD4^+$ T lymphocytes. The remaining 2 $SHIV_{AD8}$-infected macaques (CK5H and DB93) experienced a more rapid clinical course associated with very high levels of plasma viremia and very low levels of circulating and effector site memory $CD4^+$ T cells and required euthanasia at weeks 23 and 24 p.i., respectively.

Mucosal transmission of new $SHIV_{AD8}$ swarm stocks. A major aim of this study was to determine the minimal $SHIV_{AD8}$ inoculum resulting in the establishment of infection in rhesus macaques challenged by the i.r. route. As shown in Table 1, one (CL5E) of two animals inoculated with 100 $TCID_{50}$ of the
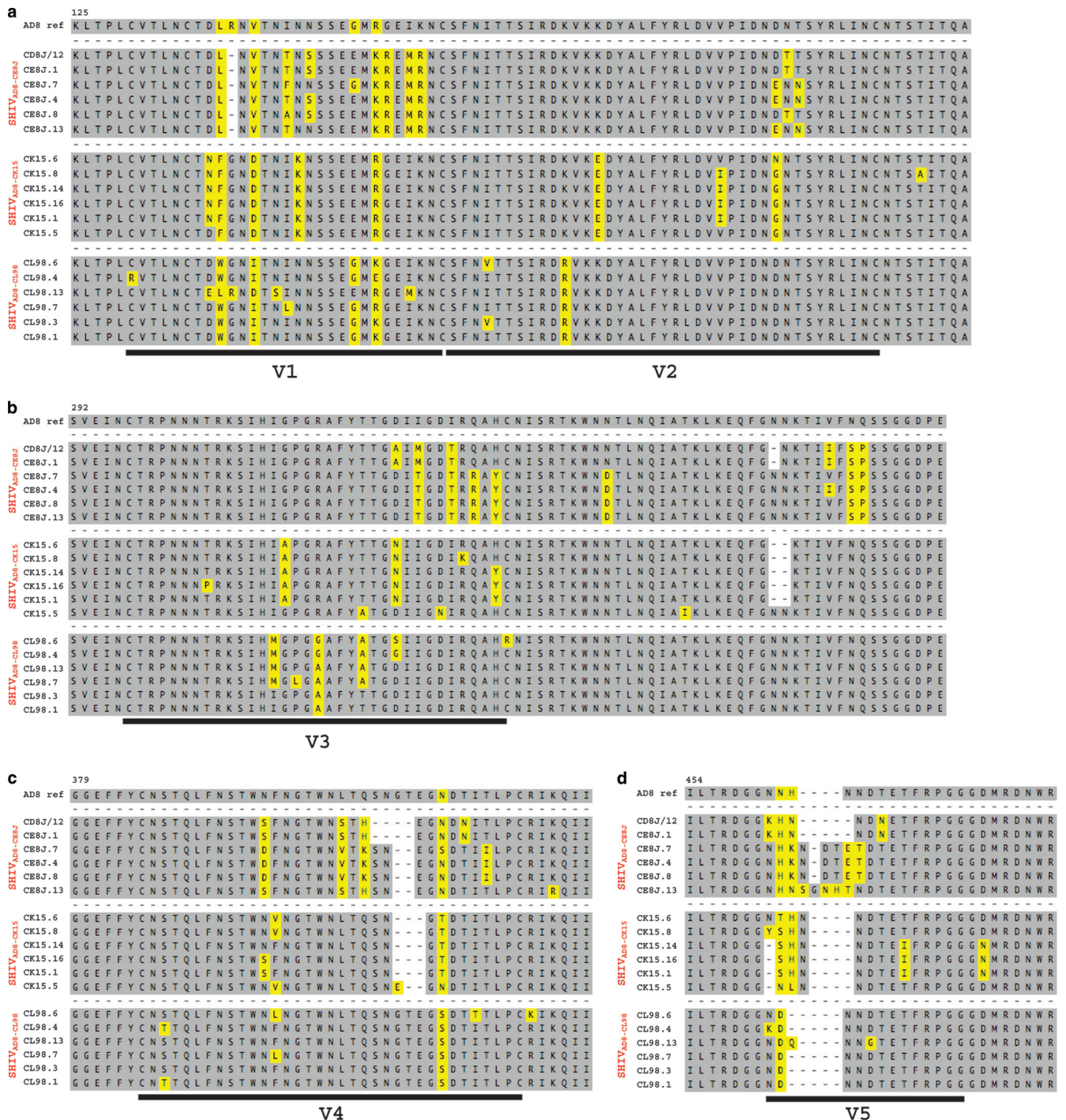
FIG 1 Gp120 sequence alignments of SHIV$_{AD8}$ viral stocks (CE8J, CK15, and CL98). Six independent clones amplified from each virus stock by RT-PCR were sequenced, and the deduced amino acid sequences of the V1 and V2 (a), V3 and C3 (b), V4 (c), and V5 (d) regions of gp120 were aligned with the HIV$_{AD8}$ sequence at the top. Amino acid changes are highlighted in yellow.

SHIV$_{AD8-CE8J}$ stock became infected following a single i.r. inoculation. A second macaque (DA24), inoculated two times with 100 TCID$_{50}$ of SHIV$_{AD8-CE8J}$, failed to become infected. Infection was established in this monkey when the challenge dose was increased to 1,000 TCID$_{50}$. Assuming that 1,000 TCID$_{50}$ might represent the threshold dose for successfully in-

fecting monkeys with SHIV$_{AD8-CE8J}$ by the i.r. route, four additional macaques (DBJE, DBZL, CZH, and DBX9) were inoculated with 1,000 TCID$_{50}$, and all four became infected following a single virus challenge (Table 1). The peak virus loads in five of the six SHIV$_{AD8-CE8J}$-infected animals ranged from 1.0×10^6 to 1.1×10^7 viral-RNA copies/ml of plasma; the peak viral-RNA
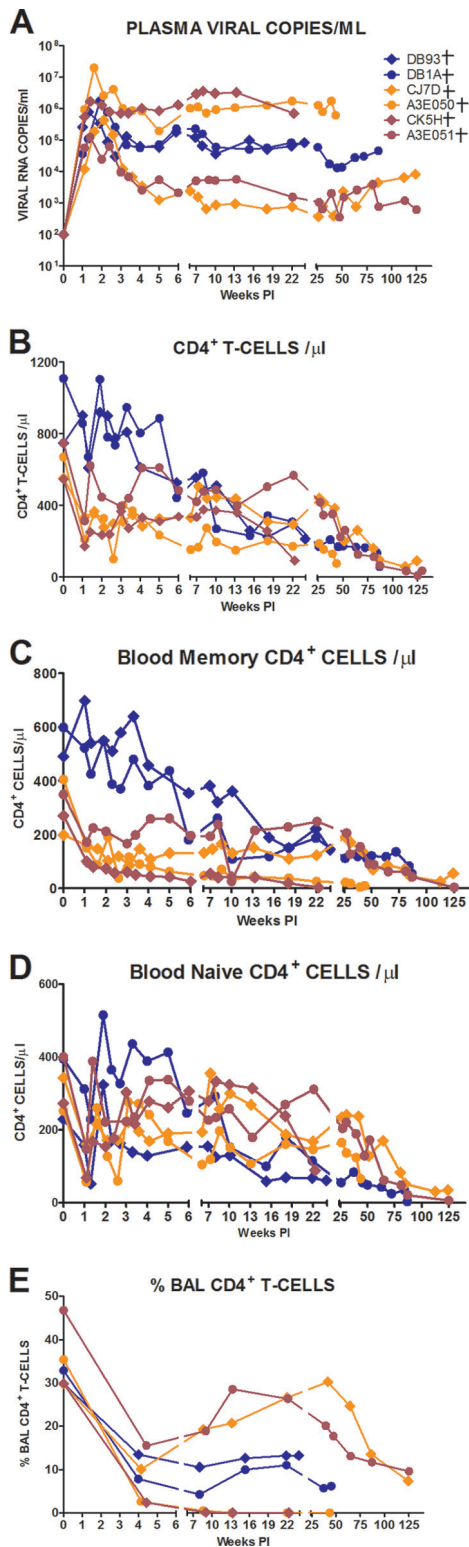
FIG 2 SHIV$_{AD8}$ induces sustained plasma viremia and loss of CD4$^+$ T cells in intravenously inoculated rhesus macaques. The levels of plasma viremia (A); absolute numbers of peripheral CD4$^+$ T cells (B), memory CD4$^+$ T cells (C), and naïve CD4$^+$ T cells (D); and percentages of BAL fluid CD4$^+$ T cells (E) are shown. Note that purple symbols denote animals infected with SHIV$_{AD8-CE8J}$, maroon symbols denote animals infected with SHIV$_{AD8-CL98}$, and orange symbols denote animals infected with SHIV$_{AD8-CK15}$. Macaques euthanized with AIDS are indicated (†).

level in the sixth macaque (DBZL) was delayed and notably lower (6×10^3 copies/ml of plasma) (Fig. 3A).

A similar approach was used to determine the minimal i.r. inoculum size for the new SHIV$_{AD8-CL98}$ and SHIV$_{AD8-CK15}$ stocks. As shown in Table 1, infection was not established in two monkeys (DA55 and DB18) inoculated with 100 TCID$_{50}$ of SHIV$_{AD8-CL98}$. Successful virus acquisition in these 2 animals required increasing the inoculum size to 1,000 TCID$_{50}$ of SHIV$_{AD8-CL98}$. Furthermore, two additional animals (CL5B and DB0F) resisted infection even following two i.r. exposures to 1,000 TCID$_{50}$, but each became infected after receiving a single inoculation of 10,000 TCID$_{50}$ of SHIV$_{AD8-CL98}$. Once infected, all four of the SHIV$_{AD8-CL98}$-inoculated macaques generated peak viral-RNA loads ranging from 1.4×10^6 to 1.2×10^7 copies/ml of plasma and levels of set point viremia ranging from 1.3×10^3 to 8.0×10^5 viral-RNA copies/ml of plasma (Fig. 3C).

Based on these results, 6 animals were inoculated by the i.r. route with 1,000 TCID$_{50}$ of the third virus stock (SHIV$_{AD8-CK15}$). As indicated in Table 1, four of six monkeys became infected following a single i.r. inoculation and developed persistent viremia. The two remaining animals (BIH and ENF) resisted infection with 1,000 TCID$_{50}$ of SHIV$_{AD8-CK15}$ but did become infected when the inoculum size was increased to 10,000 TCID$_{50}$ (Table 1). Plasma viral loads in the six SHIV$_{AD8-CK15}$-infected monkeys are shown in Fig. 3E.

No correlation was observed between inoculum size and peak viral loads/time to peak viremia following i.r. inoculation with any of the three new SHIV$_{AD8}$ preparations.

CD4$^+$ T cell depletion in macaques inoculated by the i.r. route with SHIV$_{AD8}$. The absolute numbers of CD3$^+$ CD4$^+$ T cells in peripheral blood markedly declined in a majority of animals challenged by the i.r. route during the 80- to 90-week observation period (Fig. 3), and 12 of 16 macaques sustained depletions of circulating memory CD4$^+$ T cells to the 200-cell/µl level by week 60 p.i. (Fig. 4). This was accompanied by the loss of naïve CD4$^+$ T cells to below the 100-cell/µl level in most of the monkeys in the same time frame (Fig. 4). Targeting and loss of memory CD4$^+$ T lymphocytes at an effector site (BAL samples) during acute infection was observed once again in a majority of the macaques inoculated by the mucosal route (Fig. 5). In summary, the decline of CD4$^+$ T lymphocyte subsets in the circulation or at an effector site following inoculation by the mucosal route (Fig. 3 and 5) paralleled the pattern previously observed for SHIV$_{AD8}$ administered intravenously (Fig. 2).

Immune responses to SHIV$_{AD8}$ infection. Anti-SHIV$_{AD8}$ Gag-specific CD4$^+$ and CD8$^+$ T lymphocyte responses were determined by flow cytometry by measuring intracellular staining of cells expressing tumor necrosis factor alpha (TNF-α) and/or interferon gamma (IFN-γ) following stimulation with a 15-mer peptide pool spanning SIV$_{mac239}$ Gag. The levels of virus-specific memory CD8$^+$ T cells in rhesus macaques inoculated by the i.r. route ranged from 0.38 to 1.78% (see Table S1 in the supplemental material). A similar analysis of Gag-specific responses in memory CD4$^+$ T cells in the same animals indicated that 0.55 to 4.02% expressed TNF-α and/or IFN-γ (see Table S1 in the supplemental material).

Anti-HIV-1 gp120 antibodies in macaques inoculated by the i.r. route with the three swarm SHIV$_{AD8}$ stocks became detectable by ELISA between weeks 3 and 12 p.i. (see Fig. S2 in the supplemental material). The levels of these antiviral binding antibodies
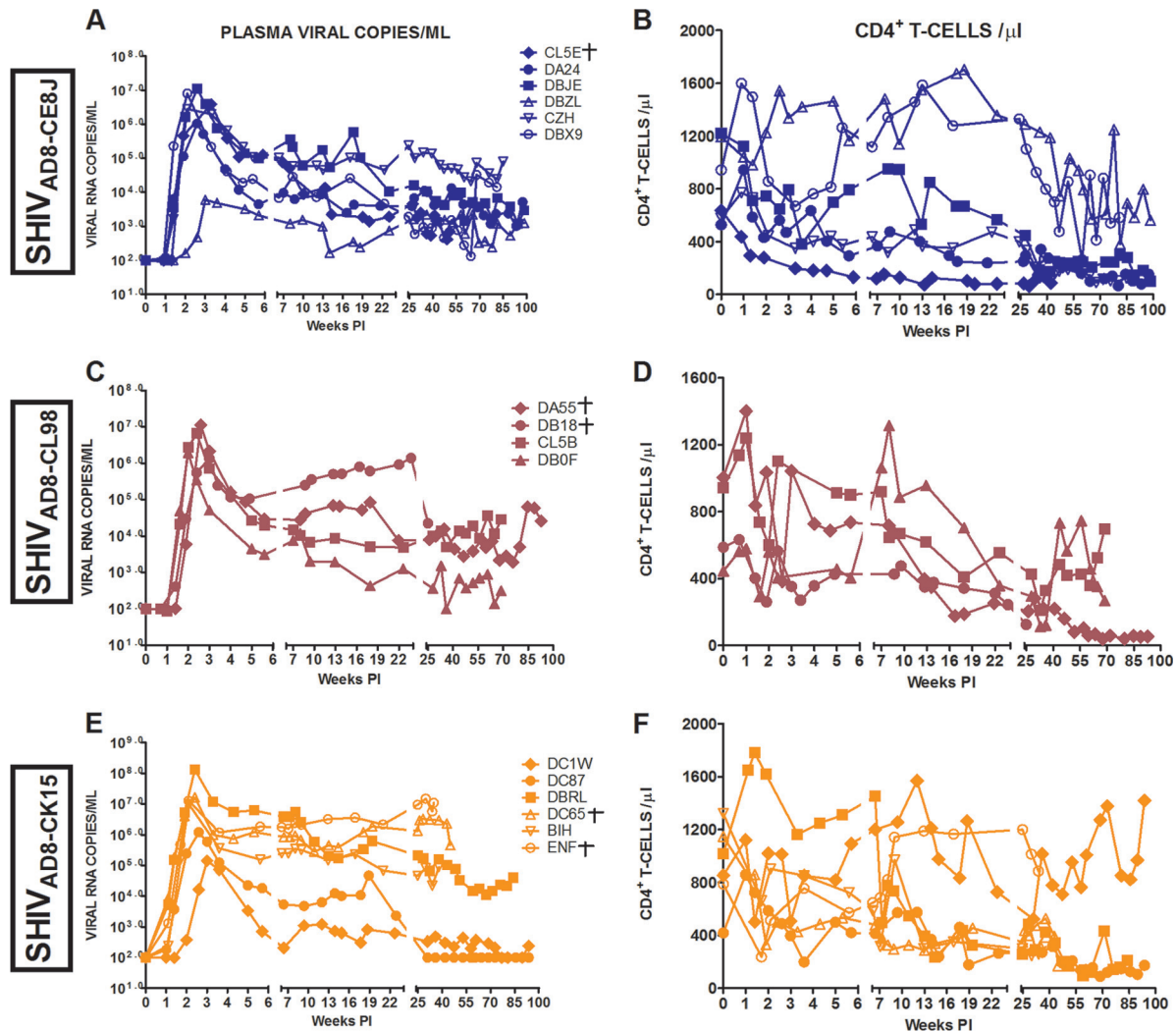
FIG 3 Intrarectal inoculation of SHIV$_{AD8}$ viral stocks in rhesus macaques. The colors in the graphs represent the challenge virus as described in the legend for Fig. 2. Macaques euthanized with AIDS are indicated (†).

remained high in most of these animals throughout the course of infection. Two macaques (DB18 and ENF) that developed very high levels of set point viremia and experienced a rapid and irreversible loss of memory CD4$^+$ T cells at an effector site (in BAL specimens) failed to generate detectable anti-gp120 antibodies and required euthanasia at weeks 25 and 35, respectively, because of their deteriorating clinical condition. This constellation of findings fulfilled the definition of "rapid progressors" assigned to SIV-infected macaques exhibiting a similar clinical phenotype (3).

A system for categorizing the neutralization sensitivities of HIV-1 isolates to plasma pools collected from individuals infected with geographically and genetically diverse virus strains has described three HIV-1 subgroups: tier 1 (very sensitive), tier 2 (moderate sensitivity), and tier 3 (neutralization resistant). In this classification system, tissue culture-adapted tier 1 viruses represent HIV-1 strains that can be neutralized by sera with minimal NAb activity, whereas tier 2 viruses exhibit sensitivities commonly observed with circulating strains of HIV-1 (44). The neutralization phenotypes of the three new SHIV$_{AD8}$ swarm stocks are shown in Table 2. In contrast to its very resistant parental HIV-1$_{AD8}$ (tier 3)

and the highly sensitive HIV-1$_{MN}$ (tier 1), all three SHIV$_{AD8}$ stocks exhibited a tier 2 phenotype. The sensitivities of these three stocks to a panel of anti-HIV-1 monoclonal antibodies are shown in Table 3. All three SHIV$_{AD8}$ stocks are resistant to the recently described PG9 and PG16 MAbs, as well as the high-mannose glycan-targeting 2G12 MAb. SHIV$_{AD8-CE8J}$ alone is resistant to the PGT121 MAb.

In a recently published study describing the extraordinarily broad and high-titer NAbs generated by one animal inoculated intravenously with an intermediate SHIV$_{AD8}$ derivative, we reported that, irrespective of the set point, only 20 to 25% of SHIV$_{AD8}$-infected monkeys develop autologous NAbs during the course of their infections (50). When detectable, autologous neutralization activity directed against SHIV$_{AD8}$ emerged after 30 to 35 weeks p.i. and was usually low titer (<1:100) and transient. In the present study, only 3 (DA24, DBJE, and DA55) of 19 monkeys inoculated with the three new SHIV$_{AD8}$ stocks developed low levels of autologous NAbs (see Table S2 in the supplemental material). In two of these macaques (DA24 and DBJE), the neutralization activity measured at weeks 41 and 42 became undetectable at
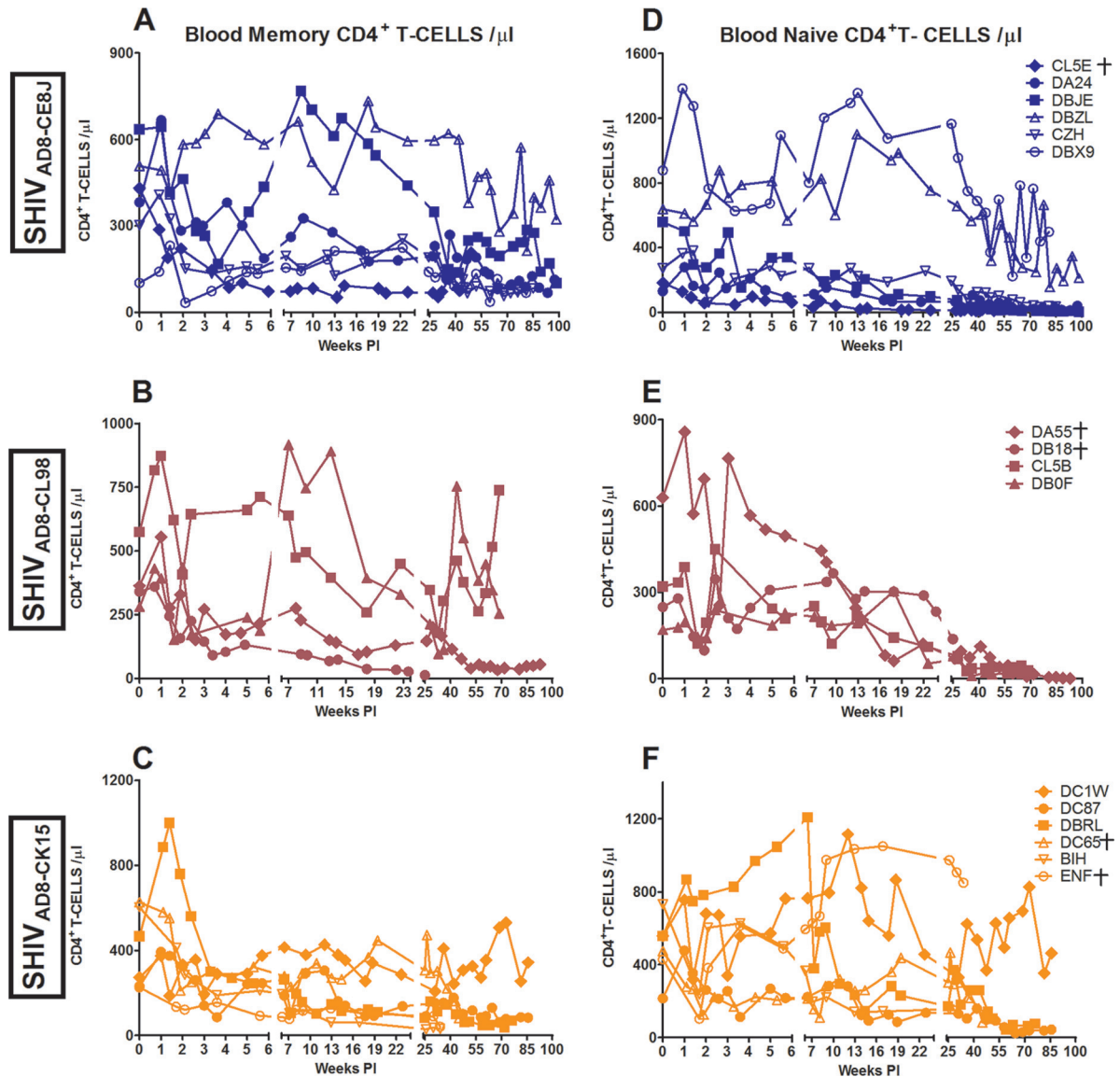
FIG 4 Loss of CD4$^+$ T cell lymphocyte subsets following i.r. inoculation of SHIV$_{AD8}$ virus stocks. Absolute numbers of memory CD4$^+$ T cells (A, B, and C), and naïve CD4$^+$ T cells (D, E, and F) are shown. The colors in the graphs represent the challenge virus as described in the legend for Fig. 2. Macaques euthanized with AIDS are indicated (†).

weeks 57 and 58 p.i. Furthermore, in this group of 19 animals, there was no obvious relationship between the levels of plasma viremia or preservation of CD4$^+$ T cell subsets, which distinguishes the three monkeys able to generate autologous NAbs from those that were not.

SGA of transmitted SHIV$_{AD8}$. The SGA-plus-sequencing approach has been previously used to determine the number of transmitted/founder viruses present in macaques following i.r. inoculation of the uncloned SIVmac251 and SIVsmE660 swarm stocks (28, 46). At low inoculum doses, one or only a few founder viruses present in the swarm virus preparation were detected in plasma collected during the early phase of acute infection of inoculated monkeys. A similar SGA analysis was performed using plasma samples collected on day 10 p.i. from two animals (DBX9 and DBJE) that became infected following a single i.r. inoculation with 1,000 TCID$_{50}$ of SHIV$_{AD8-CE8J}$ (Table 1 and Fig. 3). A total of

58 SGA-derived 3.5-kb sequences (including the entire *env* gene) were obtained from the plasma sample of each macaque or from the SHIV$_{AD8-CE8J}$ stock used for inoculation. The viral-RNA loads in macaques DBX9 and DBJE on day 14 p.i. were 8.03 × 10^6 and 1.64 × 10^6 RNA copies/ml, respectively. As shown in Fig. 6, a single SHIV *env* variant was detected in each animal. In both instances, the variant detected corresponded to an individual and unique virus component present in the SHIV$_{AD8-CE8J}$ stock, which overall contained a maximum diversity of 2.0%.

Some SHIV$_{AD8}$-infected macaques progressed to AIDS. The clinical status and disease outcomes of all 22 animals inoculated by the i.v. or i.r. route with new SHIV$_{AD8}$ stocks are presented in Table 4. Eleven animals were euthanized with symptoms of AIDS, including uncontrolled diarrhea, enteritis, typhocolitis, renal and mesenteric arteriolopathy, pulmonary thrombosis, and varying
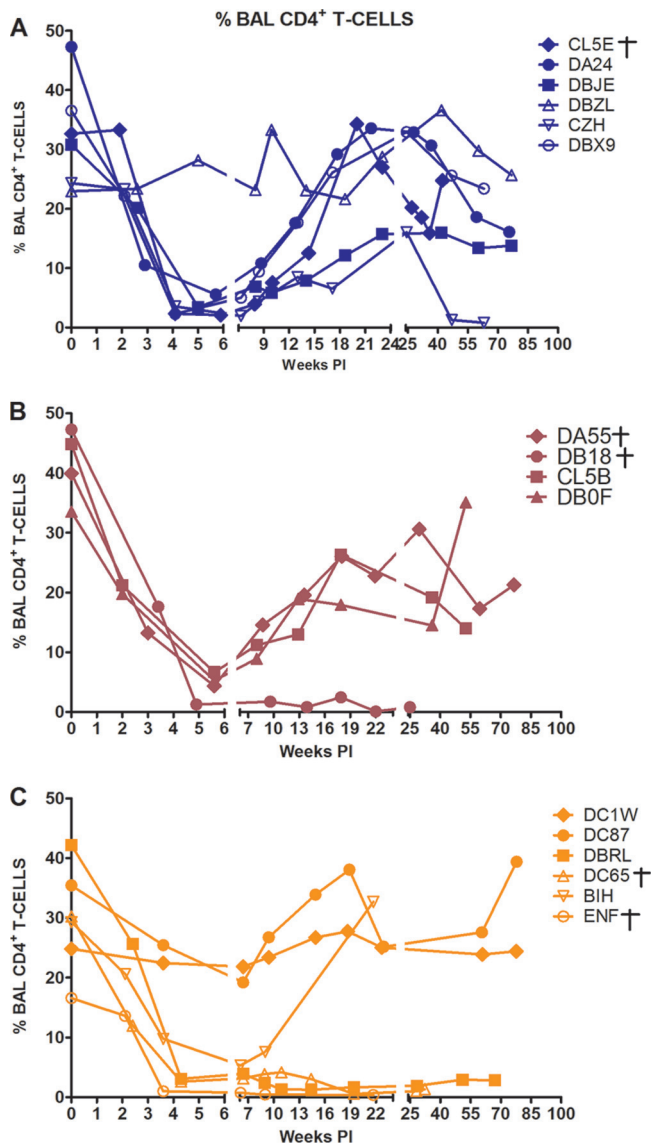
FIG 5 Loss of CD4$^+$ T cell lymphocytes at an effector site (BAL fluid). Macaques euthanized with AIDS are indicated (†).

degrees of thrombocytopenia. Histopathological studies performed on specimens collected at the time of necropsy revealed the presence of *Pneumocystis*, *Candida*, and *Mycobacterium* species in individual macaques.

DISCUSSION

In this report, we have described the biological and transmission properties of three SHIV$_{AD8}$ swarm stocks. Although each possesses distinct characteristics, their *in vivo* properties are similar to those reported (34) for "intermediate" SHIV$_{AD8}$ derivatives, which include (i) sustained but variable levels ($10^{2.5}$ to 10^6 RNA copies/ml) of set point viremia; (ii) induced depletion of circulating CD4$^+$ T cells, even in animals with modest ($<10^3$ RNA copies/ml) plasma virus loads; (iii) targeting of memory CD4$^+$ T lymphocytes for depletion at an effector site during acute infection; (iv) losses of memory and naïve CD4$^+$ T cells in the blood; (v) development of the "rapid-progressor" clinical phenotype in a few inoculated macaques; and (vi) induction of symptomatic AIDS in 9/20 normally progressing monkeys by week 125 p.i. As a consequence of their immunodeficiency, some animals acquired opportunistic infections (*Mycobacteria*, *Pneumocystis*, and *Candida* species), and several developed thrombocytopenia, with platelet counts of $<70,000/\mu l$ (Table 4).

The genetic signatures of the three SHIV$_{AD8}$ swarm stocks permitted an SGA analysis of founder viruses transmitted following i.r. inoculation. In two animals administered 1,000 TCID$_{50}$, a single and different SHIV variant, present in the original uncloned virus stock, was transmitted to each monkey. Although an exhaustive *in vivo* titration of the three new SHIV$_{AD8}$ stocks was not performed, the virus acquisition results presented in Table 1 suggest that the SHIV$_{AD8-CE8J}$ and SHIV$_{AD8-CK15}$ preparations have higher infectivity titers for i.r. inoculation of rhesus macaques than the SHIV$_{AD8-CL98}$ stock. When the method of Reed and Muench (40) is used to determine the *in vivo* infectivity of the SHIV$_{AD8-CE8J}$ and SHIV$_{AD8-CK15}$ stocks from the acquisition data, we calculate that 1,000 TCID$_{50}$, measured *in vitro*, is equivalent to approximately 3 50% animal infectious doses (AID$_{50}$). Thus, a single inoculation of 1,000 TCID$_{50}$ of SHIV$_{AD8-CE8J}$ and SHIV$_{AD8-CK15}$ might be close to the minimal challenge dose required for successful virus acquisition. Interestingly, the 1,000-TCID$_{50}$ ($\sim10^7$ RNA copies) threshold for establishing SHIV$_{AD8-CE8J}$ and SHIV$_{AD8-CK15}$ infections is also in the same range as that needed for virus acquisition with a single inoculation of SIVmac251 and SIVsmE660 (11, 22, 28).

We previously determined the coreceptor properties of the viruses used to prepare the three swarm SHIVs (SHIV$_{AD8-CE8J}$, SHIV$_{AD8-CK15}$, and SHIV$_{AD8-CL98}$) described in this study following their isolation from animals CE8J, CK15, and CL98 at the time of their euthanasia with clinical symptoms of immunodeficiency (see Fig. S2 in reference 34). All three SHIV stocks exclusively utilized CCR5. Because CCR5 is not expressed on naïve CD4$^+$ T lymphocytes, one could ask why this T cell subset is lost during SHIV$_{AD8}$ infections of macaques. It is now recognized that deple-

TABLE 2 Neutralization phenotype of SHIV$_{AD8}$ viral stocks

Sample ID	ID$_{50}$ in TZM-bl cells[a]							HIV IG (μg/ml)	Tier phenotype
	S321	C500	B520	G435	T520b	M263	M600c		
SHIV$_{AD8-CK15}$	63	112	145	36	145	85	64	>2,500	2
SHIV$_{AD8-CE8J}$	91	94	112	43	147	137	65	>2,500	2
SHIV$_{AD8-CL98}$	77	54	47	63	198	92	89	1,870	2
HIV$_{AD8}$	189	<20	<20	<20	<20	<20	25	2,032	3
MN.3	13,944	9,152	822	8,432	3,968	43,722	1,709	1.81	1
CAAN5342.A2	84	<20	27	<20	<20	77	185	638	2

[a] ID$_{50}$, serum dilution at which relative luminescence units were reduced 50% compared to virus control wells (no test sample).

TABLE 3 Neutralization assessment of SHIV$_{AD8}$ virus stocks by known MAbs targeting independent epitopes on the Env glycoprotein

Virus	Parameter	Value (µg/ml)[a]											
		HIV-IG	b12	VRC01	VRC03	VRC-PG04	CD4-Ig	PG9	PG16	2G12	PGT121	PGT123	PGT128
SHIV$_{AD8-CE8J}$	IC$_{50}$	2,412	8.04	0.917	0.088	0.317	1.89	>50	0.098	>50	>50	34.4	0.148
	IC$_{80}$	>5000	26.5	2.41	0.315	1.48	5.95	>50	>50	>50	>50	>50	2.76
SHIV$_{AD8-CK15}$	IC$_{50}$	1,331	4.37	2.34	0.184	1.73	1.72	>50	>50	>50	0.086	0.116	0.081
	IC$_{80}$	>5000	11.9	6.61	0.542	7.03	3.98	>50	>50	>50	0.232	0.324	0.201
SHIV$_{AD8-CL98}$	IC$_{50}$	1,805	6.16	2.84	0.468	2.23	1.69	30.6	22.9	>50	0.097	0.123	0.048
	IC$_{80}$	>5000	18.4	8.06	1.59	7.33	4.01	>50	>50	>50	0.254	0.306	0.123
SHIV$_{SF162 (P3)}$	IC$_{50}$	2,884	2.19	2.01	0.388	>50	3.06	>50	>50	>50	ND	ND	ND
	IC$_{80}$	>5000	4.94	5.87	0.893	>50	8.25	>50	>50	>50	ND	ND	ND

[a] ND, not determined.

tion of this T lymphocyte subset is a prominent feature of chronic HIV-1 infections in the absence of coreceptor switching (23, 42) and has been reported to occur in SIVsm543-infected rhesus macaques (25, 32), but not in SIVmac239-infected monkeys (39). Loss of naive CD4$^+$ T cells is also characteristic of R5 SHIV$_{AD8}$.

FIG 6 Composite phylogenic tree of *env* gene sequences present in the SHIV$_{AD8-CE8J}$ stock and in plasma from macaques (DBJE and DBX9) on day 14 after i.r. inoculation. Animal-derived sequences from plasma at peak viremia are color coded, and inoculum-derived sequences are shown in black.

infections in normally progressing rhesus macaques (34). In contrast, coreceptor switching has been observed only during the rapid and irreversible loss of naïve CD4$^+$ T cells in some R5-tropic SHIV rapid progressors (14, 15, 34, 47), but never in SIV rapid progressors.

The presence of the HIV-1 envelope glycoprotein on all SHIVs makes them particularly attractive challenge viruses to evaluate the protective effects of NAbs in the macaque model. In this regard, we recently reported that a single rhesus monkey (CE8J), inoculated with an intermediate SHIV$_{AD8}$ derivative, generated extremely high-titer and unusually broad autologous NAbs directed against viruses from different HIV-1 clades (50). Mapping studies revealed that the HIV-1 gp120 epitopes targeted for neutralization were dependent on the glycan present at amino acid 332. In the present study, our characterization of the neutralization properties of the three new swarm SHIV$_{AD8}$ challenge stocks indicated that each exhibited a tier 2 phenotype (Table 2) and would therefore be an ideal surrogate for circulating HIV-1 strains in vaccine experiments. Furthermore, the three SHIV$_{AD8}$ preparations exhibited similar sensitivities to several broadly reacting anti-HIV-1 neutralizing MAbs (Table 3). However, SHIV$_{AD8-CE8J}$, isolated at the time of euthanasia from macaque CE8J, the animal producing potent cross-clade anti-HIV-1 NAbs, was uniquely resistant to the recently described PGT121 and PGT123 MAbs (49). Perhaps the previously reported emergence of neutralization escape SHIV variants in monkey CE8J (50) also conferred the resistance of SHIV-$_{AD8-CE8J}$ to the PGT121 and PGT123 MAbs.

At present, we have no explanation for the relatively small fraction of SHIV$_{AD8}$-infected animals that develop sustained levels of autologous NAbs (see Table S2 in the supplemental material). We previously noted that only 9 macaques in our cohort of 40 monkeys inoculated with SHIV$_{AD8}$ (intermediate derivatives, swarm stocks, or a pathogenic molecular clone) generated detectable autologous antibodies. One of these 9 infected animals was macaque CE8J, described above, which developed high-titer and cross-HIV-1 clade autologous neutralizing activity. The low frequency of SHIV$_{AD8}$-infected monkeys generating autologous NAbs contrasts with results obtained for X4-tropic SHIVs (8, 26) and the R5-tropic SHIV$_{SF162P4}$ (24), all of which develop high levels of autologous NAbs. This property of the R5-tropic SHIV$_{AD8}$ could reflect the tier 3 neutralization phenotype of its HIV-1$_{AD8}$ parent (Table 2) and is reminiscent of the failure of SIVmac239-infected monkeys to produce significant levels of autologous NAbs (21). It
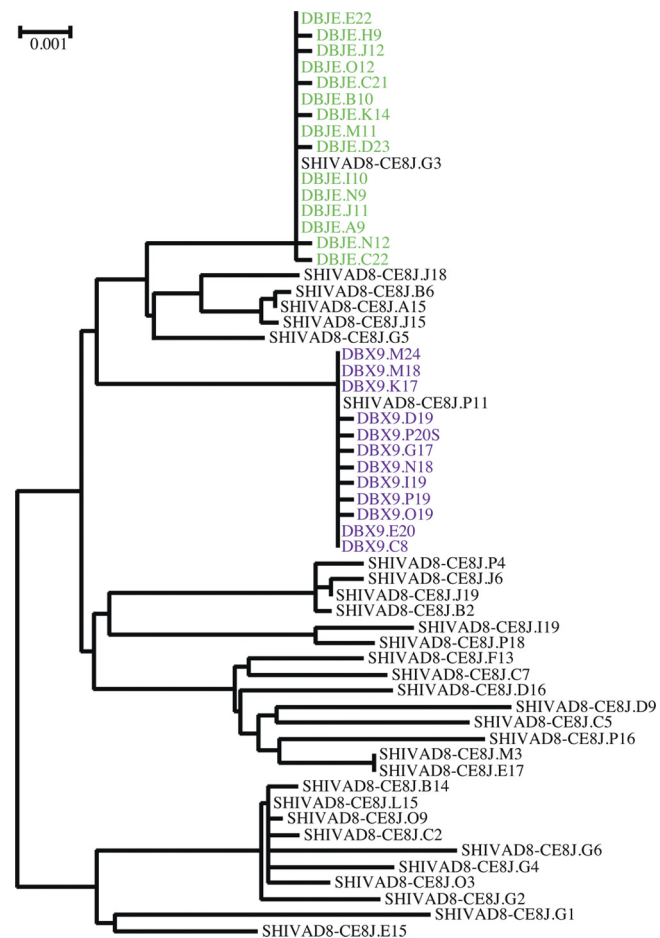
TABLE 4 Clinical and pathological findings in rhesus macaques infected with SHIV$_{AD8}$ virus stocks

Animal	Inoculum	Clinical data/pathological findings	No. of platelets[b] (10^3/μl)
DB93[a]	SHIV$_{AD8-CE8J}$	Euthanized (wk 24); rapid progressor	29
DB1A[a]	SHIV$_{AD8-CE8J}$	Euthanized (wk 87); arteriolopathy in kidneys and mesentery	37
CL5E[a]	SHIV$_{AD8-CE8J}$	Euthanized (wk 51); pulmonary thrombosis	66
DA24	SHIV$_{AD8-CE8J}$	Total CD4$^+$ T cells, 79/mm^3 (wk 94)	223
DBJE	SHIV$_{AD8-CE8J}$	Total CD4$^+$ T cells, 183/mm^3 (wk 95)	196
DBZL	SHIV$_{AD8-CE8J}$	Total CD4$^+$ T cells, 796/mm^3 (wk 95)	341
CZH	SHIV$_{AD8-CE8J}$	Total CD4$^+$ T cells, 106/mm^3 (wk 81)	183
DBX9	SHIV$_{AD8-CE8J}$	Total CD4$^+$ T cells, 583/mm^3 (wk 81)	423
CJ7D[a]	SHIV$_{AD8-CK15}$	Euthanized (wk 125); enteritis	482
A3E050[a]	SHIV$_{AD8-CK15}$	Euthanized (wk 44); uncontrolled diarrhea	201
DC1W	SHIV$_{AD8-CK15}$	Total CD4$^+$ T cells, 1419/mm^3 (wk 94)	464
DC87	SHIV$_{AD8-CK15}$	Total CD4$^+$ T cells, 173/mm^3 (wk 94)	301
DBRL	SHIV$_{AD8-CK15}$	Total CD4$^+$ T cells, 211/mm^3 (wk 84)	66
DC65[a]	SHIV$_{AD8-CK15}$	Euthanized (wk 43); *Mycobacterium intracellulare*	48
BIH	SHIV$_{AD8-CK15}$	Total CD4$^+$ T cells, 312/mm^3 (wk 38)	110
ENF[a]	SHIV$_{AD8-CK15}$	Euthanized (wk 35), *Mycobacterium avium*	125
CK5H[a]	SHIV$_{AD8-CL98}$	Euthanized (wk 23); rapid progressor; uncontrolled diarrhea, viral enterotyphocolitis	65
A3E051[a]	SHIV$_{AD8-CL98}$	Euthanized (wk 125); enterotyphocolitis	358
DA55[a]	SHIV$_{AD8-CL98}$	Euthanized (wk 95); lymphoma	36
DB18[a]	SHIV$_{AD8-CL98}$	Euthanized (wk 25); *Candida* and *Pneumocystis*	126
CL5B	SHIV$_{AD8-CL98}$	Total CD4$^+$ T cells, 696/mm^3 (wk 69)	372
DB0F	SHIV$_{AD8-CL98}$	Total CD4$^+$ T cells, 268/mm^3 (wk 69)	261

[a] Euthanized with simian AIDS.
[b] Normal range is 260×10^3 to 361×10^3/μl.

is worth noting, however, that all SHIV$_{AD8}$-infected macaques are able to generate cross-reactive NAbs against some tier 1 and 2 HIV-1 strains (M. Shingai and M. A. Martin, unpublished data).

Our data indicate that these new SHIV$_{AD8}$ viruses (SHIV$_{AD8-CE8J}$, SHIV$_{AD8-CK15}$, and SHIV$_{AD8-CL98}$) reproducibly establish infections in rhesus macaques following the mucosal transmission of one founder virus, consistently cause the loss of memory CD4$^+$ T lymphocytes, possess a tier 2 phenotype, and are sensitive to several potent cross-reacting anti-HIV-1 neutralizing MAbs. Although other R5-tropic SHIVs have been constructed, the levels of sustained viremia, depletion of CD4$^+$ T cells, and development of immunodeficiency in inoculated animals are frequently variable (35–37). These inconsistencies could affect the outcomes and interpretations of passive transfer or vaccine experiments when such R5 SHIVs are used as challenge viruses. We are also in the process of deriving a pathogenic molecular clone from SHIV$_{AD8}$-infected animals. This would provide a reagent that can readily be used to identify neutralization escape virus variants that emerge following a SHIV$_{AD8}$ challenge or during the course of vaccine experiments.

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