JOURNAL OF VIROLOGY, Mar. 2006, p. 2771–2783 Vol. 80, No. 6
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.6.2771–2783.2006

Th-1-Type Cytotoxic CD8$^+$ T-Lymphocyte Responses to Simian Immunodeficiency Virus (SIV) Are a Consistent Feature of Natural SIV Infection in Sooty Mangabeys

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Received 25 August 2005/Accepted 14 December 2005

Sooty mangabeys are a natural host of simian immunodeficiency virus (SIV) that remain asymptomatic and do not exhibit increased immune activation or increased T-lymphocyte turnover despite sustained high levels of SIV viremia. In this study we asked whether an altered immune response to SIV contributes to the lack of immunopathology in sooty mangabeys as opposed to species with pathogenic lentivirus infection. SIV-specific cellular immune responses were investigated in a cohort of 25 sooty mangabeys with natural SIV infection. Gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay responses targeting a median of four SIV proteins were detected in all 25 mangabeys and were comparable in magnitude to those of 13 rhesus macaques infected with SIVmac251 for more than 6 months. As with rhesus macaques, Th2 ELISPOT responses to SIV were absent or >10-fold lower than the IFN-γ ELISPOT response to the same SIV protein. The SIV-specific ELISPOT response was predominantly mediated by CD8$^+$ T lymphocytes; the frequency of circulating SIV-specific CD8$^+$ T lymphocytes ranged between 0.11% and 3.26% in 13 mangabeys. Functionally, the SIV-specific CD8$^+$ T lymphocytes were cytotoxic; secreted IFN-γ, tumor necrosis factor alpha, and macrophage inflammatory protein 1β; and had an activated effector phenotype. Although there was a trend toward higher frequencies of SIV-specific CD8$^+$ T lymphocytes in mangabeys with lower viral loads, a significant inverse correlation between SIV viremia and SIV-specific cellular immunity was not detected. The consistent detection of Th1-type SIV-specific cellular immune responses in naturally infected sooty mangabeys suggests that immune attenuation is neither a feature of nor a requirement for maintenance of nonpathogenic SIV infection in its natural host.

Sooty mangabeys (*Cercocebus atys*) are African Old World primates that are naturally infected with simian immunodeficiency virus (SIV) but typically do not develop AIDS despite evidence of persistent high-level SIV viremia (47). In contrast to human immunodeficiency virus (HIV)-infected humans and SIV-infected rhesus macaques, where the level of viremia is a predictor of disease progression (38, 43, 52), sooty mangabeys with a wide range of SIV RNA concentrations in plasma (10^3 to 10^7 copies/ml) remain asymptomatic over a long period of time. The mechanisms underlying maintenance of nonpathogenic SIV infection in sooty mangabeys are not well understood. The rapid kinetics of viral decay on administration of a reverse transcriptase inhibitor (R. Grant and M. Feinberg, unpublished data; reviewed in reference 28), as well as the detection of a high mutation fixation rate (47), suggest that, as with AIDS-susceptible species, SIV infection in naturally SIV-infected sooty mangabeys is maintained by ongoing viral replication in cytopathically infected cells with a short half-life. In striking contrast to pathogenic lentivirus infection, the high rate of viral replication in naturally SIV-infected sooty mangabeys is not accompanied by increased immune activation or apoptosis (50). Neither is there evidence of increased CD4$^+$ and CD8$^+$ T-lymphocyte turnover in SIV-infected sooty mangabeys (11; also A. Kaur, unpublished data). Finally, unlike with chronic pathogenic lentivirus infection, the structure and function of peripheral lymph nodes is preserved in sooty mangabeys with chronic SIV infection (47, 50). Despite these differences, the presence of lower peripheral CD4$^+$ T-lymphocyte counts in SIV-infected versus SIV-negative sooty mangabeys (11 and the recent report of a case of AIDS in a naturally SIV-infected sooty mangabey (39) underscore the fact that lentivirus infections are not entirely silent in their natural host.

Immune activation, as evidenced by increased expression of the activation-associated molecules CD38 and HLA-DR on the surface of CD4$^+$ and CD8$^+$ T lymphocytes, is a hallmark of uncontrolled viremia in HIV-infected humans and has been shown to be a major predictor of disease progression independent of viral load (15, 22, 35, 51). The mechanisms underlying increased immune activation in pathogenic lentivirus infection are not well understood. Immune activation is directly correlated to viral load in untreated HIV-infected individuals and is reduced following suppression of viremia with antiretroviral therapy, suggesting a virus-driven mechanism (13–15, 19, 53). However, viral replication is not the only predictor of immune activation in HIV infection. Thus, HIV type 1 (HIV-1)- and HIV-2-infected individuals with significantly different levels of viremia can display comparable levels of immune activation (51). Furthermore, treated, HIV-infected individuals with incomplete viral suppression show less immune activation than

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do untreated individuals with comparable levels of HIV viremia (14). In this respect, the difference in immune activation between SIV-infected rhesus macaques and sooty mangabeys, despite comparable levels of SIV viremia, appears to mimic that observed between untreated and treated HIV-infected viremic individuals. In this study we asked whether an altered host immune response to SIV infection in sooty mangabeys compared to rhesus macaques contributes to the lack of immune activation and immunodeficiency in nonpathogenic SIV infection.

We have previously shown that sooty mangabeys infected with a pathogenic molecular clone of SIV mount a robust and sustained SIV-specific cytotoxic T-lymphocyte (CTL) response and show evidence of CTL escape, suggesting that, as in rhesus macaques, CTLs exert immune selective pressure in vivo in experimentally SIV-infected mangabeys (27, 29). Although our earlier studies also documented the presence of SIV-specific CTL activity in naturally SIV-infected sooty mangabeys (28, 31), the absence of a quantitative assay precluded precise comparison to pathogenic lentivirus infection or determination of its effect on SIV replication. In this study we have used the enzyme-linked immunospot (ELISPOT) and intracellular cytokine-staining (ICS) assays to comprehensively evaluate the quality and the magnitude of the total SIV-specific cellular immune response in naturally SIV-infected sooty mangabeys and to investigate its relationship to viral load. Using pools of overlapping peptides corresponding to the sequence of SIV mac239, ELISPOT responses to all nine expressed SIV proteins were measured repeatedly over a 15-month period in a cohort of naturally SIV-infected sooty mangabeys. Gamma interferon (IFN-γ) ELISPOT responses to SIV proteins, largely mediated by $CD8^+$ T lymphocytes, were detected in 25 of 25 naturally SIV-infected sooty mangabeys and were comparable in magnitude to those in SIV-infected rhesus macaques. As with rhesus macaques, the SIV-specific cellular immune response was predominantly or exclusively a Th1-type response in sooty mangabeys. In the majority of mangabeys, two-thirds or more of the total SIV-specific T-cell response was directed toward structural SIV proteins. Consistent with data concerning HIV-infected humans (1), neither the magnitude nor the breadth of the IFN-γ ELISPOT response correlated significantly with the level of plasma SIV viremia. However, there was a trend toward higher frequencies of circulating SIV-specific $CD8^+$ T lymphocytes in sooty mangabeys with lower viral loads, suggesting the possibility that $CD8^+$ T lymphocytes play a role in limiting SIV replication in vivo in the setting of natural SIV infection. The presence of a broadly directed Th1-type cellular immune response to SIV in naturally SIV-infected sooty mangabeys closely resembles the host response to pathogenic lentivirus infection and suggests that immune attenuation is neither a feature of nor a requirement for maintenance of nonpathogenic lentivirus infection in its natural host.

MATERIALS AND METHODS

Animals. All sooty mangabeys were housed at the Yerkes National Primate Research Center (YNPRC), Atlanta, Ga., while all rhesus macaques were housed at the New England Primate Research Center (NEPRC), Southborough, Mass. All animals were maintained in accordance with institutional and federal guidelines for animal care (4). The study animals included 25 SIV-seropositive sooty mangabeys with naturally acquired SIV infection, nine SIV-seronegative sooty mangabeys, 13 SIV-infected rhesus macaques infected with SIVmac251 for more than 6 months (range, 25 to 111 weeks), and six SIV-negative rhesus macaques. Blood from sooty mangabeys housed at YNPRC was collected in heparin CPT vacutainer tubes (Becton Dickinson Vacutainer systems, Franklin Lakes, N.J.), centrifuged at 2,000 × *g* for 30 min within a half hour of collection in order to separate the peripheral blood mononuclear cells (PBMC) from the erythrocytes and granulocytes, and shipped overnight on ice to NEPRC, where it was processed on the day of arrival. This method of processing was necessary to avoid the functional loss of CTL activity observed with delayed isolation of PBMC from heparinized blood (29). Blood from rhesus macaques was collected in heparin Vacutainer tubes and subjected to centrifugation over a Ficoll gradient (Lymphocyte Separation Medium; ICN Biomedicals Inc., Aurora, Ohio) on the day of collection.

Separation of $CD4^+$ and $CD8^+$ T lymphocytes. Fractionated CD4-enriched and CD8-enriched T-lymphocyte populations were obtained by negative cell selection using the StemSep system (StemCell Technologies, Vancouver, Canada). Rhesus monkey anti-CD8 or anti-CD4 monoclonal antibodies bound to antidextran in a tetrameric antibody complex (StemSep) were incubated with PBMC on ice for 30 min, followed by a magnetic colloid consisting of dextran-coated magnetic particles. Antibody- and dextran-bound unwanted cells were subsequently removed by passage through a high-gradient magnetic column of stainless steel mesh. The eluted, negatively selected cell fraction was used in ELISPOT assays. Enriched $CD4^+$ or $CD8^+$ T-cell fractions contained <1% of the depleted cell population, as determined by flow cytometry.

Establishment of BLCL. Autologous B-lymphoblastoid cell lines (BLCL) were established as previously described (29). Briefly, B cells were transformed by incubating PBMC with herpesvirus papio (baboon lymphocryptovirus) derived from the supernatant of S594 cells (provided by Norman Letvin, Beth Israel Hospital, Boston, Mass.) in the presence of 1 μg/ml of cyclosporine at 37°C in a 5% CO_2 incubator. BLCL were propagated in RPMI 1640 medium (Gibco) supplemented with 20% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.), 10 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 50 IU/ml of penicillin (Gibco), and 50 μg/ml of streptomycin (Gibco).

Plasma SIV RNA. The concentration of SIV RNA in plasma was determined as previously described (27). Briefly, blood was collected in tubes containing the anticoagulant EDTA and centrifuged at 1,200 × *g* for 10 min within 3 h of collection. Removed plasma was centrifuged again at 1,200 × *g* for 10 min, and aliquots of cell-free plasma were stored at −80°C. Plasma RNA was extracted with the QIAamp Viral RNA kit (QIAGEN), and SIV RNA was measured by real-time reverse transcriptase PCR (7700 Sequence Detection System; PE Applied Biosystems). Random hexamers were used to prime reverse transcription. Primers and probe targeting a highly conserved region in the 5′ untranslated region were used, and the SIV RNA copy number was determined by comparison to an external standard curve consisting of virion-derived SIVmac239 RNA.

Peptides. Fifteen-amino-acid (aa) peptides overlapping by 11 aa and spanning all nine SIV proteins corresponding to the sequence of SIVmac239 were synthesized at the Massachusetts General Hospital peptide core facility (Charlestown, Mass.) by 9-fluorenylmethoxy carbonyl chemistry using an automated peptide synthesizer (MBS 396; Advanced Chemtech, Inc., Louisville, Ky.); they were also obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. Individual peptides were suspended at 100 mg/ml in 100% dimethyl sulfoxide and subsequently pooled together for each SIV protein. A total of 10 peptide pools representing Gag, Pol (2 pools), Env, Rev, Tat, Nef, Vpr, Vpx, and Vif were used to stimulate PBMC and measure the total SIV-specific response. Peptides were used at a final concentration of 1 to 2 μg/ml, with the dimethyl sulfoxide concentration being maintained at <0.5% in all functional assays.

Antibodies. The following anti-human monoclonal antibodies (MAbs) were obtained from BD Biosciences Pharmingen (San Jose, Calif.), unless otherwise indicated: anti-CD3 (clone SP34) fluorescein isothiocyanate (FITC) or phycoerythrin (PE), anti-CD4 (clone L200) allophycocyanin (APC) or peridinin chlorophyll protein cychrome 5.5, anti-CD8 (clone SK1) peridinin chlorophyll protein and anti-CD20 (clone L27) FITC or APC, anti-CD11a (clone H111) FITC, anti-CD28 (clone CD28.2) FITC or PE, anti-CD45RA (clone L48) FITC, anti-β_7 integrin (clone FIB 504) PE, and anti-CCR7 (clone 150503; R&D Systems, Inc., Minneapolis, Minn.) PE. Anti-IFN-γ (clone B27) APC or FITC, anti-tumor necrosis factor alpha (TNF-α) (clone Mab11) APC or FITC, anti-interleukin 2 (IL-2) (clone MQ1-17H12) FITC or APC, anti-macrophage inflammatory protein 1β (MIP-1β) (clone D21-1351) PE, anti-CD69 (clone FN50) PE, anti-Granzyme B (clone GB12; Caltag Labs, Burlingame, Calif.) PE, and anti-Ki67 (clone B56) FITC were used for intracellular staining. Purified anti-CD28 (clone 28.2; 10 μg/ml) and anti-CD49d (clone 9F10; 10 μg/ml) were used for costimu-

lation in ICS assays. Affinity-purified $F(ab')_2$ fragments of goat anti-mouse immunoglobulin G (heavy plus light chains) (GAM; Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was used for cross-linking the costimulatory antibodies in ICS assays.

ELISPOT assay. IFN-γ, IL-4, IL-5, and IL-13 ELISPOT kits for humans that were cross-reactive with monkeys were kindly provided by Niklas Ahlborg (Mabtech, Nacka, Sweden). The capture- and biotinylated-detector-matched MAb pairs are as follows: IFN-γ, clones GZ-4 and 7-B6-1; IL-4, clones IL-4-I and IL-4-II; IL-5, clones TRFK-5 and 5A10; and IL-13, clones IL-13-I and IL-13-II. ELISPOT assays were performed on unfractionated or fractionated PBMC suspended in R-10 medium supplemented with 50 μM 2-β-mercaptoethanol. Sterile 96-well polyvinylidene difluoride ELIIP10SSP plates (Millipore, Bedford, Mass.) coated with anti-cytokine MAb were plated with cells at concentrations ranging from 300,000 to 500,000 cells/well for SIV-specific stimulation. Stimulation with phytohemagglutinin (10 μg/ml; Sigma, St. Louis, Mo.) or concanavalin A (ConA) (5 μg/ml; Sigma, St. Louis, Mo.) or with staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) (100 ng/ml each; Sigma, St. Louis, Mo.) was used as a positive control, while stimulation with medium alone was used as a negative control. After overnight stimulation at 37°C in a 5% CO_2 incubator, cells were removed by extensive washing and incubated for 2 h at room temperature with biotinylated detector MAb (Mabtech). Spots were developed by successive incubation with streptavidin-alkaline phosphatase followed by the substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate buffer (Bio-Rad Laboratories, Hercules, Calif.). Spots were counted on a KS ELISPOT Automated Reader System (Carl Zeiss, Inc., Thornwood, N.Y.) using KS ELISPOT 4.2 software (performed by ZellNet Consulting, Inc., Fort Lee, N.J.). Frequencies of responding cells obtained after subtracting background spots in negative control wells were expressed as spot-forming cells (SFC) per million PBMC. ELISPOT responses to individual SIV proteins that were more than twofold above those of negative control wells and greater than two standard deviations (SD) above the mean in SIV-seronegative sooty mangabeys were considered positive.

ICS assay. SIV-specific T lymphocytes were analyzed by four-color flow cytometry. The ICS assay was performed as previously described (30). Briefly, PBMC were stimulated overnight at 37°C overnight in 12- by 75-mm polystyrene tubes placed at a 5° angle above horizontal in the presence of R-10 medium alone (unstimulated) or SEA/SEB (100 ng/ml each) or SIV peptide pools in the presence of the cross-linked costimulatory antibodies anti-CD28 and anti-CD49d. Brefeldin A (GolgiPlug; BD Pharmingen) was added after 2 h and continued for the remaining period of stimulation. After overnight stimulation, cells were washed with phosphate-buffered saline, incubated with 0.02% EDTA for 15 min at 37°C, washed again, and stained for 30 min at 4°C with fluorochrome-conjugated MAbs specific for cell surface molecules. Surface-stained cells were fixed by incubation with FIX & PERM Medium A (Caltag Labs, Burlingame, Calif.) for 15 min at room temperature followed by permeabilization with FIX & PERM Medium B (Caltag Labs, Burlingame, Calif.) along with fluorochrome-conjugated anti-CD69 and anti-cytokine MAbs for another 30 min at 4°C. After a final wash, cells were fixed in fresh 2% paraformaldehyde. At least 100,000 lymphocyte events (200,000 or more total events) were collected by FACSCalibur (BD Biosciences, San Jose, Calif.), and data were analyzed by CellQuest (BD Biosciences) and FlowJo (Tree Star, Inc., San Carlos, Calif.) software.

CTL assay. Effector cells for CTL assays were generated by in vitro stimulation of PBMC with SIV peptides for 10 to 14 days. For stimulation, one-third of the PBMC were pulsed with peptide at a final concentration of 1 to 2 μg/ml for 90 min at 37°C. Peptide-pulsed PBMC were then mixed with the remaining two-thirds of the PBMC, resuspended in R-10 medium at 2×10^6 cells/ml, and incubated at 37°C in a 5% CO_2 incubator. Cells were half-fed with R-10 medium twice a week, and recombinant human IL-2 (10 IU/ml; Hoffmann-La Roche Inc., Nutley, N.J.) was added to the feeding medium after 4 to 5 days. CTL activity was measured by chromium release assay after 10 to 14 days of stimulation. Unpulsed or peptide-pulsed autologous BLCL that had been labeled with radioactive chromium (^{51}Cr) were used as target cells, as previously described (31). Target cells were dispensed in duplicate at 10^4 cells/well and incubated with effectors at different effector/target cell ratios in 96-well U-bottomed plates (Corning Inc., Corning, N.Y.). ^{51}Cr release in the supernatant was assayed after 4 h of incubation at 37°C in a 5% CO_2 incubator. Plates were spun at 1,000 rpm for 10 min at 4°C, and 30 μl of supernatant was harvested from each well onto wells of a LumaPlate-96 (Packard) and allowed to dry overnight. Emitted radioactivity was measured in a 1450 Microbeta Plus Liquid Scintillation Counter (Wallac, Turku, Finland). Spontaneous release was measured from wells containing only target cells and medium. Maximum release was measured from wells containing target cells and 0.1% Triton X-100 (Sigma). The percent specific cytotoxicity was calculated as follows: [(test release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release of target cells was <20% in all assays.

Statistical analysis. Differences in the magnitudes of cellular immune responses between two groups were analyzed by the nonparametric Mann-Whitney U test, while correlation between viral load and the frequency of SIV-specific $CD8^+$ T lymphocytes was determined by the Spearman rank correlation test with Statview software (Abacus Concepts, Inc., Berkeley, Calif.). Since repeated measurements of ELISPOT responses and viral load were performed on individual mangabeys (total, 85 data points on 25 animals), a mixed (or random)-effects linear regression analysis was performed to evaluate the correlation between the log of viral load and the frequency of IFN-γ ELISPOT responses, with individual mangabeys as the random effects, using S-Plus (Insightful Corporation, Seattle, Wash.).

RESULTS

Detection of Th1 and Th2 cytokine responses by ELISPOT assay in sooty mangabeys. Previous studies with mitogen-stimulated cloned T-cell lines have suggested a Th2 cytokine bias in sooty mangabeys compared to rhesus macaques (5). In order to determine the cytokine appropriate for evaluation of SIV-specific cellular immunity in sooty mangabeys, we first investigated secretion of Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-13) cytokines in SIV-negative and SIV-infected sooty mangabeys and rhesus macaques by the ELISPOT assay. Th1 and Th2 ELISPOT responses were detected following mitogen (phytohemagglutinin and ConA) and superantigen (SEA and SEB) stimulation in both sooty mangabeys and rhesus macaques (Fig. 1). In both species, the magnitude of the IFN-γ response to mitogen or superantigen stimulus was 2- to 29-fold greater than the corresponding Th2 response (Fig. 1). We did not observe a clear Th2 cytokine bias in sooty mangabeys compared to rhesus macaques. On the contrary, IL-4 ELISPOT responses were significantly higher in SIV-negative rhesus macaques than in SIV-negative sooty mangabeys following SEA and SEB stimulation (Fig. 1). Curiously, IL-5 ELISPOT responses were undetectable or uniformly low in rhesus macaques (Fig. 1). Human IL-5 has 99.3% amino acid homology with rhesus macaque IL-5 and 97.8% homology with sooty mangabey IL-5 (55); thus, it is unlikely that the low IL-5 ELISPOT response in rhesus macaques was related to poor cross-reactivity with the anti-human IL-5 antibodies used in the ELISPOT assay.

Although Th2 cytokine responses were readily detected following mitogen or superantigen stimulation, they were seldom detected after SIV peptide stimulation (Fig. 1). When detected, SIV-specific IL-4 responses were more than 10-fold lower than the IFN-γ response to the same SIV peptide pool (Fig. 1). As with the results with mitogen stimulation, there was no difference in cytokine bias for SIV-specific cellular immune responses between rhesus macaques and sooty mangabeys (Fig. 1). Hence, IFN-γ secretion was used as the primary readout for detailed characterization of the SIV-specific cellular immune response in naturally SIV-infected sooty mangabeys.

Magnitude and breadth of SIV-specific cellular immunity in naturally SIV-infected mangabeys. The total magnitude of the SIV-specific cellular immune response in naturally SIV-infected sooty mangabeys was determined by measuring the IFN-γ ELISPOT responses to all expressed SIV proteins. Overlapping peptides corresponding to the SIVmac239 sequence were used for stimulation of PBMC in ELISPOT and ICS assays. A cohort of 25 naturally SIV-infected mangabeys

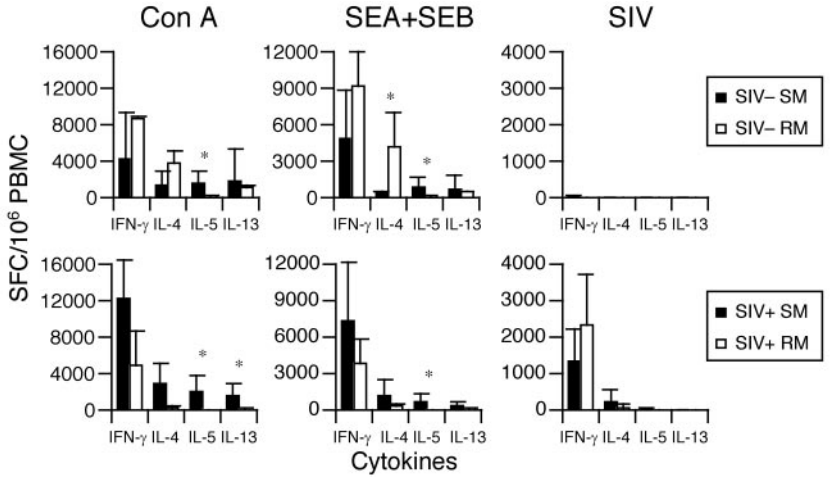

FIG. 1. IFN-γ is the dominant cytokine secreted by PBMC of sooty mangabeys (SM) and rhesus macaques (RM) in response to ConA, superantigen (SEA and SEB), and SIV peptide stimulation. IFN-γ, IL-4, IL-5, and IL-13 ELISPOT responses are shown for SIV-negative SM ($n = 9$), SIV-negative RM ($n = 6$), SIV-infected SM ($n = 5$), and SIV-infected RM ($n = 4$). Mean SFC per million PBMC ± SD, after subtraction of responses in negative control wells, are shown. The SIV-specific response depicts the sum of the ELISPOT responses to all SIV peptide pools with the exception of Pol. Asterisks denote differences between RM and SM ($P \leq 0.05$ [Mann-Whitney U test]).

were tested at three or more time points over a 15-month period. The characteristics of the cohort are shown in Table 1. Although the peripheral $CD4^+$ T-lymphocyte count ranged from <200/μl to normal levels (Table 1), none of the animals were symptomatic and none had any clinical evidence of AIDS.

The positive cutoff values for SIV-specific IFN-γ ELISPOT responses were determined by the responses to SIV peptides detected in nine SIV-seronegative mangabeys. Based on spot frequencies greater than 2 SD above the mean in SIV-seronegative mangabeys, a cutoff value of 50 SFC/10^6 PBMC was considered positive for ELISPOT responses to SIV Gag, Nef, Rev, Tat, Vpr, Vpx, and Vif, while the positive cutoff values for ELISPOT responses to Pol and Env were 95 SFC/10^6 PBMC and 118 SFC/10^6 PBMC, respectively. Positive ELISPOT responses toward one or more SIV proteins were detected reproducibly in all 25 SIV-infected mangabeys (Fig. 2 and Table 2). Low-level ELISPOT responses to single SIV proteins that exceeded the positive cutoff values were detected in two SIV-seronegative mangabeys, one to SIV Nef (60 SFC/10^6 PBMC) and another to SIV Env (140 SFC/10^6 PBMC) (Table 2).

In the naturally SIV-infected sooty mangabeys, the sum of the IFN-γ ELISPOT response to all SIV proteins ranged between 241 and 5,244 SFC/10^6 PBMC (median, 1,812 SFC/10^6 PBMC), with 19 of 25 mangabeys responding to at least three SIV proteins, demonstrating the presence of a broadly directed SIV-specific cellular immune response (Fig. 2A and B). In the majority of animals, more than 62% of the SIV-specific ELISPOT response was directed toward the Gag, Env, and Pol proteins. Gag-specific responses were detected in 23 of 25 mangabeys, 10 of whom had responses greater than 500 SFC/10^6 PBMC (Fig. 2A and Table 2). Similarly, the Env protein was also recognized by the majority of animals (20 out of 25), and in 14 animals the response was greater than 500 SFC/10^6 PBMC (Fig. 2A and Table 2). Although the structural proteins appeared to be most frequently recognized, it is noteworthy that when the response frequency was adjusted for the length of the SIV protein (percent responders divided by the number of amino acids in that protein), the accessory proteins Vpx, Vpr, and Nef were the most frequently recognized SIV proteins (Table 2). With the exception of animal 15, in which a Nef-specific response was dominant, responses to the accessory SIV proteins were lower in magnitude than were the responses to SIV structural proteins (Fig. 2A and Table 2). Since the peptides used for stimulation were based on the sequence of SIVmac239, it is likely that the immune response to the highly variable SIV accessory and regulatory proteins was underestimated. The use of peptides based on autologous virus sequence was shown to significantly increase the detection rate of responses to Tat and Vpr in HIV-infected individuals (3). Thus, our current analysis represents minimum estimates of the strength and breadth of the SIV-specific cellular immune response in naturally SIV-infected sooty mangabeys.

We then investigated whether SIV-specific cellular immune responses in naturally SIV-infected sooty mangabeys were comparable to those in species with pathogenic SIV infection. SIV-specific IFN-γ ELISPOT responses were measured in 13 rhesus macaques that had been infected for more than 6 months with pathogenic SIVmac251. Of the 13 SIV-infected rhesus macaques, 9 were asymptomatic (duration of SIV infection, 25 to 82 weeks) while 4 had progressed to AIDS

TABLE 1. Characteristics of cohort of 25 naturally SIV-infected sooty mangabeys

Characteristic	Median	Range
Age (yr)	11	8–18
Plasma SIV RNA (copies/ml)	1.0×10^5	4.3×10^2–1.7×10^6
$CD3^+$ T lymphocytes/μl	3,368	1,406–6,695
$CD8^+$ T lymphocytes/μl	1,985	883–4,574
$CD4^+$ T lymphocytes/μl	677	171–2,147
$CD20^+$ lymphocytes/μl	370	77–1,270
NK cells[a]/μl	222	51–534

[a] NK cells were phenotypically identified as $CD3^-$ $CD20^-$ $CD8^+$ lymphocytes.

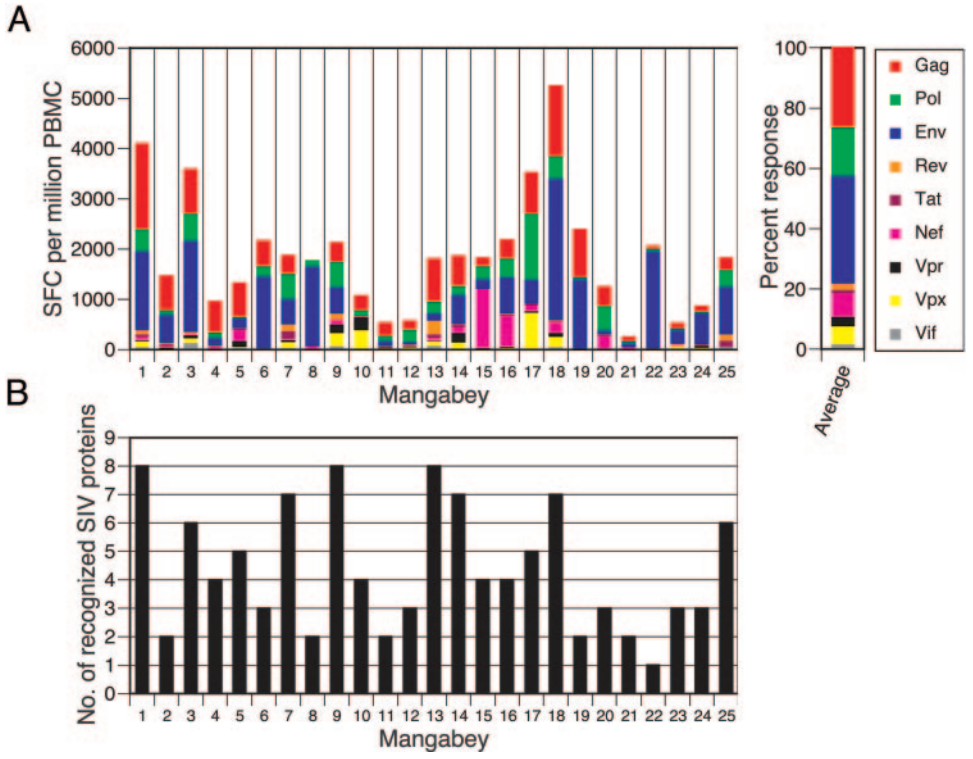

FIG. 2. IFN-γ ELISPOT responses in 25 naturally SIV-infected sooty mangabeys. (A) Magnitude of IFN-γ ELISPOT responses to SIV proteins. Individual animals are indicated as numbers on the *x* axis. Colored bars depict the frequency of the IFN-γ ELISPOT response to each SIV protein. Average percent contributions of each SIV protein to the overall IFN-γ ELISPOT response are shown adjacent to the individual animal responses. (B) Breadth of the SIV-specific response in 25 naturally SIV-infected sooty mangabeys. The numbers of SIV proteins yielding positive IFN-γ ELISPOT responses (>50 SFC/10^6 PBMC for SIV Gag, Nef, Rev, Tat, Vpr, Vpx, and Vif; >95 SFC/10^6 PBMC for SIV Pol; and >118 SFC/10^6 PBMC for SIV Env) in individual animals are shown.

(duration of SIV infection, 25 to 111 weeks) at the time of ELISPOT analysis. One of the 13 SIV-infected macaques (macaque 2 [Fig. 3A]) had undetectable SIV viremia (<200 copies of plasma SIV RNA/ml) at the time of ELISPOT analysis. In the remaining 12 macaques, plasma SIV RNA ranged between 3.1×10^4 and 2.5×10^7 and was comparable with that of the mangabey cohort (Table 1). The sum of IFN-γ ELISPOT responses to all SIV proteins in the 13 macaques ranged between 36 and 10,933 SFC/10^6 PBMC (median, 2,664 SFC/10^6 PBMC) and overlapped the responses detected in the naturally SIV-infected sooty mangabeys (Fig. 3A and B). Two macaques (no. 8 and 11 [Fig. 3A]) had undetectable or low SIV-specific ELISPOT responses (totals, 36 and 188 SFC/10^6 PBMC, respectively); macaque 8 had been infected for 25 weeks but was

TABLE 2. SIV-specific IFN-γ ELISPOT responses in sooty mangabeys

SIV protein	No. of animals with a positive IFN-γ ELISPOT response[a]		Responder frequency (%) adjusted to protein length[b]	Median (range) SFC/10^6 PBMC[c]	
	SIV$^-$ (n = 9)[d]	SIV$^+$ (n = 25)	SIV$^+$ (n = 25)	SIV$^-$ (n = 9)[d]	SIV$^+$ (n = 25)
Gag	0	23	0.18	0 (0–30)	355 (0–1,684)
Env	1	20	0.09	6 (0–140)	530 (0–2,826)
Pol	0	17	0.07	24 (14–88)	196 (26–1,316)
Nef	1	14	0.21	2 (0–60)	58 (0–1,172)
Rev	0	5	0.19	4 (0–15)	10 (0–272)
Tat	0	5	0.15	4 (0–10)	13 (0–152)
Vpr	0	6	0.24	10 (0–13)	18 (0–260)
Vpx	0	12	0.43	2 (0–12)	38 (0–720)
Vif	0	7	0.13	4 (0–14)	10 (0–142)

[a] Positive cutoff was >2 SD above the mean value in SIV$^-$ mangabeys (>50 SFC/10^6 PBMC for Gag, Nef, Rev, Tat, Vpr, Vpx, and Vif; >95 SFC/10^6 PBMC for Pol; >118 SFC/10^6 PBMC for Env).
[b] Frequency of recognition divided by the number of amino acids per SIV protein.
[c] Values are after subtraction of background in medium-only wells.
[d] With the exception of Pol (n = 6).

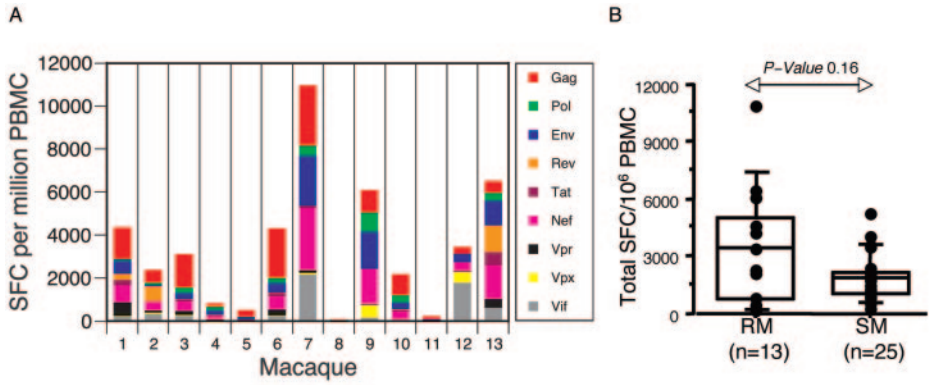

FIG. 3. SIV-specific IFN-γ ELISPOT responses in 13 SIVmac251-infected rhesus macaques. (A) Individual macaques are indicated as numbers on the *x* axis. Colored bars depict the frequency of the IFN-γ ELISPOT response to each SIV protein. (B) Comparison of IFN-γ ELISPOT responses between naturally SIV-infected sooty mangabeys (SM) and SIV-infected rhesus macaques (RM). Data on the analysis of 13 SIVmac251-infected RM and 25 naturally SIV-infected SM are shown. The sum of the IFN-γ ELISPOT responses to all nine SIV proteins is calculated as the total SIV-specific response. The Mann-Whitney U test was used to calculate the *P* value.

otherwise asymptomatic, while macaque 11 had progressed to AIDS after 111 weeks of infection. In contrast to that in the sooty mangabeys, the IFN-γ ELISPOT response in the rhesus macaques showed a broader distribution and targeted both structural and regulatory/accessory proteins (Fig. 3A). Since the sequence of regulatory/accessory proteins of SIVmac251 differs by only 36 aa from that of SIVmac239, the difference in recognition of SIV accessory proteins between mangabeys and macaques is likely related to the greater sequence variability of autologous SIVsm isolates from SIVmac239.

SIV-specific IFN-γ responses are predominantly mediated by CD8$^+$ T lymphocytes. Since 15-aa peptides overlapping by 11 aa were used for stimulation, the ELISPOT responses observed with unfractionated PBMC may represent either or both CD4$^+$ and CD8$^+$ T-lymphocyte responder cells (7, 17, 40). The contribution of CD4$^+$ and CD8$^+$ T lymphocytes to the SIV-specific IFN-γ ELISPOT response was determined by performing ELISPOT assays on fractionated PBMC in 10 of 25 mangabeys (Fig. 4 and data not shown) and by the ICS assay in 13 of 25 sooty mangabeys (Fig. 5). SIV-specific ELISPOT responses with CD8-depleted PBMC revealed a 2.2-fold to 70-fold reduction relative to CD4-depleted PBMC in the majority of mangabeys (Fig. 4 and data not shown). Mangabey 13 had a strong Gag response, which decreased at least fourfold following CD8$^+$ T-cell depletion, while there was little change after CD4$^+$ T-cell depletion (Fig. 4). Similarly, even greater reductions in Env-specific responses were observed in mangabeys 22 and 8 following CD8$^+$ T-cell depletion (Fig. 4). CD4$^+$ T-lymphocyte-mediated ELISPOT responses to Gag, Pol, Env, Rev, and Vpx were detected in 4 of 10 mangabeys; however, these were generally weak (range, 93 to 190 SFC/10^6 input cells) and significantly lower in magnitude than the corresponding CD8$^+$ T-lymphocyte responses (Fig. 4 and data not shown).

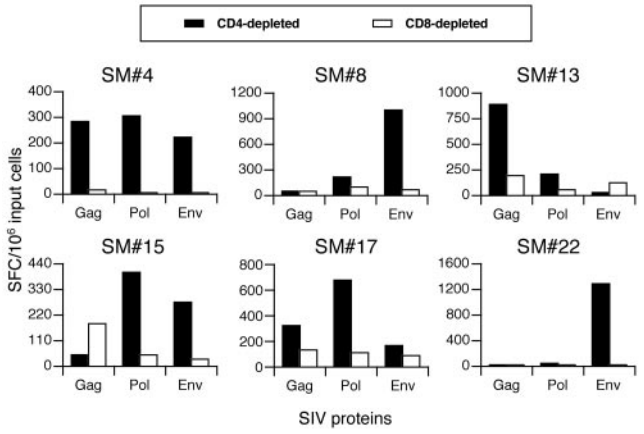

FIG. 4. Contributions of CD4$^+$ and CD8$^+$ T lymphocytes to the SIV specific IFN-γ ELISPOT response in SIV-infected sooty mangabeys (SM). IFN-γ ELISPOT responses with CD4- and CD8-enriched PBMC are shown for six naturally SIV infected SM. Gag, Pol, and Env peptide pools were used for stimulation. Animal numbers correspond to those shown in Fig. 2.

The dominance of CD8$^+$ T-lymphocyte responses to SIV proteins was confirmed by the ICS assay (Fig. 5). A moderate but significant positive correlation was observed between the ELISPOT and ICS assays (ρ, 0.68; *P*, 0.001). Gag-specific responses were detected in 9 of 13 sooty mangabeys, and the frequencies of CD8$^+$ T lymphocytes responding to SIV Gag ranged between 0.11% and 1.7% (Fig. 5B). Env-specific CD8$^+$ T-lymphocyte responses were detected in 10 of 13 sooty mangabeys, and their frequencies ranged between 0.05% and 1.56% (Fig. 5B). The frequencies of the total SIV-specific CD8$^+$ T-lymphocyte response in 13 sooty mangabeys ranged between 0.11% and 3.26% (Fig. 5B). It is noteworthy that the frequency of SIV-specific CD8$^+$ T lymphocytes detected by the ICS assay in sooty mangabeys was comparable to that reported for rhesus macaques 6 weeks after intravenous SIVmac239 inoculation (44).

IFN-γ-secreting SIV-specific CD8$^+$ T lymphocytes are cytolytic and have an effector phenotype. In order to further investigate the functional properties of IFN-γ-secreting SIV-specific CD8$^+$ T lymphocytes in sooty mangabeys, we examined their capacity for cytotoxicity as well as their ability to secrete the CCR5 inhibitor chemokine MIP-1β and other cytokines (TNF-α and IL-2).

Following in vitro stimulation with SIV peptides, strong CD8-mediated SIV-specific CTL activity was observed in four of four

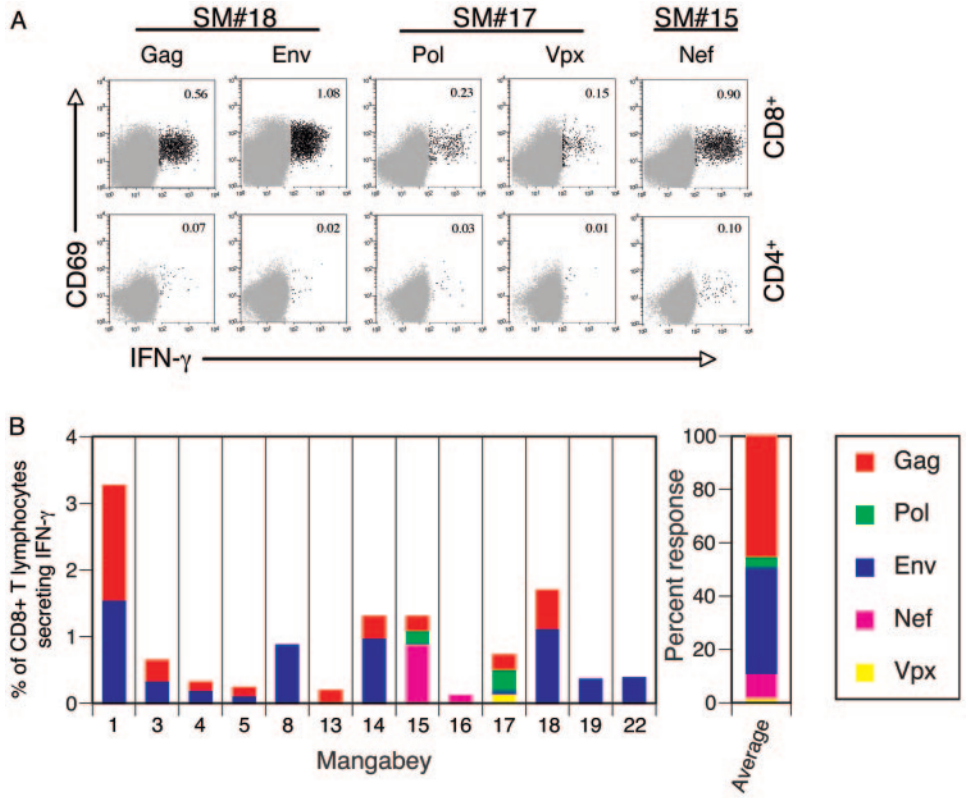

FIG. 5. SIV-specific cellular immune responses are predominantly mediated by $CD8^+$ T lymphocytes. (A) Representative ICS assay using four-color flow cytometry in three naturally SIV-infected sooty mangabeys (SM) showing responses to SIV Gag, Pol, Env, Nef, and Vpx. Animal numbers correspond to those shown in Fig. 2. The ICS assay was performed after 16 h of stimulation with SIV peptide pools in the presence of costimulatory antibodies and brefeldin A. The percentages of $CD3^+$ $CD8^+$ (top) and $CD3^+$ $CD8^-$ ($CD4^+$) (bottom) T lymphocytes coexpressing CD69 and IFN-γ are shown in the upper-right quadrant of each plot after subtraction of background. Background responses were <0.08% for $CD8^+$ and <0.05% for $CD4^+$ T lymphocytes. (B) Frequencies of SIV-specific $CD8^+$ T lymphocytes in 13 naturally SIV-infected sooty mangabeys, as determined by the ICS assay. Colored bars represent the percentages of $CD3^+$ $CD8^+$ cells secreting IFN-γ following stimulation with SIV peptides. Frequencies shown after subtraction of background responses in unstimulated cells. The average percent contribution of each SIV protein to the overall $CD8^+$ T-cell response is shown at right.

naturally SIV-infected mangabeys (Fig. 6A and data not shown). CTL clones targeting SIV Gag and Env were isolated by limiting dilution cloning of $CD8^+$ enriched T lymphocytes in all four animals, and novel CTL epitopes were mapped (Z. Wang and A. Kaur, unpublished data). CTL epitopes presented by high-prevalence major histocompatibility complex class I alleles (Wang and Kaur, unpublished) were recognized ex vivo at low peptide concentrations (1 to 100 ng/ml) in three of four mangabeys (Fig. 6B), suggesting the presence of circulating, high-avidity SIV-specific $CD8^+$ T lymphocytes in naturally infected sooty mangabeys.

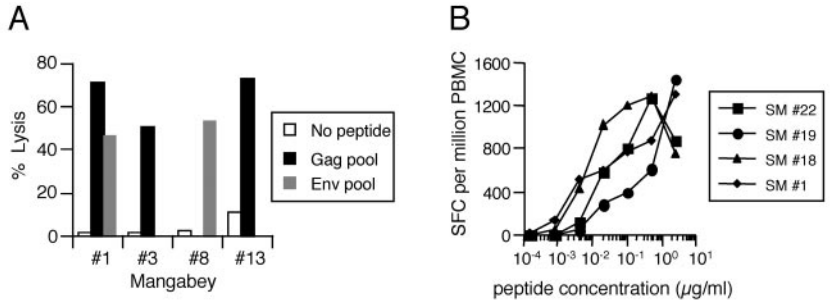

FIG. 6. SIV-specific $CD8^+$ T lymphocytes in naturally SIV-infected sooty mangabeys are cytolytic. (A) Bulk CTL responses after in vitro SIV peptide stimulation are shown for four sooty mangabeys. CTL effectors consisted of >99% $CD8^+$ T lymphocytes (data not shown). Chromium release assays were performed after 14 days of in vitro stimulation. Autologous BLCL unpulsed or pulsed with peptide were used as target cells. Effector/target ratios ranged between 15:1 and 20:1. (B) ELISPOT peptide titration of PBMC from four SIV-infected sooty mangabeys (SM) recognizing a common Env CTL epitope. Animal numbers correspond to those shown in Fig. 2.

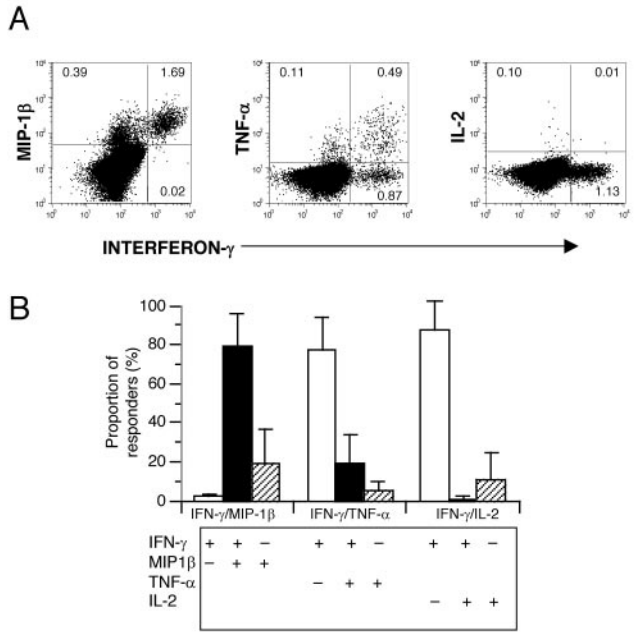

FIG. 7. Functional properties of SIV-specific $CD8^+$ T lymphocytes in sooty mangabeys. (A) Representative data of the ICS assay of PBMC stimulated with the SIV Env peptide pool in one naturally SIV-infected sooty mangabey. The four-color flow cytometric panels consisted of antibodies to CD3, CD8, and IFN-γ combined with either MIP-1β (left dot plot), TNF-α (middle dot plot), or IL-2 (right dot plot). Dot plots show gated $CD3^+$ $CD8^+$ T lymphocytes. The frequencies of SIV-specific IFN-γ-secreting, TNF-α-secreting, and MIP-1β-secreting $CD8^+$ T lymphocytes were determined after subtraction of responses obtained with unstimulated (medium) PBMC. (B) Bar graph showing the proportion of responding SIV-specific $CD8^+$ T lymphocytes exhibiting one or two functions in tubes containing IFN-γ combined with either MIP-1β, TNF-α, or IL-2. Means and SD of seven responses in six animals are shown. The open bars show the proportion of SIV-specific $CD8^+$ T lymphocytes secreting IFN-γ alone in the respective tubes, the closed bars show the double-positive population, and the hatched bars show the proportion secreting MIP-1β, TNF-α, or IL-2 in the absence of IFN-γ.

SIV-specific $CD8^+$ T lymphocytes examined by four-color flow cytometry in six mangabeys (four Gag-specific and three Env-specific responses) displayed heterogeneity in the ability to secrete IFN-γ, TNF-α, IL-2, and MIP-1β (Fig. 7A and B). Less than 50% (range, 0 to 39%) of IFN-γ-secreting SIV-specific $CD8^+$ T lymphocytes secreted TNF-α; however, the majority of TNF-α-secreting SIV-specific $CD8^+$ T lymphocytes also secreted IFN-γ (Fig. 7B). As with human subjects (16), we observed a high background of MIP-1β secretion by $CD8^+$ T lymphocytes in sooty mangabeys, thus rendering quantitation of MIP-1β-secreting SIV-specific $CD8^+$ T lymphocytes less accurate (data not shown). However, >95% of IFN-γ-secreting SIV-specific $CD8^+$ T lymphocytes concurrently secreted MIP-1β (Fig. 7B). After subtracting background responses, MIP-1β-secreting cells that did not secrete IFN-γ constituted 0 to 49% of the $CD8^+$ T cells responding to SIV peptides and were likely SIV-specific $CD8^+$ T lymphocytes (Fig. 7B). IL-2-secreting SIV-specific $CD8^+$ T lymphocytes were detected at very low levels (0.04 to 0.17%), and the majority did not concurrently secrete IFN-γ (Fig. 7B). Thus, the use of IFN-γ as a sole readout for quantitating SIV-specific $CD8^+$ T lymphocytes by ELISPOT or ICS assay may have underestimated the total SIV-specific cellular immune response in naturally infected sooty mangabeys. These data are consistent with the complex heterogeneity of HIV-specific $CD8^+$ T lymphocytes observed in chronic HIV infection (8, 16).

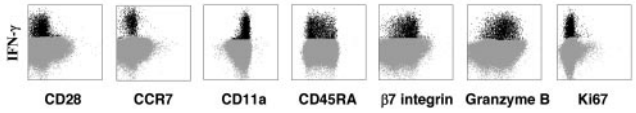

FIG. 8. Phenotypes of SIV-specific IFN-γ-secreting $CD8^+$ T lymphocytes. Representative dot plots of PBMC stimulated with SIV peptides are shown. Dot plots show gated $CD3^+$ $CD8^+$ T lymphocytes. The clusters of events shown in black correspond to IFN-γ-secreting $CD8^+$ T lymphocytes. Phenotypes were determined by four-color flow cytometry. Each panel consisted of antibodies to CD3, CD8, and IFN-γ combined with a single phenotypic marker.

The phenotype of SIV-specific $CD8^+$ T lymphocytes was characterized in seven naturally SIV-infected mangabeys by surface expression of CD11a, CD28, CD45RA, CCR7, and β_7 integrin, markers which have been used to characterize memory cells in rhesus macaques (46). The majority of Gag- or Env-specific IFN-γ-secreting $CD8^+$ T lymphocytes did not express CD28 or CCR7 and were positive for Granzyme B (Fig. 8 and Table 3), suggesting the presence of activated, effector, cytotoxic T lymphocytes. Expression of CD45RA and β_7 integrin on Gag- or Env-specific IFN-γ-secreting $CD8^+$ T lymphocytes was heterogeneous (Table 3). The presence of $CD45RA^-$ and $CD45RA^+$ cells that were $CCR7^-$ suggested that both effector memory and terminally differentiated effectors made up the SIV-specific $CD8^+$ T-lymphocyte population. Similar to the total $CD8^+$ T-lymphocyte population, <5% of the circulating SIV-specific $CD8^+$ T cells expressed the proliferation antigen Ki67 (Fig. 8 and Table 3).

Relationship between SIV-specific cellular immune responses and viral load. To investigate whether there was evidence of inhibition of SIV replication by cellular immunity, we examined the relationship between the magnitude of the SIV-specific IFN-γ ELISPOT response and plasma SIV viral load at different times in 25 naturally SIV-infected mangabeys (total of 85 data points). In view of the repeated measurements of ELISPOT responses and viral load in individual mangabeys, a mixed-effects linear regression analysis was used to evaluate the correlation between the two parameters (34). The plasma viral load ranged between 4.3×10^2 and 1.7×10^6 SIV RNA copies/ml (median, 1.1×10^5 copes/ml). In 23 mangabeys with viral load data at three different time points 2 to 11 months apart, the median variation in SIV RNA was 3.8-fold (range, 1.5-fold to 55.3-fold) and the median coefficient of variation was 0.60 (range, 0.24 to 1.27). The magnitude of the IFN-γ ELISPOT response to individual or all SIV proteins did not correlate with the level of plasma viremia (Fig. 9A). Neither was there a significant correlation between the breadth of the SIV-specific ELISPOT response and the level of plasma SIV viremia (Fig. 9B). With the more sensitive ICS assay, there was a trend toward an inverse correlation between the total frequency of circulating SIV-specific $CD8^+$ T lymphocytes and viral load ($\rho = -0.415$); however, this failed to reach statistical

TABLE 3. Phenotypes of SIV-specific CD8$^+$ T lymphocytes in naturally SIV-infected sooty mangabeys

Mangabey no.	Target specificity	% SIV specific[a]	% SIV-specific CD8$^+$ T lymphocytes[b]													
			CD11a		CD28		CD45RA		CCR7		β_7 Integrin		Ki67		Granzyme B	
			−	+	−	+	−	+	−	+	−	+	−	+	−	+
1	Gag	1.73	0	100	76	24	80	20	95	5	36	64	98	2	5	95
8	Env	1.71	0	100	99	1	51	49	98	2	24	76	99	1	8	92
13	Gag	0.28	8	92	80	20	93	7	99	1	61	39	ND	ND	27	73
14	Gag	0.14	2	98	73	27	ND	ND	100	0	58	42	ND	ND	21	79
14	Env	0.51	2	98	77	23	75	25	100	0	35	65	ND	ND	44	56
15	Nef	0.36	0	100	85	15	72	28	99	1	44	56	ND	ND	ND	ND
18	Env	1.42	0	100	94	6	22	78	98	2	38	62	98	2	4	96
22	Env	1.10	0	100	88	12	59	41	99	1	28	72	ND	ND	29	71
	Mean		2	98	84	16	65	35	98	2	40	60	98	2	20	80
	SD		3	3	9	9	23	23	2	2	13	13	1	1	15	15

[a] CD8$^+$ T lymphocytes.
[b] ND, not done.

significance (Fig. 9C). When the mangabeys were stratified based on viral load, the frequency of circulating SIV-specific CD8$^+$ T lymphocytes in six sooty mangabeys with SIV viremia of $<10^5$ SIV RNA copies/ml (median, 1.09%; range, 0.36 to 3.26%) was higher than in seven sooty mangabeys with SIV viremia of $>10^5$ SIV RNA copies/ml (median, 0.31%; range, 0.11 to 1.69%) (Fig. 9D), and this difference approached statistical significance ($P = 0.06$ [Mann-Whitney U test]).

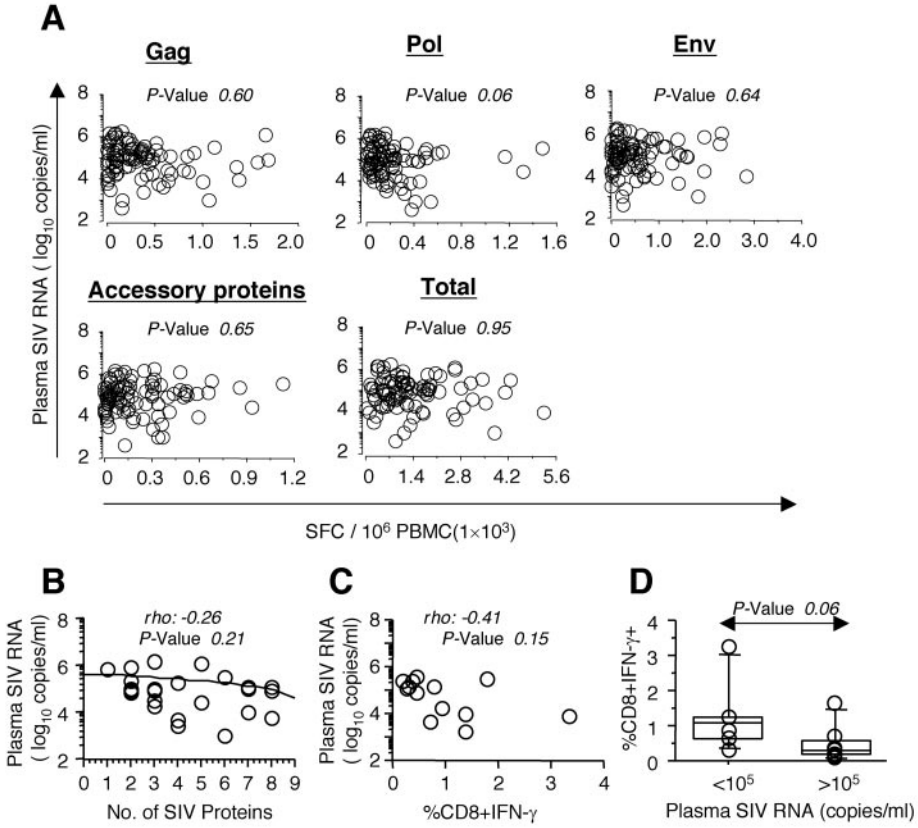

FIG. 9. Relationship between viral load and SIV-specific cellular immunity in naturally SIV-infected sooty mangabeys. (A) SIV-specific IFN-γ ELISPOT responses in 25 mangabeys assayed over a total of 85 time points. The *P* values for these correlations were determined by mixed-effects linear regression for repeated measurements. (B) Correlation between the breadth of SIV-specific ELISPOT responses and viral load in 25 sooty mangabeys. ρ and *P* were determined by the Spearman rank correlation test. (C) Correlation between viral load and frequency of SIV-specific CD8$^+$ T lymphocytes in 13 naturally SIV-infected sooty mangabeys, as determined by the Spearman rank correlation test. Sums of positive CD8$^+$ T-cell responses to different SIV proteins are shown. (D) Box plot comparing the frequencies of SIV-specific CD8$^+$ T lymphocytes in six sooty mangabeys with low-level viremia ($<10^5$ SIV RNA copies/ml) to those in seven mangabeys with high-level viremia ($>10^5$ SIV RNA copies/ml). *P* was determined by the Mann-Whitney U test.

DISCUSSION

Although the precise components of the immune response that confer protective immunity against AIDS remain a subject of intense study, there is a large body of evidence supporting a critical role of $CD8^+$ T lymphocytes in controlling HIV replication in humans (9, 18, 32, 33, 42, 45) and SIV replication in rhesus macaques (24, 48). Since SIV infection in a natural host, the sooty mangabey, is associated with high viral loads, it has been speculated that antiviral $CD8^+$ T lymphocytes are limited or absent in sooty mangabeys and that their presence might actually trigger immunopathology and disease (50). In this study we show that naturally SIV-infected mangabeys mount a Th1-type cellular immune response to SIV comparable in quality and magnitude to that mounted by SIV-infected rhesus macaques. The SIV-specific cellular immune response was broadly directed, with the majority of mangabeys recognizing three to eight SIV proteins. SIV-specific IFN-γ-secreting $CD8^+$ T lymphocytes comprised 0.11% to 3.26% of the circulating $CD8^+$ T-lymphocyte pool and recognized their cognate epitope at low peptide concentrations, suggesting that they were of high affinity. The majority of circulating SIV-specific $CD8^+$ T lymphocytes displayed an activated effector memory phenotype, secreted IFN-γ and MIP-1β, and were cytotoxic. In all, these data highlight the close similarities between the antiviral adaptive cellular immune response of a natural host of lentivirus infection and that of hosts that progress to AIDS. Our data suggest that an attenuated anti-SIV $CD8^+$ T-lymphocyte response is not a feature of natural SIV infection in sooty mangabeys and is thus unlikely to be an underlying mechanism for lack of SIV-related immune activation in this natural host.

This study is the first comprehensive and quantitative analysis of the total cellular immune response to SIV mounted by naturally SIV-infected sooty mangabeys. By using 15-aa peptides overlapping by 11 aa and spanning individual SIV proteins, we were able to evaluate both $CD4^+$ and $CD8^+$ SIV-specific T-cell responses. The cohort of sooty mangabeys ranged in age between 8 and 18 years. Since >80% of captive group-housed sooty mangabeys have acquired SIV infection by the age of 4 to 5 years (21), it is likely that the study animals had been infected for at least 3 years and for as long as 13 years. Despite the long duration of SIV viremia, IFN-γ ELISPOT responses to one or more SIV proteins (median, four proteins) were detected in all 25 animals, and SIV-specific T cells accounted for as much as 3.26% of the circulating $CD8^+$ T-lymphocyte pool. Similar frequencies of IFN-γ-secreting SIV-specific $CD8^+$ T lymphocytes (as high as 2.9%) in rhesus macaques in the first 3 to 4 weeks following pathogenic SIV mac239 infection have been reported (44). No comparable published data are available for the chronic phase of pathogenic SIV infection in rhesus macaques. The impairment of IFN-γ secretion in tetramer-positive SIV-specific $CD8^+$ T lymphocytes in rhesus macaques as early as 4 months after pathogenic SIVmac239 infection (57) suggests that the frequency of IFN-γ-secreting SIV-specific $CD8^+$ T lymphocytes in rhesus macaques with chronic SIVmac239 infection is likely to be substantially lower than that reported for acute SIVmac239 infection. In our study, the total magnitude of SIV-specific IFN-γ ELISPOT responses in 13 SIVmac251-infected rhesus macaques that had been infected for more than 6 months was comparable to that in the naturally SIV-infected sooty mangabeys. These results are especially striking since the peptides used for stimulation were derived from SIVmac239, a virus much more closely related to SIVmac251 than to natural SIVsm isolates, which demonstrate a high degree of genomic diversity and can display as much as 26% nucleotide divergence from SIVmac239 in the *gag* and *env* genes (6, 12).

SIV-specific $CD8^+$ T lymphocytes in sooty mangabeys displayed a complex heterogeneity in the ability to secrete IFN-γ, TNF-α, MIP-1β, and IL-2, which appeared similar to that described in recent studies for HIV-specific $CD8^+$ T lymphocytes in chronic HIV infection (8, 16). Thus, the frequencies of MIP-1β- and IFN-γ-secreting cells exceeded that of TNF-α-secreting cells, and IL-2-secretors comprised a very small population. Although TNF-α secretion was virtually restricted to the IFN-γ-secreting cell subset, there was a substantial population of IFN-γ-secreting cells that did not secrete TNF-α. $CD8^+$ T lymphocytes secreting MIP-1β but not IFN-γ were detected to a variable extent upon SIV peptide stimulation. However, the presence of a high background level of MIP-1β-secreting cells makes it difficult to ascertain the proportion of SIV-specific $CD8^+$ T cells that secrete MIP-1β in the absence of IFN-γ. The functional significance of the heterogeneity in cytokine and chemokine secretion within the SIV-specific $CD8^+$ T-lymphocyte population remains to be determined. A recent report demonstrated that the ex vivo cytotoxic activity of HIV-specific $CD8^+$ T lymphocytes preferentially resides in cells capable of concurrent IFN-γ and TNF-α secretion (37). Although we did not measure ex vivo cytotoxicity, high levels of cytotoxicity were detected following in vitro stimulation, a result consistent with previous observations in SIVmac239-infected mangabeys (29, 31). Moreover, several novel CTL epitopes in SIV Gag, Env, Pol, Nef, Vpr, and Vpx have been mapped (Wang and Kaur, unpublished). Importantly, the optimal CTL epitopes are recognized ex vivo in IFN-γ ELISPOT assays at low peptide concentrations, suggesting the presence of circulating, high-avidity SIV-specific $CD8^+$ T lymphocytes in naturally SIV-infected sooty mangabeys. By flow cytometry, the SIV-specific $CD8^+$ T lymphocytes displayed both an effector memory ($CD45RA^-$ $CCR7^-$) and terminally differentiated effector ($CD45RA^+$ $CCR7^-$) phenotype and expressed high levels of Granzyme B, suggesting the presence of activated effector cytotoxic T lymphocytes. In all, our data demonstrate a substantial population of functionally active, circulating SIV-specific $CD8^+$ T lymphocytes in naturally SIV-infected sooty mangabeys.

On average, more than 70% of the total anti-SIV $CD8^+$ T-lymphocyte response in naturally SIV-infected sooty mangabeys was directed toward the SIV structural proteins Gag, Pol, and Env. Gag and Env were most frequently recognized, followed by Pol, Nef, and Vpx (Table 2). Notably, strong responses toward Env were observed among two-thirds of the Env responders despite the use of peptides corresponding to the SIVmac239 sequence, suggesting that conserved regions of SIV Env were being targeted by the $CD8^+$ T lymphocytes. Compared to that for structural proteins, IFN-γ ELISPOT responses to accessory and regulatory proteins were not as frequently detected. However, when adjusted for the length of the SIV protein, Vpx was the most frequently targeted SIV

protein, followed by Vpr and Nef. A similar dominance of recognition of structural proteins has been observed in chronic, untreated HIV infection (7, 41). Similarly, when adjusted for the length of the protein, Vpr is one of the HIV-1 proteins most frequently targeted by CTL in HIV-infected individuals (1, 2). However, unlike in HIV-1 infection, where Nef is a dominant target of CTL recognition in almost all HIV-infected individuals (7, 20, 36, 41), Nef was recognized in only 56% of the naturally SIV-infected sooty mangabeys. A variety of factors can influence CTL recognition of HIV/SIV proteins, including the HLA profile, the level of expression of viral protein in infected cells, the efficiency of antigen processing, the degree of sequence variation of the viral protein, and the duration of infection (20, 23). Detection of T-cell responses to lentivirus accessory and regulatory proteins that show a great degree of sequence variation can be enhanced by using peptides based on autologous virus sequence (3); thus, it is possible that the relative skewing of recognition toward structural proteins in SIV-infected sooty mangabeys was in part related to the sequence divergence between SIVmac239 and wild-type SIVsm isolates. Another factor may be differences in CTL specificity in acute versus chronic infection. CTL recognition of SIV regulatory and accessory proteins dominates during the acute phase of SIVmac239 infection in rhesus macaques but is focused on Gag later in infection (44).

Previous studies have suggested that the host response in sooty mangabeys may differ from that in species that develop AIDS in being skewed toward a Th2-type cellular immune response. The evidence for this has been twofold. First, cloned T-cell lines established from mitogen-stimulated PBMC from sooty mangabeys displayed a higher frequency of secretion of IL-4, IL-6, and IL-10, but not of IL-2 and IFN-γ, than that of rhesus macaques (5, 56). Second, higher frequencies of IL-4 and lower frequencies of IFN-γ-secreting $CD4^+$ T cells were detected in mitogen-stimulated PBMC from SIV-infected than from SIV-uninfected sooty mangabeys (50). Our study did not detect a Th2 cytokine bias in sooty mangabeys versus rhesus macaques. Neither did we detect significant increases in Th2 cytokine responses (IL-4, IL-5, or IL-13) in SIV-infected sooty mangabeys compared to SIV-negative sooty mangabeys. Using the ELISPOT assay, which allowed evaluation of ex vivo PBMC at the single-cell level, we detected IFN-γ- and Th2-cytokine (IL-4, IL-5, and IL-13)-secreting cells following mitogen and superantigen stimulation in both rhesus macaques and sooty mangabeys. In all instances, the frequency of IFN-γ-secreting PBMC exceeded that of Th2 cytokine-secreting cells by as much as 30-fold. Furthermore, significantly higher frequencies of IL-4 responses were observed in rhesus macaques than in sooty mangabeys following superantigen stimulation. Importantly, Th2 responses to SIV peptides were absent or minimal. Thus, it is unlikely that skewing of the anti-SIV cellular immune response toward a Th2 type is a factor in maintaining nonpathogenic SIV infection in sooty mangabeys.

SIV-specific $CD4^+$ T-lymphocyte responses were detected in only a subset of the naturally SIV-infected sooty mangabeys and were significantly lower in magnitude than were the $CD8^+$ T-lymphocyte responses. A similar CD8 dominance has also been observed in chronic, untreated HIV infection (7, 26). However, these studies demonstrated a higher prevalence of HIV-specific $CD4^+$ T-cell responses than was observed in sooty mangabeys in this study. Data on HIV-specific $CD4^+$ T-lymphocyte responses has been generated with freshly isolated PBMC. Since all the immunologic assays in the current study were performed only after overnight shipment of specimens, it is possible that the SIV-specific $CD4^+$ T-cell responses in sooty mangabeys were underestimated in this study. We have previously shown that detection of cytomegalovirus-specific $CD4^+$ T-lymphocyte responses in rhesus macaques is significantly impacted by a delay in isolating and processing PBMC from whole blood (30).

The relationship between cellular immune responses and viral load in HIV-infected individuals is complex, as illustrated by conflicting results in different studies. Thus, while several studies have shown a positive or no correlation between antigen load and frequencies of anti-HIV $CD8^+$ T lymphocytes (1, 7, 10, 20), other studies have demonstrated an inverse correlation between viral load and frequencies of HIV-specific T-cell responses (18, 25, 45, 54). The discrepancies between the above studies do not negate a role for cellular immunity in the control of pathogenic lentivirus infection. Instead, they point to our incomplete understanding of the components of host immunity critical for protection against AIDS, as well as to the limitations of current immunologic assays for detecting a universal correlate of protective immunity. In this study we did not detect a significant correlation between the magnitude or breadth of the IFN-γ ELISPOT response to the total SIV proteome and plasma SIV viral load. There was a trend toward an inverse correlation between viral load and the frequency of IFN-γ-secreting SIV-specific $CD8^+$ T lymphocytes such that higher frequencies of circulating Gag- or Env-specific $CD8^+$ T lymphocytes were observed in sooty mangabeys with low-level SIV viremia ($<10^5$ SIV RNA copies/ml) than in sooty mangabeys with high-level SIV viremia ($>10^5$ SIV RNA copies/ml); however, this difference did not reach a statistically significant value ($P = 0.06$).

The failure to observe a significant inverse correlation between the magnitude of SIV-specific cellular immune responses and plasma viremia in a cross-sectional analysis does not exclude a role for $CD8^+$ T lymphocytes in immune control of SIV replication in sooty mangabeys. The importance of $CD8^+$ T cells in immune control of pathogenic lentivirus infection is suggested by the temporal relationship between the onset of CTL responses and the decline in primary viremia (9, 33) and, more directly, by the increase in viremia observed in SIV-infected rhesus macaques subjected to in vivo $CD8^+$ T-lymphocyte depletion (24, 48). Studies performed in our laboratory have shown a 2-log or greater increase in plasma SIV viremia in naturally SIV-infected sooty mangabeys following in vivo $CD8^+$ T-lymphocyte depletion (Wang and Kaur, unpublished). Furthermore, contrary to the speculation that sooty mangabeys fail to mount a primary CTL response (49), we have found that SIV-negative sooty mangabeys inoculated with plasma from a naturally SIV-infected sooty mangabey mounted a robust SIV-specific cellular immune response whose onset coincided temporally with a decline in peak SIV viremia (Wang and Kaur, unpublished). In all, these data highlight the similarities between the cellular immune response in pathogenic and nonpathogenic lentivirus infection and suggest that, as with SIV-infected rhesus macaques and HIV-infected humans, $CD8^+$ T lymphocytes are important for control of SIV repli-

cation in sooty mangabeys. Although it appears that $CD8^+$ T lymphocytes inhibit viral replication in vivo in sooty mangabeys, why then is it unable to clear the virus? Is the inhibitory effect of CTLs necessary for maintenance of nonpathogenic infection? Is persistent viremia in sooty mangabeys maintained by CTL escape? These questions are the subject of current research in our laboratory.

In summary, this study has demonstrated that the host response to natural SIV infection in sooty mangabeys is characterized by a broadly directed Th1-type anti-SIV $CD8^+$ T-lymphocyte response in chronic infection. Thus, the mechanism underlying the lack of immunopathology in SIV-infected sooty mangabeys remains to be determined but is not due to an altered SIV-specific cellular immune response.

ACKNOWLEDGMENTS

This work was supported by NIH grant RO1 AI49809 (A.K.) and PHS grant P51RR00168.

We gratefully acknowledge the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, for providing SIV mac239 peptides. We thank Silvija Staprans and David Lee for plasma SIV RNA measurements (supported by Emory CFAR Center grant P30 AI50409); Niklas Ahlborg from MabTech, Sweden, for kindly providing the IL-4, IL-5, and IL-13 ELISPOT kits for testing in sooty mangabeys; Sylvia Janetzki from Zellnet Consulting Inc. for advice on optimization of ELISPOT assays; Jackie Gillis for help with peptide reconstitution; Marie-Claire Gauduin and R. Paul Johnson for providing blood samples from SIV-infected rhesus macaques; and R. Paul Johnson for helpful discussion and critical reading of the manuscript.

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