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Nonpathogenic SIV Infection of Sooty Mangabeys Is Characterized by Limited Bystander Immunopathology Despite Chronic High-Level Viremia

Guido Silvestri,[1,2] Donald L. Sodora,[3] Richard A. Koup,[3,4] Mirko Paiardini,[1,2] Shawn P. O'Neil,[5] Harold M. McClure,[5] Silvija I. Staprans,[1,2] and Mark B. Feinberg[1,2,*]
[1]Departments of Medicine and Microbiology and Immunology and
[2]Emory Vaccine Research Center
Emory University School of Medicine
Atlanta, Georgia
[3]University of Texas-Southwestern Medical Center
Dallas, Texas
[4]NIH Vaccine Research Center
Bethesda, Maryland
[5]Yerkes National Primate Research Center
Emory University
Atlanta, Georgia

Summary

HIV-infected humans and SIV-infected rhesus macaques who remain healthy despite long-term infection exhibit exceptionally low levels of virus replication and active antiviral cellular immune responses. In contrast, sooty mangabey monkeys that represent natural hosts for SIV infection do not develop AIDS despite high levels of virus replication and limited antiviral CD8$^+$ T cell responses. We report here that SIV-infected mangabeys maintain preserved T lymphocyte populations and regenerative capacity and manifest far lower levels of aberrant immune activation and apoptosis than are seen in pathogenic SIV and HIV infections. These data suggest that direct consequences of virus replication alone cannot account for progressive CD4$^+$ T cell depletion leading to AIDS. Rather, attenuated immune activation enables SIV-infected mangabeys to avoid the bystander damage seen in pathogenic infections and protects them from developing AIDS.

Introduction

The AIDS pandemic arose from zoonotic transmission to humans of CD4$^+$ lymphocyte-tropic lentiviruses (termed Simian Immunodeficiency Virus [SIV]) that are prevalent in particular nonhuman primates. Human Immunodeficiency Virus-type 1 (HIV-1) originated from SIVcpz, a natural infection in chimpanzees (*Pan troglodytes troglodytes*), while HIV-2 originated from SIVsm present in sooty mangabey monkeys ([SMs]; *Cercocebus atys*) (Hahn et al., 2000). Importantly, the absence of any AIDS-related disease is a feature common to all known circumstances of primate lentivirus infection in their natural hosts (Hahn et al., 2000; Weiss, 2001). In contrast, transmission of SIVs to nonnatural hosts results in persistent infections associated with progressive loss of CD4$^+$ T cells and profound susceptibility to opportunistic infections. This outcome is commonly observed following HIV infection in humans and SIV infection of rhesus macaques (*Macaca mulatta* [RMs]).

Despite extensive research efforts, several key issues regarding the immunopathogenesis of AIDS have yet to be elucidated. These include the relative pathogenic contribution of "direct" (i.e., the killing of CD4$^+$ T cells that are infected by HIV) versus "indirect" (i.e., not directly caused by HIV or its gene products) mechanisms of immunodeficiency, the importance of accelerated CD4$^+$ T cell destruction versus diminished CD4$^+$ T cell production in determining the progression of CD4$^+$ T lymphocyte depletion following HIV infection, and the mechanisms responsible for increased rates of T cell turnover observed in HIV-infected individuals.

Influential studies demonstrated that the high viremia observed in the pathogenic HIV or SIV infections reflects continuous rounds of virus infection and the ongoing destruction of infected cells (Ho et al., 1995; Wei et al., 1995; Nowak et al., 1997). These observations led to formulation of a dynamic model of AIDS wherein active HIV replication induces CD4$^+$ T cell destruction at such a high rate that host compensatory mechanisms are eventually overwhelmed. This model places direct, virus-induced CD4$^+$ T cell destruction as the central event leading to the numerical and functional collapse of the immune system following HIV infection.

Although the direct cytopathic potential of HIV is well established, indirect mechanisms of T cell depletion have also been proposed as significant contributors to the development of progressive immunodeficiency that follows HIV infection (McCune, 2001; Grossman et al., 2002). Such indirect mechanisms include the loss of uninfected T cells due to increased levels of apoptosis and the impairment of lymphoid regenerative capacity. The apoptotic loss of uninfected T cells involves both CD4$^+$ and CD8$^+$ T cells and is thought to be related to the chronic high levels of generalized immune activation that accompany pathogenic HIV and SIV infections (Ameisen and Capron, 1991; Meyaard et al., 1992; Finkel et al., 1995; Gougeon et al., 1996). Consistent with this notion are the observations that the level of CD8$^+$ T cell activation is as strong a predictor of disease progression in HIV-infected individuals as the level of plasma viremia (Giorgi et al., 1999), and that in both HIV-1 and HIV-2-infections, the extent of CD4$^+$ T cell depletion correlates more closely with levels of immune activation than with viral load (Sousa et al., 2002).

In addition to the increased death rates of CD4$^+$ T cells resulting from both direct and indirect mechanisms, others have proposed that CD4$^+$ T cell decline results primarily from the inability of the infected host to adequately replace CD4$^+$ T cells, rather than from their excessive destruction alone (McCune, 2001). In this alternative model, a key factor is the extent to which the direct or indirect consequences of HIV infection interfere with effective lymphocyte proliferation necessary to compensate for the ongoing T cell destruction in the infected host (Hellerstein et al., 1999; McCune, 2001).

*Correspondence: mbf@sph.emory.edu

Table 1. Age, Age at First Available SIV$^+$ Test, Levels of SIV Plasma Viremia (copies/ml of plasma) and CD4 Counts per mm^3 in SIV-Infected and SIV-Uninfected Sooty Mangabeys

Code	Age (Years)	Age at First SIV$^+$ Test	Viremia	CD4 Count
FCP	6	2	4,479,146	1241
FOO	6	2	534,885	642
FUP	6	2	160,075	1801
FBO	7	2	197,420	819
FDO	7	4	1,961,945	1472
FJO	7	3	366,200	549
FYN	7	3	383,310	472
FEM	8	4	284,645	836
FWK	9	3	3,014,000	859
FFJ	10	5	340,070	315
FQJ	10	4	88,410	936
FBI	12	10	987,500	465
FRH	12	6	1,279,490	726
FDH	13	5	257,831	1242
FNG	13	4	244,805	631
FPH	13	9	N.A.	1023
FQG	13	6	642,435	1145
FEF	15	11	777,695	1265
FAC	22	13	107,075	565
FRV	2	N.A.	neg	2584
FCQ	5	N.A.	neg	1474
FFR	5	N.A.	neg	836
FRO	6	N.A.	neg	678
FSO	6	N.A.	neg	618
FUO	6	N.A.	neg	1620
FLN	7	N.A.	neg	1237
FZN	7	N.A.	neg	1079
FYL	8	N.A.	neg	1375
FFK	10	N.A.	neg	974

Suppression of bone marrow (BM) and thymic function, and damage to peripheral lymphoid tissue architecture, are all likely to be important factors contributing to the ultimate failure of homeostatic mechanisms that maintain lymphocyte populations (Douek et al., 1998; Moses et al., 1998; Haase, 1999). Overall, these observations suggest that AIDS is not simply due to viral factors alone, but rather reflects the outcome of a complex balance between the direct impact of virus infection and the host response to the infection.

To better understand the mechanism of T cell depletion underlying the immunopathogenesis of AIDS, we have focused our attention on the immunological features of nonpathogenic SIV infection in SMs, a natural host species for SIV infection. Although SIV infection is prevalent in SMs living in the wild and in captivity (Fultz et al., 1990), progression to AIDS is not observed despite chronic high levels of viremia (Rey-Cuille et al., 1998; Chakrabarti et al., 2000). To investigate this apparent paradox, we performed a cross-sectional phenotypic and functional analysis of lymphocyte populations in SIV-infected and uninfected SMs. In marked contrast to pathogenic HIV and SIV infections, we found that SIV-infected SMs display limited immune activation and bystander immunopathology and maintain preserved immune regenerative capacity. These results argue for a prominent, if not primary, role of the indirect mechanisms of lymphocyte depletion in the immunopathogenesis of AIDS.

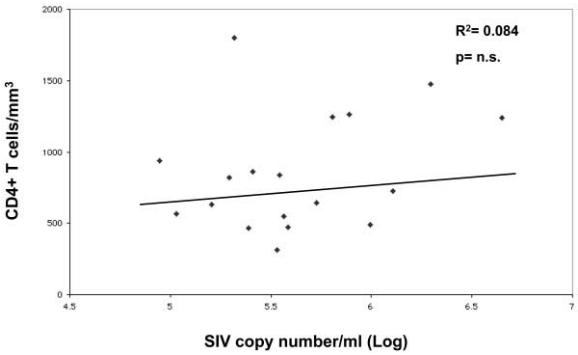

Figure 1. Lack of Correlation between CD4$^+$ T Cell Counts and SIV-Plasma Viremia in 18 Naturally SIV-Infected SMs

Best-fit line is shown.

Results

SIV-Infected SMs Remain Healthy Despite Long-Term, High-Level Virus Replication

To characterize the impact of SIV infection on immune system composition and function in naturally infected SMs, we performed a cross-sectional comparison of 19 SIV-infected and 10 uninfected SMs (Table 1). SIV-positive animals were estimated to have been infected for 3 to 19 years. All animals studied were healthy and free of any signs of immune deficiency, opportunistic infections, neoplasia, wasting syndromes, or neurological diseases.

As in HIV-infected humans and SIV-infected RMs, measurement of levels of viral RNA in the plasma of infected SMs provides a measure of the levels of ongoing virus replication in vivo. Due to the known high-sequence diversity of feral SIVsmm isolates, we developed two real-time RT-PCR assays targeting conserved *gag* or 5′-untranslated region (UTR) sequences to quantitate levels of plasma SIVsmm RNA in naturally infected SMs (S.I.S. et al., unpublished data). The *gag* and the UTR-based assay gave similar results; *gag* PCR results are reported in this study (Table 1).

SIV viral load ranged between 10^5 and 10^7 copies of SIV RNA/ml of plasma (Table 1). These levels are as high or higher than those known to be associated with rapid progression to AIDS in HIV-infected humans and SIV-infected RMs (Mellors et al., 1996; Lifson et al., 1997; Staprans et al., 1999; Watson et al., 1997). Levels of plasma viremia in infected animals did not correlate with age or length of infection. No correlation was found between CD4$^+$ T cell counts and viremia levels in SIV-infected SMs (Figure 1).

Normal Lymphocyte Immunophenotype and Function in SIV-Infected SMs

HIV infection in humans is characterized by a complex set of changes in lymphocyte subpopulations, including progressive diminution of CD4$^+$ T cell numbers, progressive depletion of naive T cells, increased levels of activated CD8$^+$ cells, loss of CD8$^+$ T cells expressing the costimulatory molecule CD28, and increased expression of CD95 by both CD4$^+$ and CD8$^+$ T cells (Choremi-

Table 2. Hematological and Immunophenotypic Analysis of Peripheral Blood- and Lymph Node-Derived Lymphocytes Isolated from SIV-Infected and Uninfected Sooty Mangabeys

	SIV-Pos (19)		SIV-Neg (10)
WBC/cmm	7836 ± 2705	ns	8355 ± 2518
Platelets/cmm	253 ± 88	ns	230 ± 47
Lymphoytes/cmm	4429 ± 1703	ns	4450 ± 1311
CD16+%	6.7 ± 4.4	$p < 0.05$	3.7 ± 2.4
CD20+%	9.8 ± 5.6	ns	11.6 ± 1.9
CD4+/cmm	895 ± 338	ns	1277 ± 602
CD4+%	20.2 ± 5.4	$p < 0.01$	28.5 ± 5.3
CD4+% (LN)	39.9 ± 14.7	ns	45.6 ± 5.5
CD8+/cmm	2151 ± 838	$p < 0.05$	1485 ± 505
CD8+%	49.0 ± 11.5	$p < 0.01$	33.7 ± 7.0
CD8+% (LN)	19.0 ± 4.4	ns	23.8 ± 4.4
CD4/CD8	0.48 ± 0.27	$p < 0.01$	0.87 ± 0.36
CD4+CD25+%	7.8 ± 3.6	ns	7.7 ± 3.6
CD4+DR+%	13.2 ± 9.2	$p < 0.05$	7.7 ± 3.8
CD4+CD69+%	0.5 ± 0.2	ns	0.4 ± 0.1
CD4+CD69+% (LN)	18.0 ± 6.3	ns	15.8 ± 3.4
CD4"naive"%	27.0 ± 14.5	ns	26.1 ± 10.3
CD4"naive"/cmm	268 ± 251	ns	359 ± 311
CD8+DR+%	51.1 ± 12.1	$p < 0.001$	23.4 ± 7.0
CD8+DR+% (LN)	22.9 ± 9.9	$p < 0.05$	13.1 ± 1.0
CD8+CD38+%	26.9 ± 10.0	$p < 0.01$	16.5 ± 5.1
CD8+CD38+% (LN)	5.4 ± 2.0	ns	5.8 ± 3.8
CD8+CD28+%	33.5 ± 9.9	$p < 0.001$	48.7 ± 10.3
CD8+CD28+/cmm	717 ± 342	ns	701 ± 217
CD8+CD28+% (LN)	88.0 ± 7.3	ns	87.0 ± 7.7
CD8+CD69+%	12.2 ± 7.1	ns	11.4 ± 6.0
CD8"naive"%	19.1 ± 10.2	$p < 0.05$	29.6 ± 11.7
CD8"naive"/cmm	416 ± 295	ns	414 ± 160
3-Thy-incorp. (cpm/10^6 cells)	102 ± 34	ns	99 ± 23
CD4+IL-2+	67.7 ± 11.0	ns	72.0 ± 5.5
CD4+IL-4+	8.4 ± 2.5	$p < 0.01$	5.3 ± 1.3
CD4+IL-10+	1.04 ± 1.07	ns	0.47 ± 0.62
CD4+IFNγ+	20.1 ± 5.3	$p < 0.01$	32.9 ± 11.4
CD4+TNF+	62.3 ± 17.2	ns	62.5 ± 9.9
CD8+IL-2+	20.7 ± 8.5	ns	24.7 ± 10.2
CD8+IL-4+	2.2 ± 1.2	ns	1.2 ± 0.4
CD8+IFNγ+	73.9 ± 9.1	ns	67.8 ± 16.4
CD8+TNF+	59.2 ± 15.0	ns	51.3 ± 12.2
CD95 MFI on CD4+	182 ± 59	ns	175 ± 65
CD95 MFI on CD4+ (LN)	84.0 ± 7.8	ns	83.0 ± 7.4
CD95 MFI on CD8+	173 ± 30	ns	154 ± 38
CD4+CD95L+	2.44 ± 1.35	ns	2.74 ± 1.70
CD4+CD95L+	1.57 ± 0.86	ns	2.12 ± 1.38
CD4+Apo2.7+	0.94 ± 1.64	ns	0.54 ± 0.61
CD8+Apo2.7+	0.83 ± 0.95	ns	0.60 ± 0.74
CD4+Ki67+ (PB)	3.12 ± 2.31	$p < 0.01$	1.49 ± 1.13
CD8+Ki67+ (PB)	1.54 ± 1.73	ns	1.53 ± 1.53
CD4+Ki67+ (LN)	1.19 ± 0.30	ns	1.08 ± 0.55
CD8+Ki67+ (LN)	1.28 ± 0.51	ns	1.18 ± 0.61
CD4+Ki67+ (BM)	10.4 ± 5.01	$p < 0.01$	4.80 ± 1.71
CD8+Ki67+ (BM)	5.41 ± 2.10	ns	4.45 ± 1.73

Papadopoulou et al., 1994; Roederer et al., 1995; Gehri et al., 1996; Ullum et al., 1997). Immunophenotypic analyses of SIV-infected and uninfected SMs revealed only relatively minor and statistically insignificant decreases in total $CD4^+$ T cell counts in SIV-infected animals (Table 2). Furthermore, equivalent numbers of naive $CD4^+$ and $CD8^+$ T cells ($CD45RA^+CD62L^+$) were present in SIV-infected and uninfected SMs (Table 2). While SIV-infected SMs showed no increase in levels of $CD4^+$ T cells expressing the activation markers CD25 or HLA-DR, an expansion of the $CD8^+$ T cells coexpressing the activation markers CD38 and HLA-DR (but not CD69) was found in SIV-infected as compared to uninfected SMs. This expansion of activated $CD8^+$ T cells contributes to significant increases in $CD8^+$ T cell percentages and absolute numbers in SIV-infected SMs (Table 2). As a result of the increased total number of $CD8^+$ T cells found in infected animals, the percentage of $CD4^+$ T cells is slightly lower in SIV-infected as compared to uninfected SMs. In contrast to the $CD8^+$ T cell lymphocy-

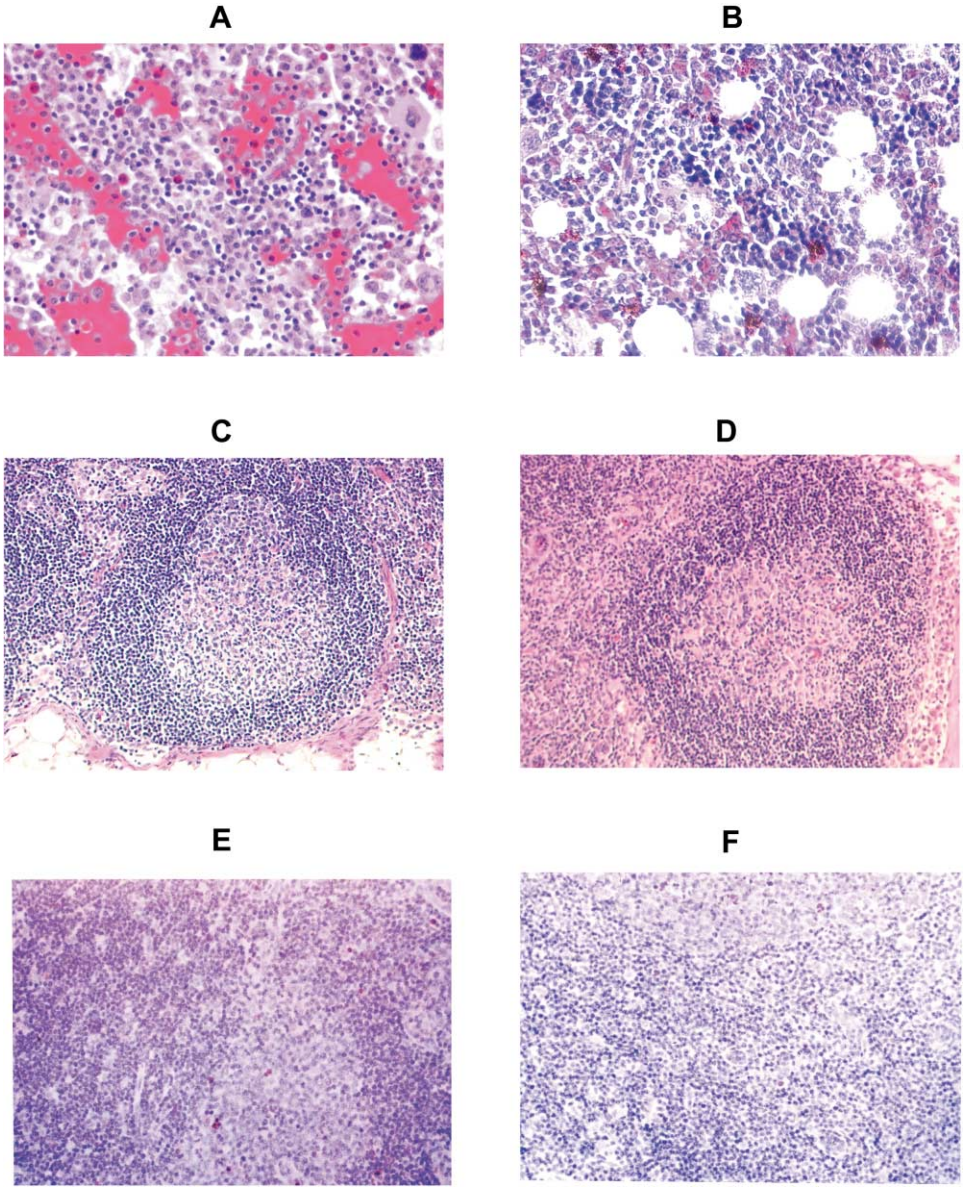

Figure 2. Bone Marrow and Lymph Node Morphology in SIV-Infected and Uninfected SMs

Histological features of a BM biopsy in an SIV-infected SM (HE 40×) (A) and an uninfected SM (B). Histological features of an inguinal LN in an SIV-infected SM (HE staining, 40×) (C) and of an uninfected animal (D). In situ detection of apoptotic cells using the TUNEL assay in a LN section from an SIV-infected SM (E) and an uninfected animal (F).

tosis seen in pathogenic HIV and SIV infections, the $CD8^+$ T cell expansion observed in SIV-infected SMs is not accompanied by a decrease in the number of naive $CD8^+$ cells or in the number of $CD8^+CD28^+$ T cells.

HIV infection leads to a defect in T cell proliferation ex vivo, the magnitude of which is predictive of risk for progression to AIDS (Meyaard et al., 1994). The genesis of this defect is likely to be multifactorial, including enhanced susceptibility to apoptosis, anergy, and decreased proliferative potential. To investigate potential differences in the ability of lymphocytes from SIV-infected and uninfected SMs to undergo proliferation ex vivo, cells were activated in culture with ConA, and T cell proliferation was quantified via incorporation of ^{3}H-thymidine. In contrast to the diminished levels of mitogen-induced proliferation seen in HIV-infected individuals, lymphocytes from SIV-infected SMs showed proliferative responses equivalent to those of uninfected animals (Table 2).

Numerous and somewhat contradictory reports have sought to link HIV infection with specific perturbations in the balance of lymphocytes that undergo differentiation toward either Th1 or Th2 patterns of cytokine production (Clerici and Shearer, 1993; Graziosi et al., 1994). To investigate if SIV infection of SMs is characterized by a specific pattern of cytokine production, we used intracellular staining to assess the production in both $CD4^+$ and $CD8^+$ T cells of IL-2, IL-4, IL-10, IFNγ, and TNF-α,

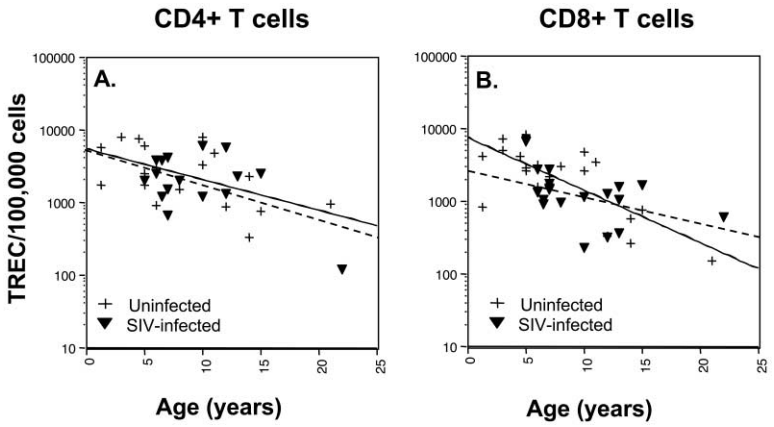

Figure 3. TREC Levels in Sorted CD4$^+$ and CD8$^+$ T Cells from SIV-Infected and Uninfected Infected SMs

Isolated CD4$^+$ (left panel, 17 animals per group) and CD8$^+$ (right panel, 19 animals per group) T cells were assessed for TREC levels. TREC levels are plotted against the age, with best-fit lines shown (solid line for uninfected, dashed line for infected SMs).

induced by brief PMA/A23187 treatment ex vivo (Table 2). CD4$^+$ T cells from SIV-infected animals showed significantly increased IL-4 production and significantly decreased IFNγ production compared to uninfected animals. The same trend was also observed when spontaneous production of IL-4 and IFNγ was studied (data not shown). These results suggest that a preferential differentiation of CD4$^+$ T cells toward a Th2 pattern of cytokine production may follow SIV infection of SMs.

Preservation of BM Function in SIV-infected SMs

HIV infection of humans and SIV infection of RMs are commonly associated with progressively severe suppression of BM function, resulting in neutropenia, thrombocytopenia, and total lymphopenia (Moses et al., 1998). BM suppression may contribute to exhaustion of CD4$^+$ T cell regenerative capacity by limiting the production of prethymic T lymphocyte progenitors, an effect that is ameliorated by antiretroviral therapy (Huang et al., 2000). BM aspirates were obtained from 10 SIV-infected and 8 uninfected animals. No signs of aplasia, dysplasia, hypercellularity, or other BM abnormalities were observed (Figure 2). Absence of measurable BM suppression is consistent with normal lymphocyte, neutrophil, and platelet counts present in the peripheral blood (PB) of infected as compared with uninfected SMs (Table 2).

Preservation of Thymic Function in SIV-Infected SMs

T cell receptor excision circles (TRECs) are episomal DNA circles that are excised during the rearrangement of the T cell receptor genes of maturing T cells within the thymus (Douek et al., 1998; Zhang et al., 1999). TRECs are maintained in resting, naive T cells, but because they are not replicated in the course of cell division, their levels decline with T cell proliferation. In humans, TREC levels decrease with age and following HIV infection (Douek et al., 1998). In both circumstances, decreased levels of TRECs could derive from decreased thymic production of naive cells, or increased proliferative drain on the pool of naive T cells, or both (Hazenberg et al., 2000).

To test whether SIV infection of SMs affects the population dynamics of the naive T lymphocyte pool, we adapted the quantitative-competitive PCR assay developed to quantify TREC levels in humans for use in SMs (Sodora et al., 2000). Since our results (Table 2) and those of others (Fleury et al., 1998; Sachsenberg et al., 1998) indicate that CD4$^+$ and CD8$^+$ T cell populations may be differentially impacted in both nonpathogenic and pathogenic lentivirus infections, we performed the quantitation of TRECs on purified CD4$^+$ and CD8$^+$ T cell subpopulations. As previously reported in humans, TREC levels decrease with age in both uninfected and SIV-infected SMs (Figure 3). Analysis of SIV-infected SMs revealed that the level of TRECs in the CD4$^+$ sorted subpopulation fits within the range of age-matched uninfected animals. Finding normal TRECs in CD4$^+$ T lymphocytes from SIV-infected SMs is consistent with the preservation of the naive CD4$^+$ T cell compartment revealed by flow cytometric analysis. These results indicate that SIV-infected SMs do not experience the decline in TREC levels observed in pathogenic SIV infections of RMs (Sodora et al., 2002). Interestingly, SIV-infected SMs display a slight but significant decrease in the levels of TRECs in CD8$^+$ T cells when compared to uninfected animals ($p < 0.05$). This difference remains significant when analysis of covariance is used to adjust for the normal decline in thymic production seen with increasing age. The observed decrease in TRECs in CD8$^+$ cells is inversely correlated with the level of prevailing immune activation in individual SMs as measured by CD38 or DR expression on CD8$^+$ cells ($p < 0.05$, data not shown), likely reflecting an increased number of CD8$^+$ T cells with a history of prior proliferation.

Preservation of Lymph Node Structure and Function in SIV-Infected SMs

LNs constitute an important anatomic compartment in the pathogenesis of HIV infection by providing primary sites for both virus replication and immunopathology (Haase, 1999). Compromise of proper lymphoid infrastructure needed for peripheral expansion of T cells has been proposed to be an important contributor to the ultimate failure of homeostatic mechanisms acting to maintain lymphocyte population sizes (Haase, 1999). To study the impact of SIV infection on lymphoid tissue structure and function in SMs, LN biopsies were performed in 10 SIV-infected and in 8 uninfected SMs. Histologic analysis of LN architecture revealed similar LN morphology in SIV-infected and uninfected SMs, with the only difference consisting of moderate follicular hyperplasia observed in some of the SIV-infected SMs

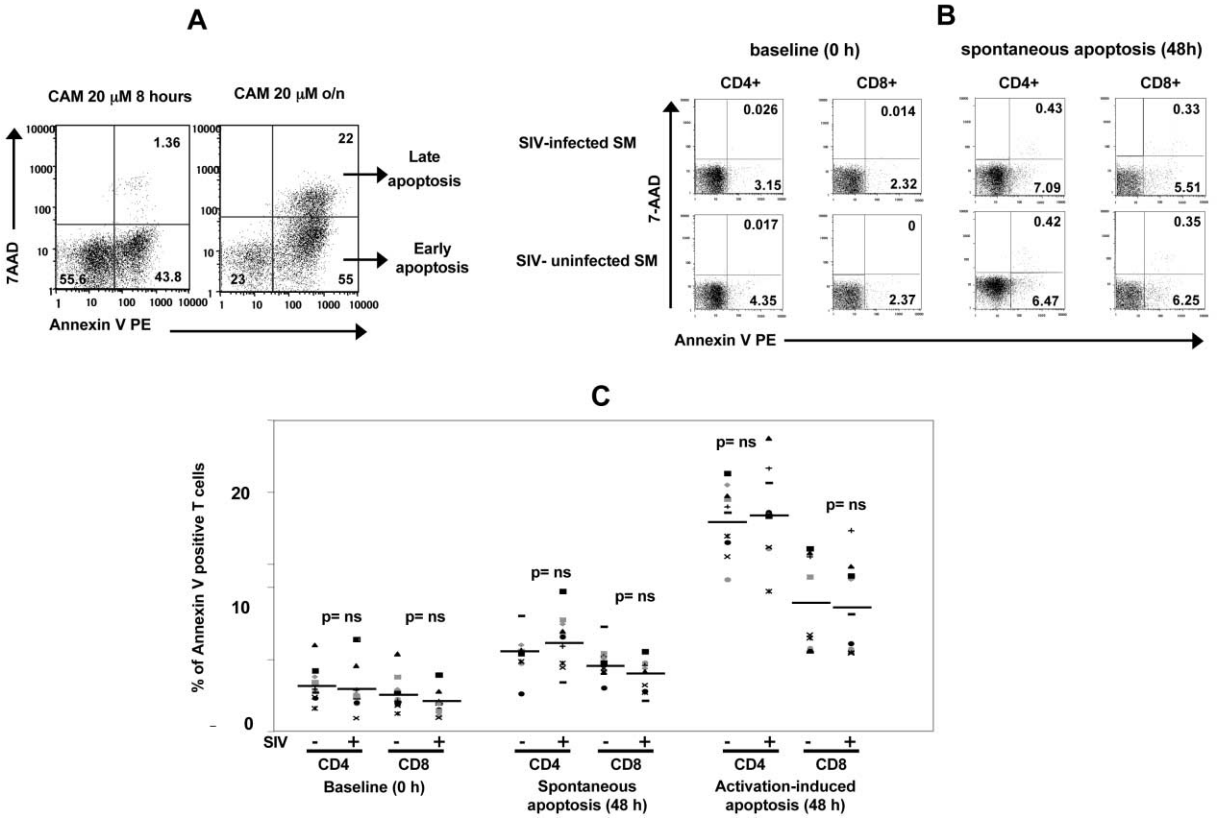

Figure 4. Levels of Baseline, Spontaneous, and Activation Induced-Apoptosis in $CD4^+$ and $CD8^+$ T Cells from SIV-Infected and Uninfected SMs Were Measured Using Annexin V and 7AAD Staining and Flow Cytometric Analysis

(A) The positive staining for Annexin V and 7AAD induced by the proapoptotic agent Campthotecin. (B) The low levels of baseline (time 0) and spontaneous (after 48 hr without any stimulus) in both SIV-infected and uninfected SMs. (C) The lack of statistically significant differences between SIV-infected and uninfected SMs in the rates of baseline, spontaneous, and activation-induced (following 48 hr of ConA stimulation) apoptosis, measured as rates of Annexin V-positive cells, in either $CD4^+$ or $CD8^+$ T cells.

(Figures 2C and 2D). Comparative immunophenotypic analysis of LN-derived lymphocytes (LNLs) and PBMCs from both SIV-infected and uninfected SMs revealed identical patterns, with LNs harboring higher levels of $CD4^+$ T cells, $CD4^+CD69^+$ T cells, and $CD8^+CD28^+$ T cells, and lower levels of $CD8^+CD38^+$ and $CD8^+DR^+$ T cells, as well as $CD4^+$ T cells with lower expression of CD95 than are seen in the peripheral circulation (Table 2). These findings are consistent with normal patterns of lymphocyte distribution seen in healthy humans and indicate that $CD4^+$ and $CD8^+$ T cell compartments are preserved despite longstanding SIV infection in the LNs of SMs. Furthermore, the quiescent LN morphology observed in SIV-infected SMs despite high-level viral replication is consistent with an attenuated host immune response to the infection.

SIV Infection of SMs Does Not Result in Increased Lymphocyte Apoptosis

An exaggerated sensitivity of uninfected lymphocytes to apoptosis is manifest in pathogenic HIV and SIV infections (Meyaard et al., 1992; Finkel et al., 1995; Gougeon et al., 1996; Haase, 1999). As increased levels of lymphocyte apoptosis can contribute to both accelerated loss of $CD4^+$ T cells and impaired lymphocyte regeneration, aberrant apoptosis is believed to be a major contributor to the progressive T cell depletion observed in AIDS. Susceptibility to apoptosis affects both $CD4^+$ and $CD8^+$ T cell populations and is correlated with the overall level of the host immune activation. To investigate whether SIV infection of SMs results in increased levels of lymphocyte apoptosis, we studied the expression of the apoptosis-related markers CD95, CD95L (Fas ligand), and 7A6 (a 38 kDa protein detected by the Apo 2.7 mAb that is expressed during early stages of apoptosis) (Koester et al., 1997) in PBMCs and LNLs. No significant increases were observed in the levels of expression of CD95 and CD95L, or in levels of Apo2.7-reactive $CD4^+$ or $CD8^+$ peripheral blood T cells from SIV-infected as compared to uninfected SMs (Table 2). Similarly, expression of CD95, CD95L, or 7A6 was not increased in either $CD4^+$ or $CD8^+$ LNLs obtained from SIV-infected SMs. In situ analysis using the TUNEL assay disclosed similarly low levels of lymphocyte apoptosis in the LNs of SIV-infected and uninfected SMs (Figures 2E and 2F). We also measured the level of spontaneous and activation-induced apoptosis in ex vivo cultures of PBMCs obtained from SIV-infected and uninfected SMs. The frequency of apoptotic $CD4^+$ and $CD8^+$ T cells was determined by enumeration of cells reactive with Annexin V

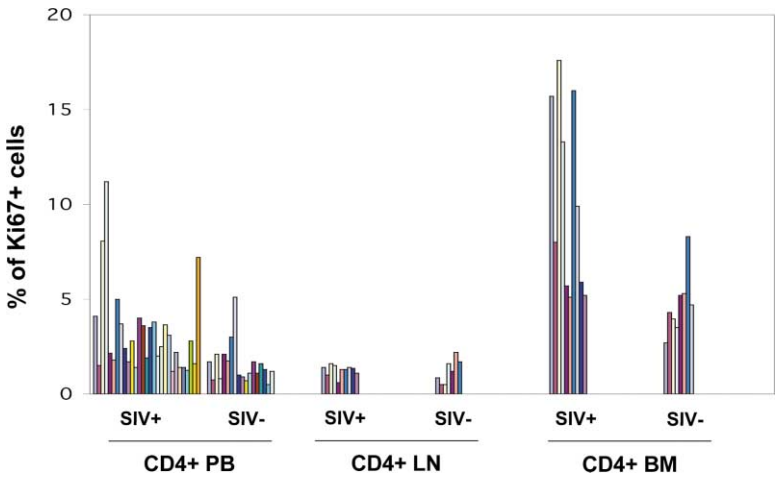

Figure 5. Expression of the Ki67 Proliferation Marker in PB-, LN-, and BM-Derived $CD4^+$ T Cells from SIV-Infected (SIV^+) and Uninfected (SIV^-) SMs

Percentage of proliferating cells are shown with each bar representing an individual animal. The analysis was completed for the following numbers of animals: SIV-infected (29 for PB; 10 for LN and BM); SIV-uninfected (18 for PB, 8 for LN and BM).

and/or 7-AAD in freshly isolated PBMCs and in PBMCs following a 48 hr incubation with either no stimulus or with ConA stimulation (Figure 4). No differences were observed between SIV-infected and uninfected SMs in the levels of baseline, spontaneous, or activation-induced lymphocyte apoptosis in either $CD4^+$ or $CD8^+$ T cells.

Increased Proliferation of $CD4^+$ T Cells in SIV-Infected SMs Is Seen in PB and BM but Not in LNs

Measurement of the frequency of $CD4^+$ and $CD8^+$ T cells that express the nuclear marker Ki67 provides a simple method to assess the rate of proliferating cells in vivo and correlates well with the results of direct measurements using BrdU and 2H-glucose labeling (Mohri et al., 2001; Pitcher et al., 2002). Higher levels of $Ki67^+$ $CD4^+$ T cells have been reported in the LNs and PB of HIV-infected individuals with higher $CD4^+$ T cell count (i.e., >500/cmm), compared to uninfected persons (Sachsenberg et al., 1998; Haase, 1999; Orendi et al., 1998; Hazenberg et al., 2000). In humans with advanced HIV disease, Ki67 expression in $CD4^+$ T cells has been reported to be reduced, suggesting possible failure of compensatory $CD4^+$ T cell proliferation (Fleury et al., 1998). Importantly, the levels of $CD8^+$ T cell, NK cell, and B cell proliferation are also increased in HIV and SIV infections, with the rates of $CD8^+$ T cell proliferation being even higher than that of $CD4^+$ T cells (Fleury et al., 1998; Mohri et al., 2001). The fact that $CD8^+$ T cells are not targets of HIV or SIV infection argues against the suggestion that their increased turnover represents a homeostatic compensatory mechanism and supports the proposal that increased levels of overall T lymphocyte proliferation may primarily reflect the chronic state of immune activation (Hazenberg et al., 2000; Grossman et al., 2002).

To evaluate rates of T cell proliferation in SIV-infected and uninfected SMs, we measured the Ki67 expression in $CD4^+$ and $CD8^+$ T cells from PB, LNs, and BM (Table 2; Figure 5). A modest but statistically significant 2-fold increase was observed in the number of proliferating $CD4^+$ T cells in the blood of SIV-infected SMs ($p < 0.05$). No correlation was found between $CD4^+$ T cell counts and levels of $Ki67^+$ $CD4^+$ T cells in the PB (data not shown). In contrast to pathogenic HIV and SIV infections, no differences were observed between infected and uninfected animals in the level of $CD8^+$ T cell proliferation (Table 2). Interestingly, high levels of $Ki67^+$ $CD4^+$ and $CD8^+$ T cells were found in the BM of uninfected SMs, suggesting that the BM may be an important, but previously unappreciated, site for proliferation of mature T cells (Table 2; Figure 5). In SIV-infected animals, the level of Ki67 expression of BM-derived $CD4^+$ T cells was significantly higher than in uninfected animals ($p < 0.05$). Consistent with the observed quiescent appearance of LN morphology, levels of $CD4^+$ or $CD8^+$ T cell proliferation, as assessed by Ki67 expression, were not increased in the LN in SIV-infected as compared to uninfected SMs (Table 2; Figure 5).

Decreased $CD4^+$ T Cell Levels in SIV-Infected SMs Are Associated with Increased Levels of Immune Activation

We observed significant differences between individual SIV-infected SMs with respect to $CD4^+$ T cell counts, expression of activation markers on $CD8^+$ T cells, and the degree of preservation of the naive T cell compartment. These differences suggest that the extent of host-virus accommodation may differ between individual SIV-infected SMs. As such differences may provide clues concerning the nature of salient determinants of T lymphocyte population homeostasis, we sought to delineate immunological correlates of $CD4^+$ T cell levels in the SIV-infected SMs. The relationships between different immunological parameters were assessed using a series of linear correlation analyses. In SIV-infected SMs, $CD4^+$ T cell counts were found to directly correlate to the percentage of naive $CD4^+$ T cells, the number of naive $CD8^+$ T cells, and the number of $CD8^+CD28^+$ cells (all with $p < 0.01$, data not shown). A significant inverse correlation was observed between $CD4^+$ T cell counts and the percentage of $CD8^+DR^+$ T cells (Figure 6A, $p < 0.01$). Lower $CD4^+$ T cell counts were also observed in association with elevated CD95 expression on $CD4^+$ T cells (Figure 6B, $p < 0.01$). Since increased levels of both activated $CD8^+$ T cells and expression of CD95 on $CD4^+$ T cells were associated with the decreased $CD4^+$ T cell counts, we sought a correlation between markers of $CD8^+$ T cell activation and of the propensity for apo-

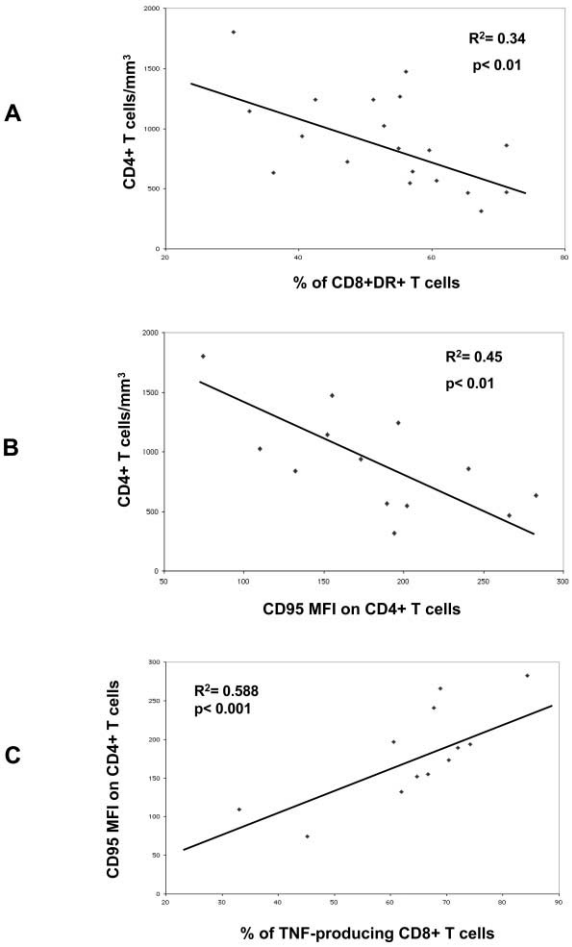

Figure 6. Decreased $CD4^+$ T Cell Counts in SIV-Infected SMs Are Correlated with Increased Levels of T Cell Activation

Inverse correlations between $CD4^+$ T cell counts and frequencies of $CD8^+DR^+$ T cells ([A], 19 animals), $CD4^+$ T cell counts and CD95 expression (MFI) on $CD4^+$ T cells ([B], 13 animals), and a direct correlation between CD95 expression (MFI) on $CD4^+$ T cells and TNF-α production by $CD8^+$ T cells ([C], 13 animals) is seen in SIV-infected SMs. Best-fit lines are shown.

ptosis of $CD4^+$ T cells. A highly significant direct correlation was found between the level of PMA/A23187-induced TNF-α production by $CD8^+$ T cells and the expression of CD95 on $CD4^+$ T cells (Figure 6C, $p < 0.001$). Overall, these analyses indicate that better preservation of $CD4^+$ T cell counts within SIV-infected SMs is associated with preservation of the naive T cell compartment, low levels of $CD8^+$ T cell activation, and a lower propensity for apoptosis of $CD4^+$ T cells.

Discussion

A very intriguing feature of SIV infection in natural host species, such as SMs and African green monkeys, is that $CD4^+$ T cell depletion and AIDS do not arise despite levels of viremia that are as high or higher than those observed in HIV-infected humans and SIV-infected RMs (this study; Rey-Cuille et al., 1998; Chakrabarti et al., 2000; Broussard, et al., 2001). In this and other studies, attenuated antiviral cellular immune responses have also been observed in SIV-infected SMs (Kaur et al., 2000; G.S. et al., unpublished data). Collectively, these observations indicate that SIV-infected SMs avoid immunodeficiency by mechanisms that are opposite to those described in HIV-infected long-term non-progressor (LTNP) humans in whom active cellular immune responses are associated with control of viral replication to exceptionally low levels (Cao et al., 1995; Pantaleo et al., 1995; Rosenberg et al., 1997; Migueles et al., 2002). Several alternative explanations exist for this seemingly paradoxical observation. First, SIV might not be cytopathic for SMs $CD4^+$ T cells (with a consequent absence of the direct loss of $CD4^+$ T cells that is characteristic of HIV infection). Second, SIV-infected SMs may not experience the bystander consequences of a generalized state of immune activation that would otherwise result in both increased apoptotic loss of uninfected T lymphocytes and suppression of compensatory T cell production (with attendant diminished levels of indirect damage). Third, SMs may be better able to replace T cells lost to the direct or indirect consequences of the virus infection than are HIV-infected humans or SIV-infected RMs (with preserved or potentially even enhanced host compensatory responses to $CD4^+$ T cell depletion, accomplished either through increasing the thymic output of naive cells or the proliferation of mature $CD4^+$ T cells).

In this work and in a series of related studies, we have sought to distinguish between these possibilities. To address whether SIV infection is cytopathic for SM $CD4^+$ T cells in vivo, we have measured turnover of virus and virus-infected cells employing the identical methods that have been used in HIV-infected patients and SIV-infected RMs (Ho et al., 1995; Wei et al., 1995; Nowak et al., 1997). These studies indicate that the high-level plasma viremia in SIV-infected SMs is maintained by multiple rounds of de novo infection of $CD4^+$ T cells whose longevity is estimated to be as short as the infected $CD4^+$ T cells present in pathogenic SIV and HIV infections (R.M. Grant and M.B.F., unpublished data). Thus, direct, virus-induced damage alone is likely not sufficient to cause lymphocyte depletion in pathogenic SIV or HIV infections.

To explore the possibility that the lack of immunodeficiency in naturally SIV-infected SMs is related to the absence of immunopathology and preserved lymphoid regenerative capacity, we performed an extensive cross-sectional study of the immunological features in a group of infected and uninfected animals. Consistent with their lack of clinically relevant immunodeficiency, SIV-infected SMs were found to maintain near normal $CD4^+$ T cell counts in both PB and lymphoid tissues despite years of infection. This is in marked contrast to the generalized depletion of $CD4^+$ T cells and the progressive disruption of the normal LN structure and function that accrue during HIV infection of humans and SIV infection of RMs (Haase, 1999).

The failure of lymphoid regenerative capacity has been proposed as an important factor in the pathogenesis of AIDS (McCune, 2001), with BM suppression, reduced thymic output, and loss of naive T cells commonly observed in HIV-infected individuals (Ullum et al., 1997; Douek et al., 1998; Moses et al. 1998; Zhang et al., 1999).

The depletion of naive T cells during HIV infection is consistent with a progressive exhaustion of the regenerative capacity of the immune system, as well as a drain on the naive T cell compartment due to the chronic state of generalized immune activation. Ultimately, the loss of naive cells exacerbates the HIV-induced immunodeficiency by limiting the potential of the host to generate primary immune responses against pathogens. Our results show that the regenerative capacity of the $CD4^+$ T cell compartment is maintained in SIV-infected SMs. In SIV-infected SMs, BM morphology and function are well preserved. Thymic function also appears to be preserved in SIV-infected SMs, who possess levels of TRECs in $CD4^+$ cells similar to those observed in age-matched uninfected animals. This result is in contrast to what has been described in the pathogenic SIV infection of RMs (Sodora et al., 2002) and indicates that thymic function is neither impaired nor augmented in SIV-infected SMs. Further, these results show that normal levels of TRECs can be maintained within the $CD4^+$ T cell pool of SIV-infected SMs, even in the presence of mildly increased rates of $CD4^+$ T cell proliferation, as long as the size of the naive T cell pool is preserved. This conclusion is consistent with the observation that the majority of proliferating $CD4^+$ T cells in SIV-infected SMs are of memory/activated phenotype (data not shown). In all, these results indicate the regenerative capacity of the lymphocyte compartment is not compromised in SIV-infected SMs.

During HIV infection, a heightened level of immune activation is commonly manifest and is associated with an expansion of $CD8^+CD38^+$ and $CD8^+DR^+$ T cells, the magnitude of which predicts rates of progression to AIDS (Giorgi et al., 1999; Sousa et al., 2002). Increased levels of $CD8^+$ T cells expressing these activation markers were also observed in SIV-infected as compared to uninfected SMs. However, in contrast with HIV-infected humans, the increased levels of $CD8^+CD38^+$ and $CD8^+DR^+$ cells present in SIV-infected SMs are not associated with decreased levels of $CD8^+CD28^+$ cells and naive $CD8^+$ T cells.

Aberrant immune activation seen following HIV infection may result in both increased rates of T cell destruction and impaired immune system regenerative capacity (Grossman et al., 2002). The substantially increased susceptibility of uninfected $CD4^+$ and $CD8^+$ T cells to undergo apoptosis is more highly correlated with the overall level of immune activation than with levels of ongoing HIV replication (Muro-Cacho et al., 1995) and is associated with high-level expression of proapoptotic factors such as CD95, CD95L, TNF, and IFNγ (Mitra et al., 1996; Sloand et al., 1996; Badley et al., 2000). We did not find any significant evidence of aberrant generalized immune activation or excessive apoptosis (measured either in vivo or ex vivo) in SIV-infected SMs. The absence of generalized immune activation and bystander immunopathology is likely of fundamental importance in explaining how natural host species avoid immunodeficiency following SIV infection.

The preservation of near normal numbers of $CD4^+$ T cells in a context of high viremia and short life span of infected cells suggests that $CD4^+$ T cells should proliferate at higher rates in order to compensate for the loss mediated by SIV. In agreement with this a priori consideration, the percentage of $CD4^+$ T cells expressing the Ki67 proliferation marker was increased in the PB and BM (but not in the LNs) of the SIV- infected SMs, whereas no significant differences were observed in the $CD8^+$ T cell compartment. The lack of increased $CD8^+$ T cell proliferation in SIV-infected SMs is consistent with the attenuated CD8-mediated anti-SIV immune responses observed in other studies on SMs (see below). The finding of increased $CD4^+$ T cell proliferation in the PB and BM of SIV-infected SMs may reflect either a compensatory $CD4^+$ T cell regeneration or the presence of $CD4^+$ T cells that are responding to SIV as an antigen. Increased rates of $CD4^+$ T cell proliferation were not observed in the lymphocyte population extant in the LNs, the primary anatomic sites where immune responses to systemic antigens are generated, suggesting that homeostatic mechanisms are primarily responsible for the increased rates of $CD4^+$ T cell proliferation observed in SIV-infected SMs. However, the fact that SMs are known to produce anti-SIV antibodies following SIV infection indicates that they do mount anti-SIV $CD4^+$ T helper responses (Fultz et al., 1990). As such, the observed increase in $CD4^+$ T cell proliferation may alternatively reflect an SIV antigen-specific T cell response. If so, it will be important to determine the true magnitude of $CD4^+$ T cell death that results from the direct cytopathic effect of SIV infection, and the physiologic impact of this process above and beyond that arising from normal T cell activation and proliferation.

SIV-infected SMs were found to exhibit substantial interindividual variation in their $CD4^+$ T cell counts. Preservation of higher CD4 levels was found to correlate directly with the degree of conservation of both $CD4^+$ and $CD8^+$ naive cells, and inversely with the levels of activated $CD8^+$ cells and the expression of CD95 on $CD4^+$ T cells. Thus, even in natural host species, increased immune activation can impact the integrity and turnover of T cell populations, but not to the extent that immune function is compromised.

Much attention has focused on the direct cytopathic consequences of HIV infection for $CD4^+$ T cells as the primary force driving T cell depletion in AIDS. However, alternative models have also been presented that posit that the indirect consequences of a maladaptive host immune response to HIV infection, rather than direct cytopathic consequences of the virus, are primarily responsible for the pathogenesis of AIDS (Zinkernagel and Hengartner, 1994). In this model, immunopathology is believed to arise as an unintentional consequence of antiviral CTLs and the proinflammatory cytokines they produce in response to a chronic infection that the immune system is unable to clear. Given that the genetic plasticity of HIV may facilitate escape from host immune responses, the fact that HIV has active mechanisms to evade CTL clearance, and the ability of the virus to infect and kill antiviral $CD4^+$ T helper cells essential for preservation of $CD8^+$ T cell function, it is perhaps not surprising that most HIV-infected persons fail to effectively control virus replication despite the presence of active antiviral $CD8^+$ T cell responses (Walker and Korber, 2001; Douek et al., 2002). Further, as HIV-specific $CD8^+$ T cells may be impaired in their ability to proliferate and to lyse infected targets but still able to produce proinflammatory cytokines (Appay et al., 2000;

Migueles et al., 2002), certain HIV-specific immune responses may actually be better able to mediate immunopathology than to control virus infection.

In summary, our findings indicate that SIV-infected SMs manifest far less of the aberrant generalized immune activation and attendant predisposition to bystander lymphocyte apoptosis than are seen in pathogenic $CD4^+$ T cell-tropic lentivirus infections. While the precise mechanisms by which SMs avoid the indirect consequences of SIV remain to be elucidated, it is notable that SMs fail to mount an active proinflammatory cellular immune response following SIV infection, and limited SIV-specific $CD8^+$ cellular immune responses are found in SMs with established SIV infections (this work; Kaur et al., 2000). Experimental infection of SMs with uncloned SIVsmm results in overall levels of immune activation (especially in the $CD8^+$ T cell population) during primary infection that are considerably lower than those observed when RMs are infected with the identical virus inoculum, despite similarly high levels of virus replication in both species (G.S. et al., unpublished data). That attenuated host cellular immune responses to SIV are evident in SMs from the first days of primary infection suggests that their innate pathogen recognition processes may fail to recognize SIV as a "dangerous" pathogen. As a primary event or as a default pathway, SMs may mount anti-SIV immune responses skewed toward a Th2-like or T regulatory pattern, which may not control viral replication effectively but would also not engender a deleterious proinflammatory state. In the absence of significant indirect immunopathology, SIV-infected SMs are able to maintain rates of peripheral mature $CD4^+$ cell proliferation and levels of production of naive lymphocytes via normal BM and thymic pathways sufficient to compensate for increased rates of destruction of $CD4^+$ T cells arising from the direct cytopathic consequences of virus infection. In contrast, during HIV infection of humans and SIV infection of RMs, the generation of active but incompletely effective host antiviral immune responses leads to the establishment of a proinflammatory milieu wherein deleterious indirect consequences of the host immune response arise. These indirect consequences of the host immune response precipitate accelerated T cell depletion via activation-induced apoptosis, that in conjunction with increased rates of $CD4^+$ T cell destruction resulting from the direct cytopathic consequences of virus infection and impaired T cell regenerative responses underlie the inexorable depletion of $CD4^+$ T cells and progression to AIDS. In the course of the evolution of the natural host species for SIV infection, natural selection appears to have favored the development of attenuated virus-specific host immune responses, wherein the direct consequences of infection may be tolerated as long as the deleterious indirect consequences of host immune responses are not manifest.

Experimental Procedures

Animals

SMs used in this study are housed at the Yerkes National Primate Center and maintained in accordance with NIH guidelines. In uninfected animals, negative SIV PCR of plasma and negative HIV-2 serology confirmed the absence of SIV infection. Based on longitudinal serologic surveys, the majority of infected SMs studied are known to have acquired their SIV infection by 3 to 4 years of age. For analysis of parameters that may be affected by age, including recent thymic emigrants and others, age-matched animals were used.

Determination of Plasma Viral RNA

Plasma viral RNA levels were quantitated using real-time PCR as described (Kaur et al., 2001). SIV RNA copy number is determined by comparison to an external standard curve consisting of virion-derived SIVmac239 RNA previously quantified by the SIV bDNA method (P. Dailey, personal communication).

Hematological Studies and Cell Separation

WBC, platelet, and total lymphocyte counts were measured using standard methods. PBMCs were isolated by density gradient centrifugation. LNLs were isolated from biopsied LNs. BMMCs were isolated from BM aspirates via density gradient centrifugation.

Flow Cytometry for Cell Surface Markers

Staining was performed with the following monoclonal antibodies (all shown, in preliminary studies, to crossreact with SM cells): αCD20, αCD25, αCD45RA, αCD69, and αHLA-DR (FITC); αCD2, αCD62L, αCD95 (PE); αCD4 and αCD8 (PE and PerCP, all from Becton Dickinson, San Jose, CA), FITC-labeled α-monkey CD3 (Biosource Int, Camarillo, CA); αCD4 and αCD38 FITC (Ortho Diagnostic System, Raritan, NJ); αCD16 and αCD28 FITC, αCD95L biotin (Coulter, Miami, FL). PE-labeled Apo2.7 MoAb (Coulter) was used to detect the apoptotic cells in vivo. FITC-labeled αKi67MAb was used to detect actively proliferating cells (Coulter). Samples used for Apo2.7 and Ki67 staining were surface stained for CD4 and CD8, then fixed and permeabilized using the Pharmingen CytoFix/Perm-Kit, and stained intracellularly with the proper MAbs and controls. Flow cytometric acquisition and analysis of samples was performed on at least 10,000 acquired events on a FACScaliber flow cytometer driven by the CellQuest software package (Becton Dickinson).

Detection of DNA Fragmentation In Situ

Apoptotic cells were identified within paraffin sections of LNs by TUNEL staining according to manufacturer's instructions (Boehringer Mannheim).

Studies of Lymphocyte Apoptosis In Vitro

The level of spontaneous and activation-induced apoptosis was determined in freshly isolated PBMCs (baseline), and after a 48 hr incubation either with no stimulus (spontaneous apoptosis) or with ConA (activation-induced apoptosis). Rates of apoptotic cells were determined by multicolor flow cytometry in both $CD4^+$ and $CD8^+$ T cells as percentage of cells reactive to Annexin V alone (early apoptosis) or annexin V and 7-AAD (late apoptosis) (all reagents from Becton Dickinson).

Detection of Signal Joint TRECs in Lymphocytes

PBMCs were separated into $CD4^+$ and $CD8^+$ T cell populations using a FACS Vantage cell sorter (Becton Dickinson). Purity of the sorted subpopulations was greater than $>95\%$. The number of signal joint TRECs in purified lymphocyte subpopulations were quantitated as described in (Sodora et al., 2000, 2002).

Intracellular Cytokine Staining

Cytokine production by PBMCs was assessed by flow cytometry. MAbs against human IL-2, IL-4, IL-10, IFNγ, and TNF (Pharmingen) were shown to be crossreactive with SMs cytokines. PBMCs were incubated for 5 hr in medium containing PMA (10 ng/ml), A23187 (200 ng/ml), and either monensin (10 nM) or brefeldin A (5 nM) (Sigma). Cells were first surface stained with anti-CD4 and CD8 Mabs, fixed and permeabilized using the CytoFix/CytoPerm Kit (Pharmingen), and finally stained intracellularly with the proper MAbs and controls

Statistical Analysis

A two tailed, two-sample Student's T test was used to calculate P values for differences in means between SIV infected and uninfected

SMs. To evaluate associations between lymphocyte immunophenotypic parameters, Pearson's correlations were calculated. Since age was negatively correlated with levels of TRECs, we used analysis of covariance (ANCOVA) to adjust for the age effect. Assumptions involving ANCOVA were checked and found to be satisfied.

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