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Correlates of Preserved CD4⁺ T Cell Homeostasis during Natural, Nonpathogenic Simian Immunodeficiency Virus Infection of Sooty Mangabeys: Implications for AIDS Pathogenesis

Beth Sumpter,* **Richard Dunham,*†** **Shari Gordon,*†** **Jessica Engram,†** **Margaret Hennessy,‡** **Audrey Kinter,‡** **Mirko Paiardini,†** **Barbara Cervasi,†** **Nichole Klatt,*†** **Harold McClure,§** **Jeffrey M. Milush,¶** **Silvija Staprans,*§** **Donald L. Sodora,¶** **and Guido Silvestri[1]*†§**

In contrast to HIV-infected humans, naturally SIV-infected sooty mangabeys (SMs) very rarely progress to AIDS. Although the mechanisms underlying this disease resistance are unknown, a consistent feature of natural SIV infection is the absence of the generalized immune activation associated with HIV infection. To define the correlates of preserved CD4⁺ T cell counts in SMs, we conducted a cross-sectional immunological study of 110 naturally SIV-infected SMs. The nonpathogenic nature of the infection was confirmed by an average CD4⁺ T cell count of $1,076 \pm 589/mm^3$ despite chronic infection with a highly replicating virus. No correlation was found between CD4⁺ T cell counts and either age (used as a surrogate marker for length of infection) or viremia. The strongest correlates of preserved CD4⁺ T cell counts were a low percentage of circulating effector T cells (CD28⁻CD95⁺ and/or IL-7R/CD127⁻) and a high percentage of CD4⁺CD25⁺ T cells. These findings support the hypothesis that the level of immune activation is a key determinant of CD4⁺ T cell counts in SIV-infected SMs. Interestingly, we identified 14 animals with CD4⁺ T cell counts of $<500/mm^3$, of which two show severe and persistent CD4⁺ T cell depletion ($<50/mm^3$). Thus, significant CD4⁺ T cell depletion does occasionally follow SIV infection of SMs even in the context of generally low levels of immune activation, lending support to the hypothesis of multifactorial control of CD4⁺ T cell homeostasis in this model of infection. The absence of AIDS in these "CD4^{low}" naturally SIV-infected SMs defines a protective role of the reduced immune activation even in the context of a significant CD4⁺ T cell depletion. *The Journal of Immunology,* 2007, 178: 1680–1691.

I t is now well established that the AIDS pandemic originated from zoonotic transmission to humans of simian CD4⁺ T cell-tropic lentiviruses that infect African monkey species and are collectively defined as simian immunodeficiency viruses or SIV (1, 2). In marked contrast to HIV-infected humans who, if untreated, almost invariably develop progressive CD4⁺ T cell depletion and AIDS, the African monkey species naturally infected with SIV are generally spared from any signs of disease (reviewed in Refs. 3–5). Importantly, experimental SIV infection of nonnatural host Asian monkey species, such as rhesus macaques (*Macaca mulatta*), causes symptoms similar to those described in AIDS patients (simian AIDS) (reviewed in Ref. 6). At present, it is still unclear why SIV infection is relatively nonpathogenic in natural host monkey species but induces immunodeficiency in nonnatural

or recent hosts (including humans). A better understanding of the mechanisms underlying the lack of disease in natural hosts for SIV infection will likely provide important clues as to the pathogenesis of AIDS in HIV-infected individuals (7, 8).

Among the natural hosts for SIV infection, the sooty mangabeys (SMs)[2] (*Cercocebus atys*) are particularly relevant for two reasons. First, SIVsmm, the virus infecting SMs, is the origin of the HIV-2 epidemic in humans (1). Second, SIVsmm was used to generate the various rhesus macaque-adapted viruses (SIVmac; e.g., SIVmac239 and SIVmac251) that are commonly used for studies of AIDS pathogenesis and vaccines (reviewed in Ref. 6). SIV-infected SMs typically maintain normal CD4⁺ T cell counts and do not develop AIDS despite intensive virus replication, with levels of plasma viremia that are as high, or even higher, than those observed in HIV-infected individuals (9–11). At present, there is only one report of AIDS in a naturally SIV-infected SM (12). In all, these observations are in marked contrast with the known pathogenicity of both the HIV infection of humans and the experimental SIV infection of RMs, where high levels of virus replication are a strong predictor of more rapid disease progression (13–16).

In previous studies conducted in a smaller subset of animals, we found that natural SIV infection of SMs is characterized by near-normal CD4⁺ T cell counts in both blood and lymph nodes

*Department of Medicine and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322; †Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; ‡Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; §Yerkes National Primate Research Center of Emory University, Atlanta, GA 30329; and ¶Department of Medicine, University of Texas Southwestern, Dallas, TX 75390

[1] Address correspondence and reprint requests to Dr. Guido Silvestri, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 705 Stellar-Chance Laboratories, 422 Curie Boulevard, Philadelphia, PA 19104. E-mail address: gsilvest@mail.med.upenn.edu

[2] Abbreviations used in this paper: SM, sooty mangabey; BAL, bronchioalveolar lavage; IEL, intraepithelial lymphocyte; LN, lymph node; LPL, lamina propria lymphocyte; RB, rectal biopsy; SIVmac, SIV infecting rhesus macaques; SIVsmm, SIV infecting SMs; T$_E$, naïve T cell; T$_M$, memory T cell; T$_N$, naive T cell; Treg, regulatory T.

(LNs), preserved bone marrow and thymic function, and the absence of the generalized immune activation and bystander T cell apoptosis that are typical of HIV-infected individuals (11). Similarly, no signs of disease progression and limited immune activation were observed in SMs experimentally infected with uncloned SIVsmm (17). Taken together, these results led us to hypothesize that the attenuated immune activation in SIV-infected SMs is a mechanism that favors the preservation of $CD4^+$ T cell homeostasis and, thus, the nonpathogenic state of this chronic infection.

In this report, we present the results of a comprehensive virological and immunological cross-sectional study of the vast majority (i.e., 110 animals) of naturally SIV-infected SMs hosted at the Yerkes National Primate Research Center (Atlanta, GA). This study is, to our knowledge, the largest of its kind ever performed on a natural SIV host species, thus giving us the unprecedented opportunity to establish the correlates of preserved $CD4^+$ T cell counts seen during natural, nonpathogenic SIV infection. We found that, in naturally SIV-infected SMs, high $CD4^+$ T cell counts best correlate with low levels of effector T cells (i.e., $CD28^-$ $CD95^-$ and/or $CD127^-$) and high levels of $CD4^+CD25^+$ regulatory T (Treg) cells. Importantly, we also discovered and characterized a subset of SIV-infected SMs with decreased $CD4^+$ T cell counts, including two animals with $CD4^+$ T cell counts of $<50/$ mm^3. The absence of AIDS in these "$CD4^{low}$" SIV-infected SMs underlines the protective role of reduced immune activation in maintaining normal immune system function even in the context of significant $CD4^+$ T cell depletion.

Materials and Methods

Animals

Thirty uninfected SMs and 110 naturally SIV-infected SMs were used in this study; at least one 30-ml blood sample was collected from all animals. All SMs were housed at the Yerkes National Primate Research Center and maintained in accordance with National Institutes of Health 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 SIV-infected SMs are known to have acquired their infection by 3 to 4 years of age (18).

Determination of plasma viral RNA

For SIVsmm RNA quantitation, RNA was extracted and reverse transcribed as described (17). Real-time PCR was performed by amplification of 20 μl of cDNA in a 50-μl reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 0.2 μM forward primer, 0.3 μM reverse primer, 0.1 μM probe, and 5 U of AmpliTaq Gold DNA polymerase (reagents from Applied Biosystems). Primer and probe sequences were targeted to the 5′ untranslated region of the SIVsmm genome; forward primer sequence was 5′-GGCAGGAAAATCCCTAGCAG-3′, the reverse primer sequence was 5′-GCCCTTACTGCCTTCACTCA-3′, and the probe sequence was 5′-FAM-AGTCCCTGTTCRGGCGCCAA-TAMRA. Amplicon accumulation was monitored with an ABI PRISM 7700 sequence detection system (Applied Biosystems) with the cycling conditions of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 93°C for 30s and 59.5°C for 1 min. RNA copy number was determined by comparison to an external standard curve consisting of in vitro transcripts representing bases 216–2106 of the SIVmac239 genome.

Lymphocyte studies and flow cytometry

Four- to seven-color flow cytometric analysis was performed in whole blood samples according to standard procedures using a panel of mAbs that were originally designed to detect human molecules but that we and others have shown to be cross-reactive with SMs (11). The Abs used were as follows: anti-CD4-PE, -allophycocyanin, or -PerCP-Cy5.5 (clone SK3); anti-CD8-allophycocyanin or -PacBlue (clone SK1); anti-CD25-PE or PE-Cy7 (clone 2A3); and anti-CD28-PE or PE-Cy7 (clone L293) (all from BD Biosciences); Ki67-FITC (clone B56); anti-CD3-PE or Alexa700 (clone SP34-2), anti-CD69-CyChrome (clone FN50); anti-CD95-CyChrome (clone DX2); and anti-HLA-DR-CyChrome (clone G46-6) (all from BD Pharmingen); anti-Foxp3 (clone PCH-101) (eBioscience); and anti-CD127-PE (clone R34.34) (Beckman Coulter). Samples assessed for Ki67

were surface stained first with the appropriate Abs, then fixed and permeabilized using the CytoFix/CytoPerm kit (BD Pharmingen), and stained intracellularly with Ki67. Flow cytometric acquisition and analysis of samples was performed on at least 100,000 events on a FACSCaliber (four-color) or a LSR-II (seven-color) flow cytometer driven by either the CellQuest or the DiVa software package, respectively (BD Biosciences). Analysis of the acquired data was performed using Flow Jo software (Tree Star).

Cytokine production

SM PBMCs were isolated using density gradient centrifugation according to standard procedures and resuspended to 1×10^6 cells/ml in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 1.7 mM sodium glutamate). Cells were then incubated for 6 h at 37°C in medium containing *Staphylococcus* enterotoxin B (1 mg/ml final concentration, Sigma-Aldrich) or PMA plus A23187 (positive controls), medium alone (negative control), or SIV peptides in the presence of brefeldin A (Sigma-Aldrich). Following incubation, the cells were washed once in PBS containing 1% BSA and 0.1% sodium azide and then surface stained with directly conjugated Abs against CD3, CD4, CD8, CD28, and CD95 for 20 min in the dark at room temperature followed by fixation and permeabilization using the CytoFix/CytoPerm Kit (BD Pharmingen). After permeabilization, the cells were washed twice in the supplied kit buffer and then stained intracellularly with anti-human IFN-γ-allophycocyanin (clone B27), anti-human TNF-α-PE (clone MAB11), and anti-IL-2-FITC (clone MQI-17H12) Abs (all from BD Biosciences) for 20 min in the dark at room temperature. All used Abs were previously shown to be cross-reactive with SM molecules (11). Following staining, the cells were washed a final time and then fixed in PBS containing 1% paraformaldehyde. The fixed cells were stored at 4°C until the time of FACS analysis. In all experiments at least 100,000 T cells were acquired.

Depletion of $CD4^+CD25^+$ T cells and proliferative response to nominal Ags and mitogens

SM PBMCs were isolated using density centrifugation according to standard procedures. $CD4^+$ T cells were obtained by negative selection using a mixture of goat anti-mouse Dynal beads coated with anti-human CD8, CD20, CD56/14 (BD Pharmingen), and anti-NKG2A (Zin199; a gift of D. Mavilio, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Bead-to-target cell ratios and methods were executed as per the manufacturer's recommendation. $CD25^-$ and $CD25^+$ subsets of PBMC or $CD4^+$ T cells were obtained by incubating cells with anti-CD25 PE mAb (BD Pharmingen) followed by anti-PE microbeads (Miltenyi Biotec) as per the manufacturer's recommendation. APCs (i.e., $CD2^-CD25^-$ PBMCs) were obtained from PBMCs depleted of $CD2^+$ cells using anti-human CD2-coated beads (Dynal) along with anti-CD25PE/anti-PE beads. $CD2^-$ $CD25^-$ PBMCs were gamma irradiated at 5,000 rad before use. All subpopulations were stained and analyzed for purity using a BD Biosciences FACSCalibur. For the lymphoproliferative assay, unfractionated PBMCs, total $CD4^+$ T cells, $CD25^-$, and $CD25^+CD4^+$ T cells were resuspended in RPMI 1640 (containing 1 mM HEPES buffer, penicillin/streptavidin antibiotic, and L-glutamine) plus 10% human sera and plated in 96-well round-bottom plates at 2×10^5 cells/well. All wells containing purified $CD4^+$ T cell subsets also received irradiated APCs at 2×10^5/well. Cells were left unstimulated or stimulated with 5 μg/ml SIV p27 (Applied Biosystems) or *Candida* (Greer Laboratories). On day 7 poststimulation cells were pulsed (16 h) with 1μCi/well [^{3}H]thymidine and harvested on glass fiber filters. Net cpm was determined by subtracting the cpm of unstimulated cells from the cpm of p27- or *Candida*-stimulated cells.

In vitro T cell apoptosis

The level of spontaneous and activation-induced apoptosis in peripheral blood lymphocytes was determined in freshly isolated PBMCs (baseline) and after a 48-h incubation either with no stimulus (spontaneous apoptosis) or with ConA (activation-induced apoptosis). The rate of apoptotic cells was determined by multicolor flow cytometry in both $CD4^+$ and $CD8^+$ T cells as the percentage of cells reactive to annexin V.

LN biopsy, rectal biopsy (RB), and bronchoalveolar lavage (BAL)

For a LN biopsy the animals were anesthetized with ketamine or Telazol and the skin over the axillary or inguinal region was clipped and surgically prepared. An incision was made over the LN, which was exposed by blunt dissection and excised over clamps. For RBs, fecal material was removed from the rectum by finger manipulation; a rectal scope/sigmoidoscope was

FIGURE 1. CD4+ T cell counts in naturally SIV-infected SMs. *A*, Comparison of CD4+ T cell counts (cells/mm³) in naturally SIV-infected and uninfected SMs (*p* value as determined by the Mann-Whitney *U* test). *B*, Lack of correlation between CD4+ T cell counts (cells/mm³) and ages of the animals (years) in the same cohort of 110 SIV-infected SMs. For *B*, p = ns indicates that the *p* value of the correlation coefficient was <0.05 (Spearman rank correlation test).
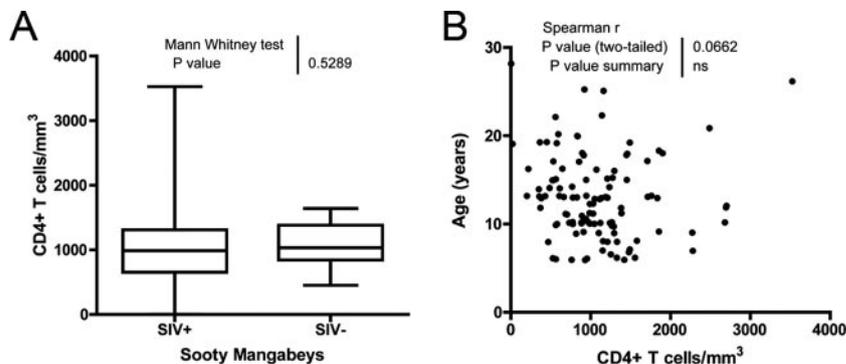
then placed a short distance into the rectum. RBs were obtained with biopsy forceps and placed in tissue culture fluid for immunophenotypical studies. For BAL, a fiber optic bronchoscope was placed into the trachea after local anesthetic was applied to the larynx. The scope was directed into the right primary bronchus and wedged into a distal subsegmental bronchus. Up to four 35-ml aliquots of warmed normal saline were instilled into the chosen bronchus through the bronchoscope. The saline was collected by aspiration between each individual lavage before a new aliquot was instilled. Immunophenotypical studies were performed by multicolor flow cytometry (as detailed above) on mononuclear cells derived from LN biopsy, RB, and BAL isolated by gradient centrifugation. In the case of RB, to separate intraepithelial lymphocytes (IELs) from lamina propria lymphocytes (LPLs), HBSS containing 5 mM EDTA solution was added to the mucosal tissue and incubated with gentle shaking for 1 h. The supernatant, which contains IELs, was then collected while the remaining tissue was digested to obtain LPLs (two sequential 30-min incubations at 37°C in RPMI 1640 containing 0.75 mg/ml collagenase). The digested cell suspension was passed through 70-µm cell strainers to remove residual tissue fragments. In all cases, cells were stained the day of sampling and cell suspensions were kept on ice between each incubation so that no changes in cell surface expression or apoptosis occurred after tissues were harvested.

Statistical analysis

Correlation analyses within the same group of animals for two different parameters were performed using the Spearman's rank correlation test with $\alpha = 0.05$. The Mann-Whitney *U* test (two-tailed; $\alpha = 0.05$) was used to analyze the difference between the median percentage of specific immunological markers between SIV-infected and uninfected SMs. Statistics were performed using GraphPad Prism 4.0b (GraphPad Software) for Macintosh.

Results

Natural SIV infection of SMs is usually associated with normal CD4+ T cell count despite high levels of viral replication

We conducted a cross-sectional study of 110 naturally SIV-infected SMs hosted at the Yerkes National Primate Research Center of Emory University (Atlanta, GA). This group of 110 SMs represents the vast majority (>90%) of the animals housed at the center. The overall lack of pathogenicity of this infection was confirmed by the observation that all animals were asymptomatic with generally well-preserved CD4+ T cell counts (average of 1,076 ± 589 cells/mm³) despite high levels of viral replication (average viral load of 170,000 ± 205,000 copies/ml). As shown in Fig. 1*A*, the average CD4+ T cell count in SIV-infected SMs was only slightly decreased compared with that in uninfected animals (median of 987 ± 589 for SIV-infected, and 1034 ± 329 for uninfected SMs). As naturally SIV-infected animals are older than the uninfected SMs (data not shown), it is possible that the modest decline of CD4+ T cells observed in the SIV-infected animals is related to their age rather than being a consequence of SIV infection. Interestingly, we observed significant variability in the CD4+ T cell counts between different individual animals, with 14 of 110 naturally SIV-infected SMs (12.7%) possessing a CD4+ T cell

count of <500 cells/mm³. Two of these 14 animals had a CD4+ T cell count of <50 cells/mm³. This finding indicates that the typically nonpathogenic natural SIV infection of SMs can be associated, in a minority of animals, with moderate-to-severe CD4+ T cell depletion and is consistent with the results of another study where two of six SMs experimentally infected with uncloned SIVsmm developed significant CD4+ T cell depletion within two years of infection (J. M. Milush, J. D. Reeves, S. Gordon, D. Zhou, A. Muthukumar, D. A. Kosub, E. Chacko, L. Giavedoni, C. C. Ibegbu, K. S. Cole et al., manuscript in preparation). Further details on these "CD4low" naturally SIV-infected SMs are provided in the last subheading of this section (*Results*).

In naturally SIV-infected SMs, CD4+ T cell counts are not correlated with the length of infection or the level of plasma viremia

We next sought to determine whether, during natural SIV infection of SMs, there is a tendency to develop CD4+ T cell depletion over time. As natural SIV infection occurs most often at the time of sexual maturity, i.e., 3–5 years (17), age may be used as a surrogate marker for length of infection (which was estimated, in this cohort of animals, to be an average of 10 years). Based on this assumption, we examined the relationship between the age of the animals and their CD4+ T cell counts and found only a nonsignificant ($p = 0.06$) trend toward an inverse correlation between these two parameters (Fig. 1*B*). As CD4+ T cell counts tend to decline with age even in the uninfected animals (data not shown), we concluded that it is unlikely that the CD4+ T cell count in naturally SIV-infected SMs simply reflects the duration of the infection. Consistent with our previous observation in a smaller group of animals (11), we found no association between CD4+ T cell count and viral load (data not shown), confirming that the level of virus replication is unlikely to be the key determinant of CD4+ T cell counts in this nonpathogenic model of infection. In addition, in a separate study (19) we also have found a lack of correlation between CD4+ T cell counts and the magnitude or breadth of the SIV-specific T cell response mediated by either CD4+ or CD8+ T cells. Taken together, these results indicate that, in naturally SIV-infected SMs, there is not a significant association between length of infection, viral replication, or level of SIV-specific T cell responses and CD4+ T cell counts.

Characterization of the subsets of naive, central memory, and effector memory T cells in SMs

We have shown previously that a trend toward lower CD4+ T cell counts was associated with increased expression of markers of immune activation in naturally SIV-infected SMs (11). To better define the relationship between CD4+ T cell counts and immune

activation during natural SIV infection of SMs, we sought to determine whether, in these animals, there is a correlation between CD4$^+$ T cell counts and the relative proportions of naive (T$_N$), memory (T$_M$), and effector (T$_E$) subsets of CD4$^+$ and CD8$^+$ T cells. To this end, we first performed a series of preliminary experiments to phenotypically define the functional subsets of T$_N$, T$_M$, and T$_E$ CD4$^+$ and CD8$^+$ T cells in SMs using seven-color flow cytometric analysis and adopting the principles and techniques that have been used to define the same T cell subsets in rhesus macaques (20). To the best of our knowledge, this is the first report of such a characterization in SMs. We used various combinations of phenotypic markers, including CD28, CD95, CD27, CD45RA, CD62L, CCR7, CD69, HLA-DR, Ki67, and CD127. The naive SM T cells (functionally defined here as T cells producing IL-2 but not IFN-γ or TNF in response to *Staphylococcus* enterotoxin B or PMA/ionomycin) were found to be comprised of the CD28$^+$CD95$^-$ population and expressed CCR7, CD62L, CD27, and CD127 but did not express CD69, Ki67, HLA-DR, or granzyme B (Fig. 2*A* and data not shown). Consistent with their naive phenotype, these cells were found in blood and LNs but not in the MALT (i.e., from RB and BAL; Fig. 2*B*). The fraction of these naive T cells declined with age in both SIV-infected and uninfected SMs (data not shown). The IFN-γ- and/or TNF-producing "nonnaive" T cells belong to either the CD28$^+$CD95$^+$ or the CD28$^-$CD95$^+$ subsets (Fig. 2*A*). The CD28$^+$CD95$^+$ subset includes a significant fraction of cells that express CCR7, CD127, CD27, and CD62L (data not shown); these cells produce IL-2 as well as IFN-γ and TNF (Fig. 2*A* and data not shown), are common in both peripheral blood and LNs, but less common in the MALT (Fig. 2*B*). Based on an immunophenotypic, functional, and anatomic analogy with human and rhesus macaque T cells, we propose that this CD28$^+$CD95$^+$ subset represents true T$_M$. The CD28$^-$CD95$^+$ subset includes cells that do not express CCR7, CD27, or CD127, do produce IFN-γ and TNF, but, differently from T$_M$ cells, do not produce IL-2 (Fig. 2*A* and data not shown). Importantly, these cells are highly represented in the MALT, are found particularly among the IELs rather than among LPLs (Fig. 2*C*), and likely represent true T$_E$. Interestingly, while circulating CD8$^+$ T cells include a large fraction of T$_E$ cells in naturally SIV-infected SMs, only a small percentage (typically <5%) of circulating CD4$^+$ T cells displays the T$_E$ phenotype (Fig. 3*A*). A summary of the relative proportions of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T$_N$, T$_M$, and T$_M$ in lymphocytes isolated from peripheral blood, LN biopsy, RB, and BAL of 13 SIV-uninfected SMs is shown in Fig. 2*D*.

In all, these studies indicate that in SMs, as in RMs, the subsets of T$_N$, T$_M$, and T$_E$ CD4$^+$ and CD8$^+$ T cells can be defined using the CD28/CD95 pattern of staining. Specifically, T$_N$ cells are defined as CD28$^+$CD95$^-$, T$_M$ cells are defined as CD28$^+$CD95$^+$, and T$_E$ cells are defined as CD28$^-$CD95$^+$.

Natural SIV infection of SMs is associated with an absolute expansion of effector CD8$^+$ T cells

We next sought to determine whether natural SIV infection of SMs is associated with specific changes in the relative proportions of circulating CD4$^+$ and CD8$^+$ T$_N$, T$_M$, and T$_E$ cells. As shown in Fig. 3*A*, we compared the percentages of these three T cell subsets in our cohorts of 110 SIV-infected and 30 uninfected animals and found that SIV infection of SMs is associated with a decline in the fraction of CD4$^+$ and CD8$^+$ T$_N$ cells, an increase in the fraction of CD4$^+$ T$_M$ cells, and an increase in the fraction of CD8$^+$ T$_E$ cells. We then compared, in SIV-infected vs uninfected SMs, the absolute count of these T cell subsets and found that SIV-infected SMs are characterized by an expansion of CD8$^+$ T$_E$ cells (Fig. 3*B*). We did not find a significant difference in the absolute number

of circulating T$_N$ and/or T$_M$ cells per mm^3 when comparing the infected and uninfected animals (Fig. 3*B*).

In a previous study (21) we determined that HIV infection is associated with an expansion of CD8$^+$CD127$^-$ T cells expressing phenotypical and functional features of effector cells. In this study we find that natural SIV infection of SMs is also characterized by an expansion (both in terms of the percentage of CD8$^+$ T cells and the absolute number of circulating cells) of CD8$^+$CD127$^-$ T effector-like cells (Fig. 3, *C* and *D*), although the magnitude of the expansion is less prominent that what we have observed in HIV-infected individuals. Interestingly, a relative (but not absolute) expansion of CD4$^+$CD127$^-$ T cells was also observed in SIV-infected SMs compared with uninfected SMs (Fig. 3*D*).

In SIV-infected SMs there is a significant inverse correlation between the level of CD4$^+$ T cells and the percentage of either CD4$^+$ or CD8$^+$ T$_E$ cells

We next sought to determine whether, in SIV-infected SMs, the levels of circulating CD4$^+$ T cells are correlated with the fraction of T$_N$, T$_M$, or T$_E$ CD4$^+$ and/or CD8$^+$ T cells. To this end, we performed a series of regression analyses and found a strong significant inverse correlation between the percentage of CD4$^+$CD28$^-$CD95$^+$ or CD8$^+$CD28$^-$CD95$^+$ T$_E$ cells and the percentage of CD4$^+$ T cells (Fig. 4, *A* and *B*). In addition, we found a strong significant inverse correlation between the percentage of CD4$^+$CD127$^-$ or CD8$^+$CD127$^-$ T$_E$-like cells (Fig. 4, *C* and *D*) and the percentage of CD4$^+$ T cells. This latter finding is of interest as an inverse correlation between CD8$^+$CD127$^-$ T cells and CD4$^+$ T cells was also found in HIV-infected individuals (21).

As expected, given the reciprocal changes in the proportion of T$_N$ and T$_E$ cells shown in Fig. 3 we also found that the percentage of circulating CD4$^+$ T cells was directly correlated with the fraction of CD4$^+$ or CD8$^+$ T$_N$ cells (Fig. 4, *E* and *F*). Interestingly, while the percentage of CD4$^+$ T cells was also inversely correlated with the absolute number of CD4$^+$ or CD8$^+$ T$_E$ cells, no association with the absolute number of T$_N$ cells, either CD4$^+$ or CD8$^+$, was observed (data not shown). In all, these findings indicate that the lower levels of circulating T$_E$ cells are the strongest correlate of preserved CD4$^+$ T cell homeostasis during natural SIV infection of SMs.

In SIV-infected SMs there is significant direct correlation between CD4$^+$ T cell count and the percentage of CD4$^+$CD25$^+$ Treg-like cells

We hypothesized that the state of attenuated immune activation observed in naturally SIV-infected SMs, when compared with that in HIV-infected humans, could at least in part be determined and/or maintained by an immunological mechanism involving Treg cells, particularly those included in the CD4$^+$CD25$^+$ T cell subset. Although expression of CD25 follows in vitro activation of CD4$^+$ T cells, the majority of resting CD4$^+$CD25$^+$ T cells found in vivo in human subjects are considered to be Treg cells (22–24). Similar to what has been described in humans, CD4$^+$CD25$^+$ T cells in SMs comprise the vast majority of Foxp3-expressing cells (Fig. 5*A*), and in vitro depletion of CD4$^+$CD25$^+$ T cells is associated with increased proliferative responses to nominal Ags and mitogens (Fig. 5*B* and data not shown). When we measured the fraction of CD4$^+$CD25$^+$ T cells in the peripheral blood of 110 SIV-infected and 30 SIV-uninfected SMs, we found that SIV-infected SMs have significantly higher fractions of CD4$^+$CD25$^+$ T cells (17.56 ± 12.19% of CD4$^+$ T cells) than uninfected SMs (11.52 ± 4.63% of CD4$^+$ T cells) ($p < 0.0001$) (Fig. 5*C*).
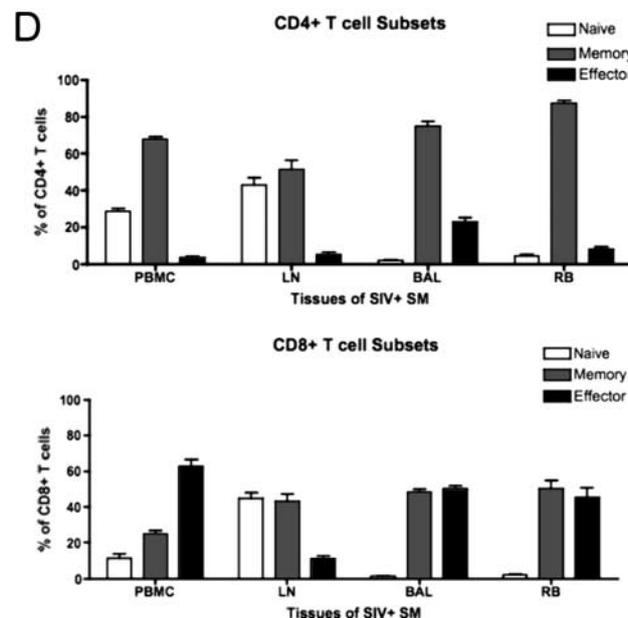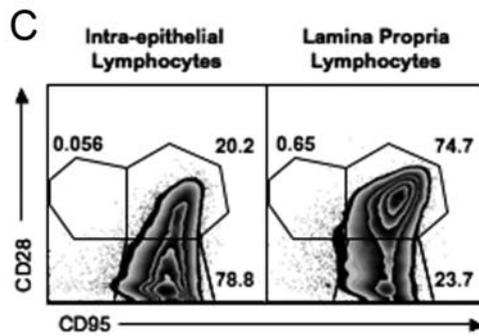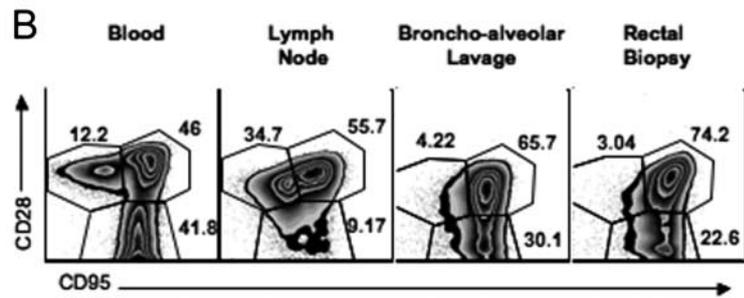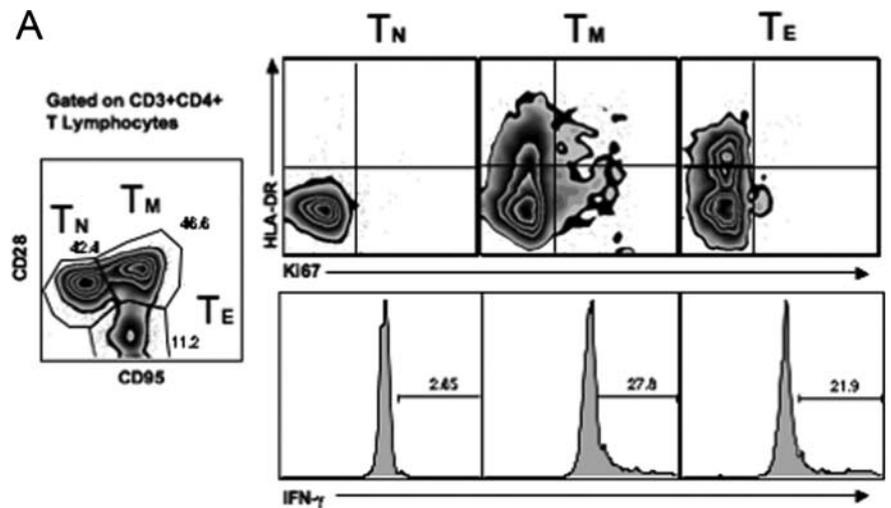
FIGURE 2. Classification of the subsets of naive, memory, and effector T cells in SMs. *A*, Representative dot plot showing CD28 and CD95 staining (gated on CD3+CD4+ T cells) in an SIV-infected SM. The *right panels* include dot plots showing the expression of Ki67 and HLA-DR in the three subsets of CD4+ T_N, T_M, and T_E cells (*top*) and histograms showing the production of IFN-γ by the same cell subsets (*bottom*). *B*, Representative dot plots indicating the relative proportion of CD3+ T_N, T_M, and T_E cells in peripheral blood, LNs, BAL, and RB in an SIV-infected SM. *C*, Representative dot plots indicating the relative proportion of CD3+ T_N, T_M, and T_E cells in lymphocytes isolated from the same rectal biopsy and separated into IELs and LPLs. *D*, Summary of the relative proportion of CD3+CD4+ and CD3+CD8+ T_N, T_M, and T_E cells in lymphocytes isolated from peripheral blood, LNs, RB, and BAL of 13 SIV-uninfected SMs.
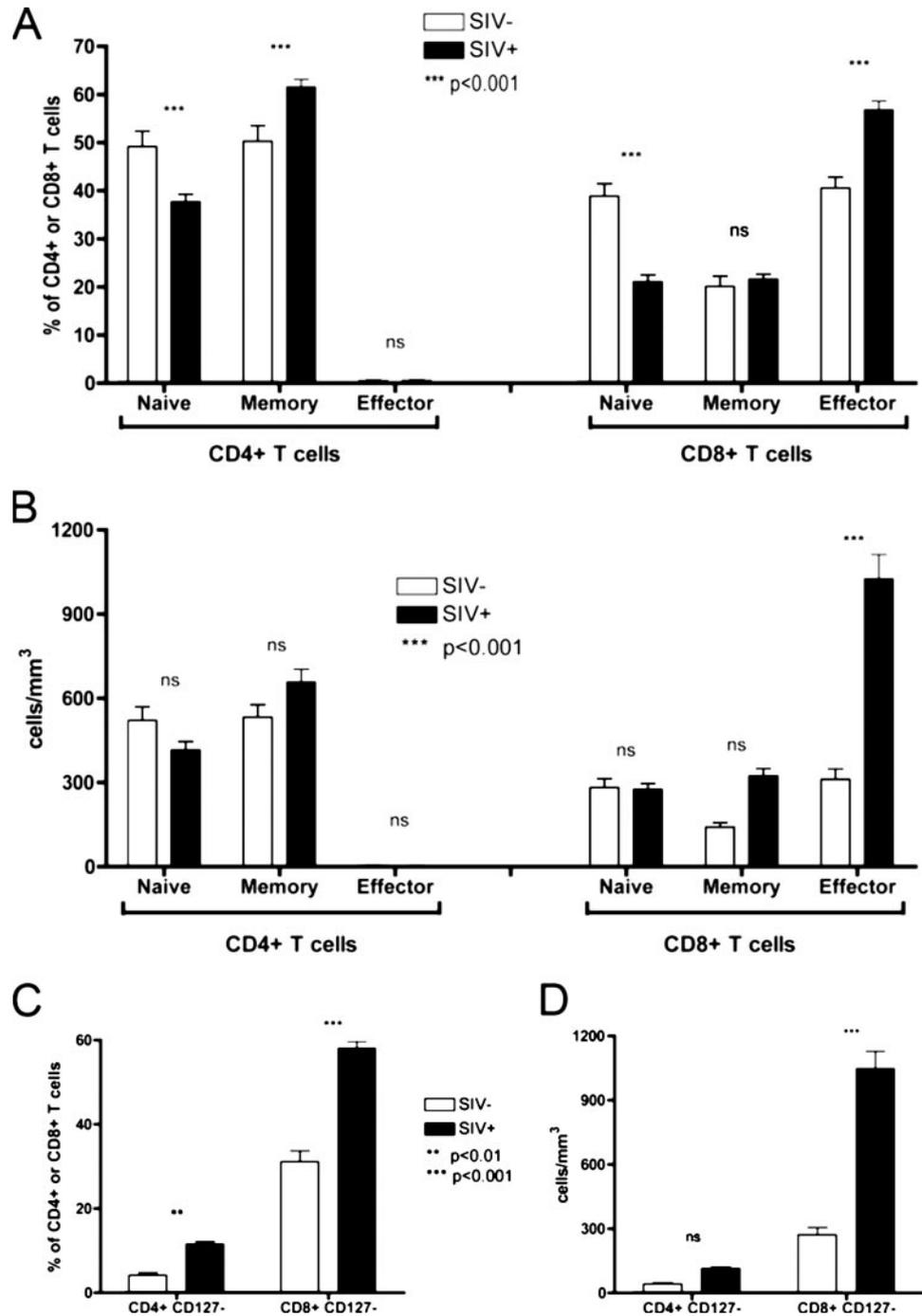
FIGURE 3. Natural SIV infection of SMs is associated with expansion of T_E cells that do not express CD28 or CD127. *A*, Comparison of the relative proportion of $CD3^+CD4^+$ (*left*) and $CD3^+CD8^+$ (*right*) T_N, T_M, and T_E cells in naturally SIV-infected and uninfected SMs. *B*, Comparison of the absolute number (cells/mm^3) of $CD3^+CD4^+$ (*left*) and $CD3^+CD8^+$ (*right*) T_N, T_M, and T_E cells in naturally SIV-infected and uninfected SMs. *C*, Comparison of the relative proportion of $CD3^+$ $CD4^+CD127^-$ (*left*) and $CD3^+CD8^+$ $CD127^-$ (*right*) T cells in naturally SIV-infected and uninfected SMs. *D*, Comparison of the absolute number (cells/mm^3) of $CD3^+CD4^+$ $CD127^-$ (*left*) and $CD3^+CD8^+$ $CD127^-$ (*right*) T cells in naturally SIV-infected and uninfected SMs (**, $p < 0.01$; ***, $p < 0.001$; as determined by the Mann-Whitney U test).

To next investigate whether a potential relationship may exist between the CD4$^+$ T cell count and the percentage of circulating CD4$^+$CD25$^+$ Treg-like cells, we performed a linear regression analysis using the 110 SIV-infected SMs in our cohort. As shown in Fig. 5D, we found a strong direct correlation between CD4$^+$ T cell count and the percentage of CD4$^+$CD25$^+$ T cells, suggesting that Treg-like cells may play a role in maintaining the CD4$^+$ T cell homeostasis in SIV-infected SMs, perhaps by reducing the level of T cell activation. Interestingly, the percentage of CD4$^+$CD25$^+$ T cells correlated inversely with the level of circulating CD4$^+$ and CD8$^+$ T$_E$ cells (i.e., CD28$^-$CD95$^+$ and/or CD127$^-$ cells; data not shown). In all, these data indicate that the state of attenuated T cell activation typical of SIV-infected SMs that likely is central to the preservation of CD4$^+$ T cell homeostasis may be, at least in part,

related to the presence of increased circulating CD4$^+$CD25$^+$ Treg-like cells.

In SIV-infected SMs there is no correlation between CD4$^+$ T cell count and the percentage of proliferating T cells

In HIV-infected individuals the fraction of proliferating T cells is consistently increased, and this increase is directly correlated with the level of plasma viremia and inversely correlated to CD4$^+$ T cell counts (25, 26). This increased level of T cell proliferation is thought to be a reflection of the generalized immune activation that is associated with HIV infection rather than representing a homeostatic mechanism aimed at compensating for the loss of T cells induced by the virus (27).
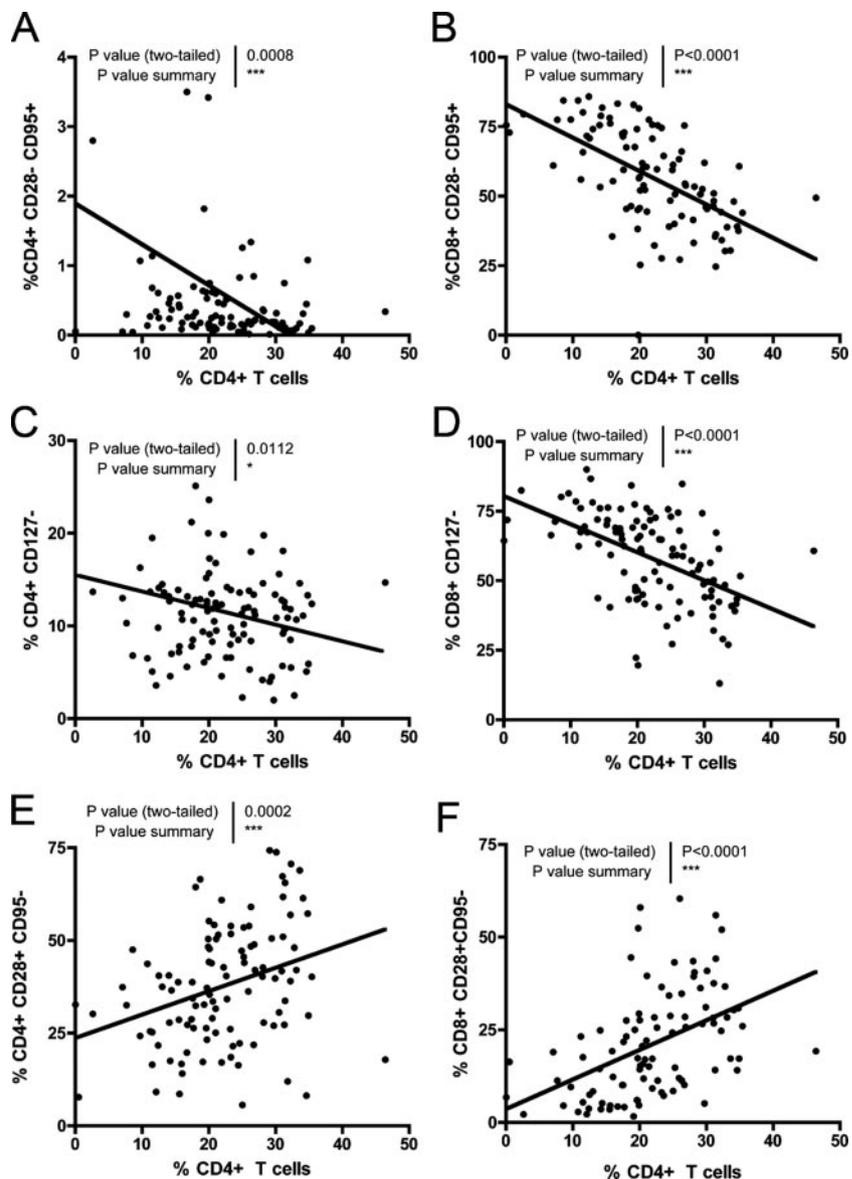
FIGURE 4. In SIV-infected SMs the percentage of CD4⁺ T cells is inversely correlated with the percentage of T_E cells and directly correlated with the percentage of T_N cells. Shown are the correlations between percentage of CD3⁺CD4⁺ T cells (within the total lymphocyte gate) and the percentages of CD3⁺CD4⁺CD28⁻CD95⁺ T_E cells (*A*), CD3⁺CD8⁺CD28⁻CD95⁺ T_E cells (*B*), CD3⁺CD4⁺CD127⁻ T cells (*C*), CD3⁺CD8⁺CD127⁻ T cells (*D*), CD3⁺CD4⁺CD28⁺CD95⁻ T_N cells (*E*), and CD3⁺CD4⁺CD28⁺CD95⁻ T_N cells (*F*) (*, $p <$ 0.05; ***, $p <$ 0.001; as determined by the Spearman rank correlation test).

In previous studies (11) we showed that natural SIV infection of SMs is associated with only a modest increase in proliferating (i.e., Ki67⁺) CD4⁺ T cells and no change in the level of CD8⁺Ki67⁺ T cells. In this study we sought to determine whether, in a large cohort of 110 naturally SIV-infected SMs, any relationship exists between CD4⁺ T cell count and the percentage of proliferating T cells. As shown in Fig. 6, we found no correlation between the percentage of CD4⁺Ki67⁺ (Fig. 6*A*) or CD8⁺Ki67⁺ (Fig. 6*B*) T cells and the absolute CD4⁺ T cell count. Interestingly, no correlation was found between the percentage of CD4⁺Ki67⁺ T cells and the level of viral replication (Fig. 6*C*), whereas a significant direct correlation was present between CD8⁺Ki67⁺ T cell percentage and viral load (Fig. 6*D*). Collectively, these data identify an interesting difference between HIV-infected individuals and naturally SIV-infected SMs in that, in SMs, the prevailing level of CD4⁺ T cell proliferation does not seem to be a direct reflection of higher viral antigenic load or CD4⁺ T cell depletion. The absence of a clear correlation between CD4⁺ T cell count and T cell proliferation also suggests that, in SIV-infected SMs, the level of either T_E (i.e., CD28⁻CD95⁺ and/or CD127⁻) or Treg-like (i.e., CD4⁺CD25⁺Foxp3⁺) cells

is a better marker of the type of T cell activation that is linked to the loss of CD4⁺ T cell homeostasis.

Immunologic characterization of a subset of naturally SIV-infected SMs with moderate-to-severe CD4⁺ T cell depletion ("CD4^low")

As mentioned above, the current study revealed that 14 of 110 SIV-infected SMs had a CD4⁺ T cell count lower than 500/mm³ at the time of investigation, and two of these animals had fewer than 50 CD4⁺ T cells per mm³ of blood (a level that indicates high risk of AIDS in HIV-infected individuals). These low CD4⁺ T cell counts are specific to SIV-infected SMs, as all of the 30 uninfected animals tested showed CD4⁺ T cell counts of >500/mm³. Representative dot plots showing the levels of CD4⁺ T cells in the peripheral blood and LNs of one of these "CD4^low" SIV-infected SMs are shown in Fig. 7*A*. Interestingly, none of the "CD4^low" SIV-infected SMs manifested any sign of clinical illness and, in fact, all have remained asymptomatic 18–36 mo after the first detection of a CD4⁺ T cell count of <500/mm³. As shown in Fig. 7, *B* and *C*, these "CD4^low" SIV-infected SMs maintained levels of viral replication that are comparable to "CD4^high" animals and

FIGURE 5. In SIV-infected SMs the CD4+ T cell count is directly correlated to the percentage of CD4+CD25+ Treg-like cells. *A*, Representative dot plot showing the expression of CD25 on CD4+ T cells from a SM (*left*) and a representative histogram overlay from the same animal showing that Foxp3+ cells (in red) are mostly included within the subset of CD4+ CD25+ T cells (*right*). *B*, In vitro depletion of CD4+CD25+ T cells results in increased proliferative response to SIVp27 by SM PBMCs; experiments were performed in triplicate. *C*, Comparison of the percentage of CD4+CD25+ T cells in naturally SIV-infected and uninfected SMs (***, *p* < 0.001; as determined by the unpaired *t* test with Welch's correction). *D*, Correlation between CD4+ T cell count (cells/mm³) and the percentage of CD3+CD4+CD25+ T cells. (***, *p* < 0.001; as determined by the Spearman rank correlation test.
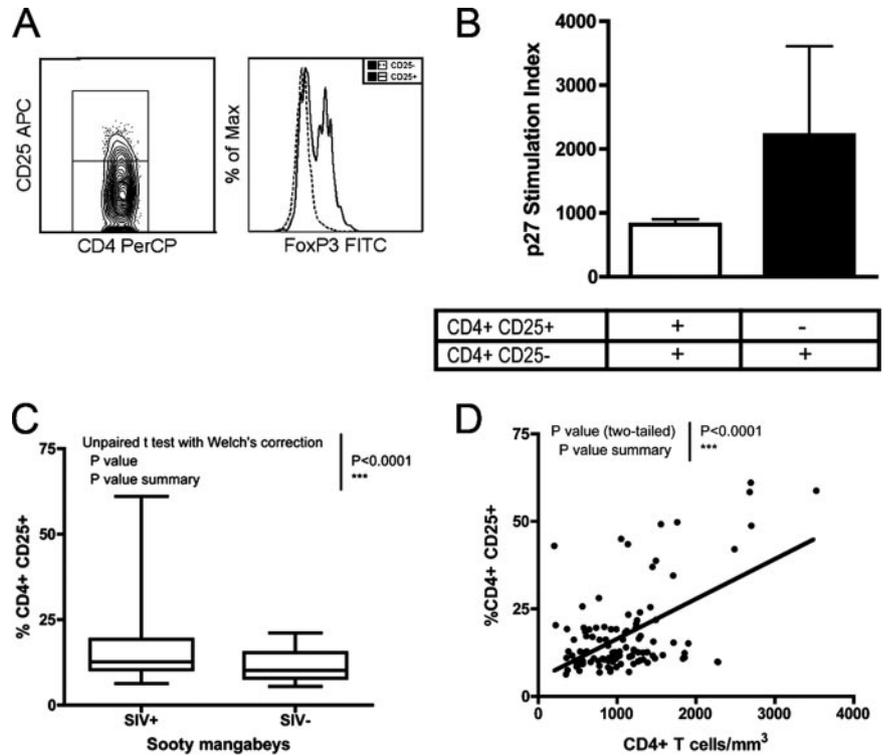
were, on average, older than the "CD4high" animals. However, because the number of CD4+ T cells tends to decrease with age even in uninfected SMs (data not shown), it appears unlikely that the "CD4low" phenotype is only related to the fact that these animals were infected for a longer time. Interestingly, the "CD4low" naturally SIV-infected SMs exhibited a trend toward an increased proportion of CD4+ and CD8+ T cells expressing markers of T_E differentiation (i.e., loss of CD28 and/or CD127; Fig. 7, *D* and *E*) and a concomitant reduction in T_N cells (Fig. 7*D*) when compared with naturally SIV-infected SMs with CD4+ T cell counts >500/ mm³. Overall, these findings support the hypothesis that the presence of increased levels of immune activation (specifically, the percentage of circulating T_E cells) is an important correlate of CD4+ T cell decline during natural SIV infection of SMs.

We next focused on the two asymptomatic "CD4low" naturally SIV-infected SMs that displayed severe and persistent CD4+ T
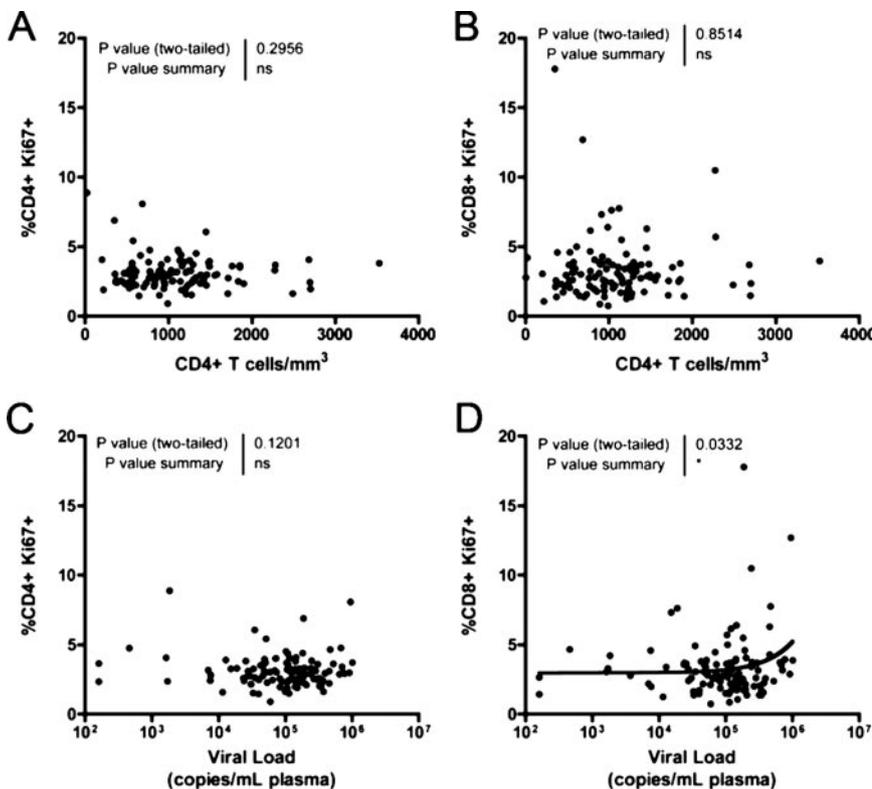
FIGURE 6. Lack of correlation between CD4+ T cell count and percentage of proliferating T cells. *A* and *B*, Correlation between CD4+ T cell counts (cells/mm³) and the percentage of CD3+CD4+Ki67+ T cells (*A*) and CD3+ CD8+Ki67+ T cells (*B*). *C* and *D*, Correlation between viral load (copies/ml plasma) and percentage of CD3+CD4+Ki67+ T cells (*C*) and CD3+CD8+Ki67+ T cells (*D*) (p = ns; *, *p* < 0.05; as determined by the Spearman rank correlation test.)
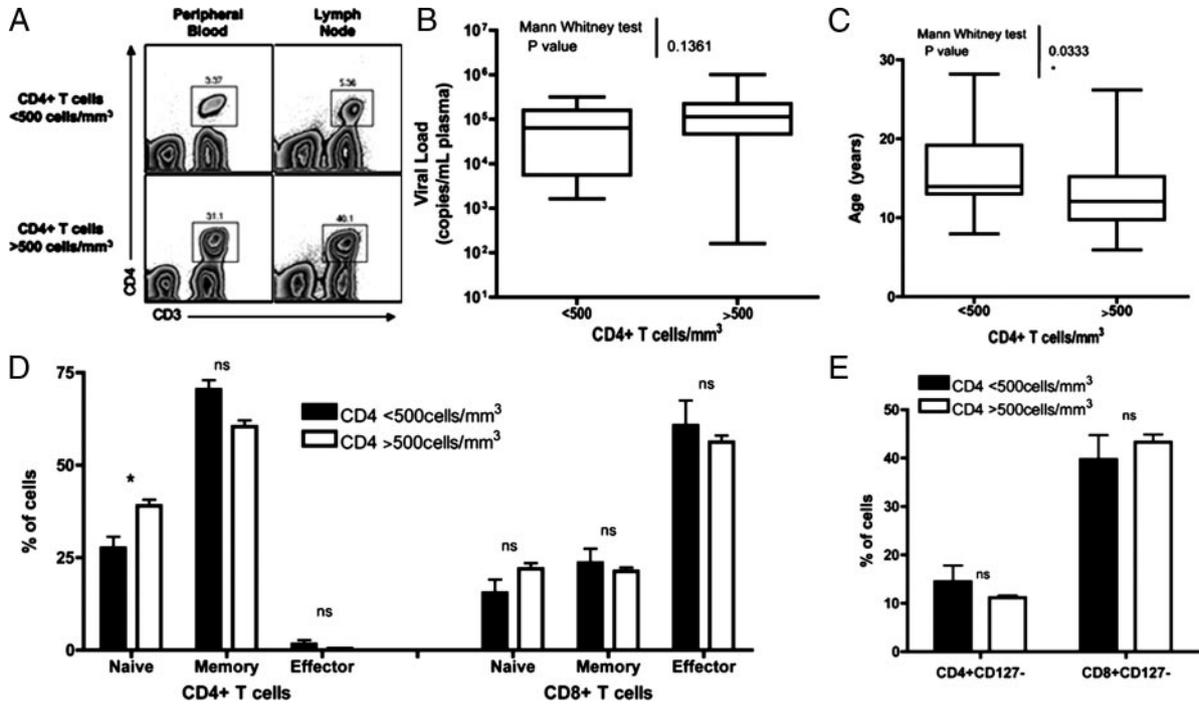
FIGURE 7. Identification of a subset of naturally SIV-infected SMs with moderate to severe CD4+ T cell depletion. *A*, Representative dot plots showing the percentage of CD3+CD4+ T cells (gated on total lymphocytes) in the blood and LNs of a "CD4low" (*top*) and "CD4high" (*bottom*) SIV-infected SM. *B*, Comparison of plasma viral load (copies/ml) in "CD4low" (i.e., <500) and "CD4high" (i.e., >500) SIV-infected SMs. *C*, Comparison of age (years) in "CD4low" and "CD4high" SIV-infected SMs. *D*, Comparison of the relative proportion of CD3+CD4+ (*left*) and CD3+CD8+ (*right*) T_N, T_M, and T_E cells in "CD4low" and "CD4high" SIV-infected SMs. *E*, Comparison of the percentage of CD4+CD127− (*left*) and CD8+CD127− (*right*) T cells in "CD4low" and "CD4high" SIV-infected SMs. For *B–E*, p = ns; ∗, $p < 0.05$; as determined by the Mann-Whitney *U* test.

cell depletion (CD4+ T cell count of <50/mm³ for over 2 years of followup). In Fig. 8*A* we have summarized the main immunological and virological data available on these two SIV-infected SMs (SM-84 and SM-103, with CD4+ T cell counts of 20 and 3 cells/mm³, respectively). Flow cytometric data showing the very low

levels of CD4+ T cells in both the peripheral blood and the LNs of SM-84 are presented in Fig. 8*B*. It should be noted that both SM-83 and SM-103 show relatively low levels of viremia and, except for a moderate expansion of CD8+CD28−CD95+ and CD8+ CD127− T_E cells, do not exhibit unusually high levels of T cell
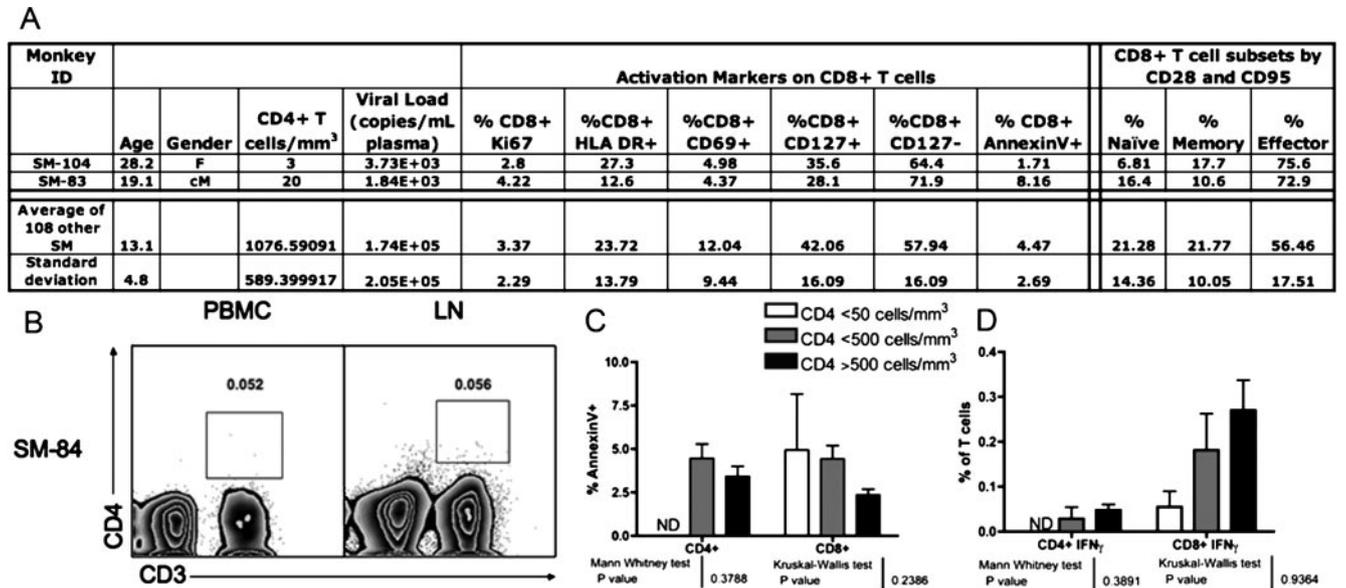
FIGURE 8. Immunological characterization of two naturally SIV-infected SMs with severe CD4+ T cell depletion (<50 cells/mm³). *A*, Demographics and main immunological and virological data available on the two SIV-infected SMs (SM-84 and SM-103) with severe CD4+ T cell depletion. *B*, Dot plots showing the very low percentage of CD3+CD4+ T cells in the peripheral blood and LNs of SM-84. *C*, In vitro susceptibility to apoptosis in SIV-infected SMs grouped by CD4+ T cell count (□, <50 cells/mm³, n = 2; ▨, 50–500 cells/mm³, n = 12; and ■, >500 cells/mm³, n = 10). *D*, Production of IFN-γ by CD4+ and CD8+ T cells in response to SIV peptides in SIV-infected SMs grouped by CD4+ T cell count (□, <50 cells/mm³, n = 2; ▨, 50–500 cells/mm³, n = 12; and ■, >500 cells/mm³, n = 20).

activation when compared with the rest of the cohort of naturally SIV-infected SMs (Fig. 8A). Similarly, there was no difference between SM-84 and SM-103 and the animals with higher CD4$^+$ T cell counts in either the level of in vitro T cell susceptibility to spontaneous and activation-induced apoptosis (Fig. 8C) or the production of IFN-γ by T cells in response to SIV peptide stimulation (Fig. 8D). These findings suggest that this very unusual phenotype manifested by severe CD4$^+$ T cell depletion may have a complex pathogenesis that is not simply related to high viral replication and/or chronic generalized immune activation. Perhaps more importantly, the discovery of these two asymptomatic SIV-infected SMs with extreme CD4$^+$ T cell depletion underscores the potential protective effect of low levels of immune activation even in the presence of very low CD4$^+$ T cell counts. Attenuated immune activation may thus protect naturally SIV-infected SMs from developing AIDS not only by helping them maintain a healthy CD4$^+$ T cell level but also by preserving the function of other non CD4$^+$ immune cell types (i.e., CD8$^+$ T cells, B cells, NK cells, $\gamma\delta$ T cells, and others) in the event of CD4$^+$ T cell depletion.

Discussion

In stark contrast to HIV-infected humans and SIV-infected rhesus macaques, natural hosts for SIV, including SMs, only very rarely develop AIDS despite chronic high levels of viral replication. Defining the mechanisms of AIDS resistance in these animals is a key challenge for contemporary HIV/AIDS research, and many in the field believe that a better understanding of these mechanisms may reveal important molecular and cellular determinants of CD4$^+$ T cell depletion and disease progression that define pathogenic HIV/SIV infections (7, 8).

Based on a series of previous studies conducted in relatively small cohorts of naturally and experimentally SIV-infected SMs, we proposed that attenuated immune activation may be instrumental in maintaining the AIDS-free state of these animals (11, 17, 28). From an evolutionary perspective, these studies suggested that SMs (and perhaps other natural hosts for SIV) have adapted to the selective pressure of SIV infection and have reached a disease-free equilibrium that does not require strict control of viral replication (with viral loads that are as high if not higher than those seen during pathogenic HIV infection) but instead results from a general attenuation of the overall level of immune activation.

In this article, we report data relative to a comprehensive clinical, virological, and immunological survey of 110 naturally SIV-infected SMs hosted at the Yerkes Primate Center of Emory University, which is home to the largest colony of captive SMs in the world. To the best of our knowledge, this work represents the largest study ever performed in natural hosts for SIV infection. It is important to note that, more than just confirming previous findings from smaller groups of animals (i.e., lack of disease, high levels of viremia, generally preserved CD4$^+$ T cell counts, and low levels of immune activation), this study led us to a number of intriguing new observations. First, we were able to identify a previously unrecognized subset of animals with moderate to severe CD4$^+$ T cell depletion ("CD4^{low}"), including two animals with <50 CD4$^+$ T cells/mm^3. We believe that these animals are of particular interest as they may represent an intermediate immunologic outcome between the typically nonpathogenic infection observed in the majority of SIV-infected SMs and the typically pathogenic infection observed in HIV-infected patients. Second, this study provided a better definition of the relationship between T cell activation and CD4$^+$ T cell counts. We initially validated by

differential cytokine production and tissue homing in SMs a classification of T_N, T_M, and T_E cells based on the expression of CD28 and CD95 and then observed that the relative and absolute expansion of CD28$^-$CD95$^+$ T_E cells (as well as the related expansion of CD4$^+$CD127$^-$ and CD8$^+$CD127$^-$ T cells that also show phenotypic and functional features of T_E cells) was the best immunologic correlate of CD4$^+$ T cell loss. Third, this study revealed, for the first time, an interesting direct correlation between the percentage of circulating CD4$^+$CD25$^+$ T cells (that include Foxp3-expressing Treg-like cells) and the preservation of CD4$^+$ T cell counts in a natural host for SIV infection. It is important to note that this relationship is also consistent with the hypothesis that lower levels of T cell activation are instrumental in maintaining the normal immune system of SIV-infected SMs and suggest a role for Treg cells in establishing this state of attenuated T cell responses.

By studying such a large cohort of animals, we were able to determine unequivocally that there is no correlation between the level of circulating CD4$^+$ T cells and either the level of SIV replication or years of age (used as a surrogate marker for length of infection). In particular, we found only a nonsignificant trend ($p = 0.06$) toward lower CD4$^+$ T cell counts in older animals that was also observed in uninfected SMs. This finding suggests that the development of profound CD4$^+$ T cell depletion and/or clinical AIDS is not likely to occur in all animals even if they were to live longer than the average SM lifespan (i.e., 20–25 years). Instead, our work indicates very clearly that the best correlates of CD4$^+$ T cell decline, when present, during natural, nonpathogenic SIV infection of SMs are: 1) an increased percentage of effector T cells that have lost the expression of CD28 and/or CD127; and 2) a decreased percentage of CD4$^+$CD25$^+$ Treg-like cells. These results suggest that the nonpathogenic SIV infection of SMs is similar to pathogenic HIV infection in humans in that, in both cases, a heightened level of immune activation seems to exert a net negative effect on the maintenance of CD4$^+$ T cell homeostasis. However, in contrast to HIV-infected humans, SIV-infected SMs display an overall lower level of immune activation that is consistent with the preservation of CD4$^+$ T cell homeostasis observed in the majority of animals despite high levels of viral replication. Another interesting difference between HIV-infected individuals and SIV-infected SMs is that the latter do not show the inverse correlation between CD4$^+$ T cell count and T cell proliferation that has been found during HIV infection (25, 26). The absence of this correlation in naturally SIV-infected SMs suggests that the prevailing level of T cell proliferation may have a different biological meaning in SMs as compared with HIV-infected humans. Although in HIV-infected humans the level of CD4$^+$ T cell proliferation mainly reflects the level of immune activation in response to viral replication (27), in SIV-infected SMs the level of CD4$^+$ T cell proliferation may also involve homeostatic mechanisms aimed at compensating for virus-induced cell loss. Consistent with this possibility is the observation that, in SIV-infected SMs, the suppression of viral replication with anti-retroviral therapy is not followed by a rapid decline in the levels of Ki67$^+$ T cells (S. Gordon, S. Bosinger, J. Brenchley, N. R. Klatt, J. M. Milush, J. Engram, R. M. Dunham, M. Paiardini, S. Klucking, A. Danesh, et al., manuscript in preparation) as has been observed in HIV-infected humans (27).

The absence of clinical manifestations of AIDS in naturally SIV-infected SMs is particularly intriguing in the two animals with CD4$^+$ T cell counts persistently lower than 50/mm^3, as this level of CD4$^+$ T cell depletion would almost invariably be associated

with opportunistic infections and/or cancer in HIV-infected humans. Although the "CD4^{low}" SIV-infected SMs show a significant trend toward higher levels of immune activation when compared with "CD4^{high}" SIV-infected SMs, their levels of T cell activation and proliferation are still lower than those observed in HIV-infected individuals. This latter observation suggests that, in naturally SIV-infected SMs, the attenuated immune activation is beneficial not only as it favors the maintenance of CD4$^+$ T cell homeostasis but also because it may preserve the overall integrity of the immune system even when the number of CD4$^+$ T cells has declined dramatically. In this scenario, the function of non-CD4$^+$ immune cell types (i.e., CD8$^+$ T cells, T$\gamma\delta$-cells, B-cells, NK-cells, etc) would be better preserved in the setting of low levels of immune activation and the lack of a markedly proinflammatory immune environment.

As of yet, our attempts to determine the exact etiology of the "CD4^{low}" phenotype observed in this subset of naturally SIV-infected SMs have been unsuccessful. Because severe CD4$^+$ T cell depletion has been reported in two experimentally SIV-infected SMs in association with expanded viral tropism for coreceptors such as CXCR4 and CCR8 (S. Gordon, S. Bosinger, J. Brenchley, N. R. Klatt, J. M. Milush, J. Engram, R. M. Dunham, M. Paiardini, S. Klucking, A. Danesh, et al., manuscript in preparation), we explored the possibility that these two naturally SIV-infected animals may also harbor viral quasispecies with extended viral tropism. However, sequence analyses of the gp160 envelope protein cloned from our two "CD4^{low}" naturally SIV-infected SMs ruled out this explanation (G. Silvestri, unpublished observations). We also explored the possibility that our two "CD4^{low}" animals may have lost immune control of viral replication exerted, for instance, by SIV-specific cytotoxic T-lymphocytes. Again, we found that "CD4^{low}" SIV-infected SMs show SIV-specific T cell responses comparable to those observed in animals with normal CD4$^+$ T cell counts (19). It is likely that the CD4$^+$ T cell depletion observed in a subset of SIV-infected SMs is, in fact, more complex and involves immune factors (defective CD4$^+$ T cell regeneration?) as well as viral factors (increased in vivo cytopathicity?). In particular, the advanced age (28 years) of one of these severely CD4$^+$ T cell depleted SIV-infected SMs suggests that, in this animal, the defective CD4$^+$ T cell regeneration typical of immune senescence (29–31) may play a significant role in the pathogenesis of this unusual phenotype.

In summary, this study provides the largest and most detailed characterization of the correlates of preserved CD4$^+$ T cell counts during nonpathogenic, natural SIV infection of SMs. In these animals, CD4$^+$ T cell levels are not related to the level of viral replication or the length of infection but rather to the set point level of T cell activation, that is most accurately reflected by the percentage of circulating T$_E$ cells and by the percentage of CD4$^+$ CD25$^+$ T cells. In addition, we have shown, for the first time, that a small minority of naturally SIV-infected SMs develop moderate to very severe CD4$^+$ T cell depletion but still remain AIDS free in the setting of relatively low levels of immune activation. This observation suggests that the attenuated immune activation of natural SIV hosts has a beneficial effect on the overall immune system function that goes beyond the preservation of high CD4$^+$ T cell counts. The fact that in the absence of chronic generalized immune activation very low levels of CD4$^+$ T cells are not associated with clinical immunodeficiency supports the hypothesis that, in HIV-infected humans, the level of immune activation plays a key role in the pathogenesis of AIDS.

Disclosures

The authors have no financial conflict of interest.

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