Virally Induced CD4+ T Cell Depletion Is Not Sufficient to Induce AIDS in a Natural Host


_J Immunol_ 2007; 179:3047-3056; doi: 10.4049/jimmunol.179.5.3047
http://www.jimmunol.org/content/179/5/3047

References
This article cites 50 articles, 30 of which you can access for free at:
http://www.jimmunol.org/content/179/5/3047.full#ref-list-1

Subscription
Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Virally Induced CD4+ T Cell Depletion Is Not Sufficient to Induce AIDS in a Natural Host

Jeffrey M. Milush,* Jacqueline D. Reeves,† Shari N. Gordon,‡ Dejiang Zhou,**
Alagar Muthukumar,* David A. Kosub,* Elizabeth Chacko,* Luis D. Giavedoni,‡
Chris C. Ibegbu,¶ Kelly S. Cole,¶ John L. Miamidian,‡ Mirko Paiardini,‡ Ashley P. Barry,**§
Silvija I. Staprans,¶¶ Guido Silvestri, and Donald L. Sodora2*

Peripheral blood CD4+ T cell counts are a key measure for assessing disease progression and need for antiretroviral therapy in HIV-infected patients. More recently, studies have demonstrated a dramatic depletion of mucosal CD4+ T cells during acute infection that is maintained during chronic pathogenic HIV as well as SIV infection. A different clinical disease course is observed during the infection of natural hosts of SIV infection, such as sooty mangabeys (Cercocebus atys), which typically do not progress to AIDS. Previous studies have determined that SIV+ mangabeys generally maintain healthy levels of CD4+ T cells despite having viral replication comparable to HIV-infected patients. In this study, we identify the emergence of a multispicropathic R5/X4/8-enveloped SIV infection after 43 or 71 wk postinfection in two mangabeys that is associated with an extreme, persistent (>5.5 years), and generalized loss of CD4+ T cells (5–80 cells/μl of blood) in the absence of clinical signs of AIDS. This study demonstrates that generalized CD4+ T cell depletion from the blood and mucosal tissues is not sufficient to induce AIDS in this natural host species. Rather, AIDS pathogenesis appears to be the cumulative result of multiple aberrant immunologic parameters that include CD4+ T cell depletion, generalized immune activation, and depletion/dysfunction of non-CD4+ T cells. Therefore, these data provide a rationale for investigating multifaceted therapeutic strategies to prevent progression to AIDS, even following dramatic CD4+ depletion, such that HIV+ humans can survive normal life spans analogous to what occurs naturally in SIV+ mangabeys. The Journal of Immunology, 2007, 179: 3047–3056.

The original observation that clinical AIDS was associated with peripheral blood CD4+ T cell loss due to the direct and indirect effects of HIV infection led to the paradigm that depletion of peripheral blood CD4+ T cells is the major determinant of immune dysfunction, ultimately resulting in the opportunistic infections and cancers characteristic of AIDS. Refining the paradigm in recent years has led to the inclusion of additional immunologic events that contribute to the progression to AIDS, including generalized immune activation and depletion of CD4+ T cells at mucosal sites. These multifaceted events are all occurring concurrently in HIV+ patients, making it difficult to discern the importance of any one factor from the others, although the role of HIV in triggering these events is clear and undisputed.

The envelope glycoprotein (env)3 present on a HIV virion is critical for determining the cellular tropism and coreceptor usage of the virus. HIV infects target cells (CD4+ T cells, macrophages, and brain microglia) by first binding to the CD4 receptor, which results in conformational changes that allow the envelope protein to bind a coreceptor, typically the CCR5 (R5-tropic) or CXCR4 (X4-tropic) chemokine receptors. The HIV variants that are responsible for most vertical (mother-to-child) and horizontal transmissions and predominate early in the course of infection are generally R5-tropic; however, X4-tropic variants eventually emerge in ~50% of infected individuals (1–4). This expanded coreceptor usage is generally associated with dramatic loss of CD4+ T cells and the onset of clinical AIDS (1–4). The infection of rhesus macaques with SIV pseudotyped with an X4 or R5/X4 envelope gene of HIV (simian HIV (SHIVs)) has also identified a precipitous depletion of CD4+ T cells generally culminating in progression to simian AIDS within 12–25 wk postinfection (wpi) (5–8). In lymphoid tissues, the CXCR4 receptor is present on ~86% of CD4+ T cells whereas only 10% express CCR5 (9). This high percentage of CD4+ T cells expressing CXCR4 offers a mechanism in which CD4+ T cells are depleted during an X4 or R5/X4-tropic HIV infection by direct viral killing (9). The ability of a human host to suppress replication of X4 variants may be due to an increased susceptibility of X4 HIV-infected cells to CD8+ T cell-mediated killing (10).

*University of Texas Southwestern Medical Center, Dallas, TX 75390; *Monogram Biosciences, South San Francisco, CA 94080; †University of Pennsylvania, Philadelphia, PA 19104; ‡Southwest Foundation for Biomedical Research, San Antonio, TX 78245; ¶Enzyme Vaccine Center, Atlanta, GA 30329; †University of Pittsburgh School of Medicine, Pittsburgh, PA 15261; ¶University of California San Diego, La Jolla, CA 92039; **Trimeris, Morrisville, NC 27560; and ††Merck Vaccine Division, West Point, PA 19486

Received for publication January 24, 2007. Accepted for publication June 19, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health Grants R01 AI035522 (to D.L.S.) and R21 AI060451 (to D.L.S.) and RR-00165 (Yerkes Primate Center).

2 J.M.M. and D.K. were supported in part by training grants from the National Institute of Allergy and Infectious Diseases (ST32 AI07520 and ST32 AI005284, respectively). J.D.R. was supported by Fellowship 106437-34-RFGN from the American Foundation for AIDS Research and National Institutes of Health Grant R21 AI060451 (to D.L.S.) and RR-00165 (Yerkes Primate Center).

3 Abbreviations used in this paper: env, envelope glycoprotein; SHIV, simian HIV; SM, sooty mangabey; HSP, heat shock protein; IPP, isopentenyl pyrophosphate; wpi, week postinfection.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/82.00
SIV infection of natural hosts, such as sooty mangabeys (Cercocebus atys), has provided key insights into disease pathogenesis of primate lentiviral infections. SIV infection of mangabeys generally does not result in opportunistic infections, lymphomas, or any signs of clinical AIDS (11–13). The absence of disease signs is not due to virologic differences in the virus replicating in the mangabeys, because viral loads are comparable to HIV-infected patients, $10^7$–$10^8$ copies per ml of plasma, and passage of virus to rhesus macaques induces clinical signs of simian AIDS (14). Several mechanisms have been suggested to explain the lack of disease progression in mangabeys (11, 14, 15), including a role for the earlyeral mechanisms have been suggested to explain the lack of disease progression in this species. This study describes an extreme, persistent, and generalized depletion of peripheral blood, lymphoid, and mucosal CD4$^+$ T cells in two animals during nonpathogenic SIV infection of sooty mangabeys. We demonstrate that in these two mangabeys the severe depletion coincides with an expanded coreceptor usage such that the virus can now infect a broader repertoire of CD4$^+$ T cells. Irrespective of the CD4$^+$ T cell levels, SIV-infected mangabeys maintain low levels of systemic immune activation, healthy lymphoid architecture, and preserved functionality of a non-CD4$^+$ T cell, the y6 T cell. Taken together, these data indicate that virally induced CD4$^+$ T cell depletion is not sufficient to cause clinical simian AIDS in a natural host species and indicates that aberrant immune activation and dysfunction of non-CD4$^+$ immune cells, in combination with CD4$^+$ T cell depletion, are important for triggering AIDS in HIV$^+$ patients. Therefore, these data provide a rationale for investigating multifaceted therapeutic strategies to prevent progression to AIDS, even following dramatic CD4 depletion, such that HIV$^+$ humans can survive normal life spans analogous to what occurs naturally in SIV$^+$ mangabeys.

Materials and Methods

Animals and viral infection

The sooty mangabeys (SMs) used in this study were between 3 and 5 years of age when infected with SIV. All six mangabeys were inoculated i.v. with 1 ml of plasma transferred from mangabey FQi, a naturally SIVsmm-infected mangabey at the Yerkes primate colony. The infection occurred in October 2000 and has previously been described (16). Before SIVsmm infection, virologic assessment determined that none of the mangabeys contained simian T cell lymphotropic virus or simian retrovirus. Furthermore, antisera analysis determined that all mangabeys except SM2 were seropositive for CMV/simian agent 6, and all except SM2 and SM4 contained Abs that cross-reacted with herpesvirus B. Passage of virus from one CD4-low mangabey, SM2, to three additional SIV-negative mangabeys was performed by i.v. inoculation in October 2007 (1.5 ml of plasma from the 303 wpi time point). Local animal care and use committee and National Institutes of Health protocols were strictly followed in the maintenance of the animals at the Yerkes National Primate Center (Atlanta, GA).

Lymph node biopsies, rectal biopsies, and bronchoalveolar lavage

Lymph node (axillary or inguinal) and rectal mucosal biopsies, as well as bronchoalveolar lavages were performed on two SIV$^+$ CD4-low (SM1 and SM2) and two SIV$^+$ CD4-low (SM3 and SM4) mangabeys. Rectal mucosal biopsies were obtained using a sigmoidoscope with forceps and nonnuclear cells were isolated by collagenase digestion (two sequential 30-min incubations at 37°C in RPMI 1640 containing 0.75 mg/ml collagenase). The digested suspension was passed through 70-μm cell strainers and then enriched for lymphocytes by Percoll density gradient. For bronchoalveolar lavages, a fiberoptic bronchoscope was placed into the trachea after local anesthetic was applied to the larynx. Four 35-ml aliquots of warmed saline were injected into the right primary bronchus and collected by aspiration before a new aliquot was instilled.

Viral load analysis

SIVsmm plasma viral load was determined as previously described (11, 17). All Abs were purchased from BD PharMingen unless otherwise noted. Percentage of CD4$^+$ and CD8$^+$ T cells was determined at multiple tissue sites by gating lymphocyte populations in the forward/side scatter, pulse width to ensure only single cells were analyzed and the mAb CD3-PE (SP34-2). CD4 (clone SK3) and CD8$^+$ T cells (clone RPA-T8) were then identified and the proliferating cells within the CD8$^+$ population were determined using an Ab to Ki-67 (clone B56). Multiple CD4 Ab clones were tested including S3.5 (Caltag Laboratories), SK3, SK4, L200, MT477 (BD Biosciences). All CD4 Ab clones performed similarly when assessing CD4+ healthy and CD4+ low mangabeys. Percentage of total y6 T cells within the CD4$^+$ T cell population of PBMC was determined by flow cytochemistry using anti-Pan-y6 TCR (5AE6; Pierce) and CD4 on a FACSCalibur (BD Biosciences). To assess y6 T cell proliferation, PBMC were incubated with 5 μM CFSE (Molecular Probes) at 37°C. After 5 min, the CFSE was diluted out with 10% FCS in PBS/BSA washes and cells were cultured with medium alone or with one of the following Ags/mitogens: heat-shock protein 65 (HSP; 10 μg/ml), isopentenyl pyrophosphate (IPP; 1 mg/ml), LPS (200 μg/ml), or Con A (10 μg/ml) for 7 days. Cells were stained before acquiring a minimum of 50,000 events on a FACSCalibur. Analysis was performed with FlowJo software (Tree Star).

SIV-specific CTL and envelope-specific Ab responses

SIV-specific peripheral blood cytotoxic T cell responses were assessed using SIVsmm-specific peptides in the intracellular cytokine staining protocol as previously described (18).

Ab responses to SIV envelope were measured as previously described using a Con A ELISA (19). Briefly, detergent-disrupted SIV envelope proteins from SIVsmmB7 captured on the Con A plate were exposed for 1 h at room temperature to plasma Abs, mAbs, or plasma from SIV$^+$ control mangabeys. To determine end point titers, the plates were washed with PBS and developed using peroxidase-labeled goat anti-mouse IgG Ab and TM blue (Serologicals) as the substrate. End point titers represent the last 2-fold dilution with an OD$_{540}$ twice that of SIV$^+$ control animals.

Assessment of lymph node architecture

General lymph node architecture was assessed following standard reticulin or H&E staining. Immunofluorescent staining with anti-CD3 (N1580; DakoCytomation) and anti-CD20 (clone L26; DakoCytomation) Abs was performed on 8-μm Streck-fixed, paraffin-embedded tissue sections. Briefly, slides were deparaffinized, rehydrated, and boiled for 20 min in 10 mM citrate buffer (pH 6.0). Slides were blocked in PBS containing 1% milk, 0.5% BSA, and 3% normal goat serum before staining with anti-CD3 for 1 h. Slides were then washed and stained with goat anti-rabbit Alexa Fluor 555 (5 μg/ml; Molecular Probes). Slides were washed again, blocked with 3% normal horse serum, and stained with anti-CD20 (1/200 dilution) for 1 h, washed, and counterstained with horse anti-mouse FITC (1/200 dilution; Vector Laboratories). Lastly, nuclei were identified using 4′,6′-diamidino-2-phenylindole (DAPI). Images were acquired using a Zeiss microscope and the PASCAL version 3.2 image software (512 x 512 pixel resolution).

Detection and characterization of SIV-infected cells by in situ hybridization and immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were hybridized and immunostained as described previously, with a few modifications (20). Briefly, tissue sections were dehydrated, peroxide treated for 20 min, then blocked in HCl for 20 min. Proteinase K treatment was performed for 12 min before overnight hybridization at 49°C with digoxigenin-labeled ribo-probes. Detection of the riboprobe was accomplished with sheep antidigoxi-gen-peroxidase (1/1000 dilution) followed by FITC-tyramide amplification (PerkinElmer Life Sciences). The sections were then washed, blocked, and probed with biotinylated anti-CD3 (DakoCytomation) or anti-HAM56 (1/75 dilution; DakoCytomation) Ab and detected using streptavidin/Alexa Fluor 633. Nuclei were identified using DAPI (Molecular Probes). Confocal microscopy was performed using the Zeiss LSM5 PASCAL laser scanning microscope and the PASCAL version 3.2 image software (512 x 512 pixel resolution).

Assessment of plasma cytokine/chemokines using Luminex array

The assessment of plasma cytokines and chemokines was performed as previously described (21). Briefly, frozen plasma samples were thawed rapidly at 37°C, vortexed, and spun at 10,000 x g for 5 min to remove any solid particles. Aliquots of 50-μl samples were combined with the coated
beads, and incubations and washes were performed in 1.2-μm filter membrane 96-well microtiter plates (MABV1250; Millipore) with the help of a MultiScreen vacuum manifold for 96-well plates (Millipore). After the final wash, beads in the 96-well microtiter plate were resuspended in 125 μl of Luminex sheath fluid and loaded into the Luminex instrument. An acquisition gate was set between 8,000 and 13,500 for the doublet discriminator, sample volume was 75 μl, and 100 events per region were acquired. Raw data (mean fluorescence intensity) were analyzed with the MasterPlex QT quantification software (MiraiBio) to obtain concentration values.

**Determination of coreceptor usage and env sequence analysis**

Full-length env gene was PCR amplified from plasma and cloned into pcDNA 3.1 TOPO Directional expression vector (Invitrogen Life Technologies). The V3 region was sequenced using the Applied Biosystems Big Dye Terminator 3.1 chemistry and analyzed on an Applied Biosystems capillary instrument. The coreceptor use of SIVsmm envs was determined in a cell-cell fusion assay as previously described (22). Briefly, QT6 cells were infected with a vaccinia virus encoding T7 polymerase and transfected...
with env expression vectors containing a T7 promoter upstream of env. Env-expressing cells were added to CD4/coreceptor-expressing QT6 cells that contained a luciferase reporter plasmid under the control of a T7 promoter. Fusion between env- and receptor-expressing cells resulted in T7 polymerase-driven luciferase expression that was quantified in a luminometer.

Nucleotide sequence accession numbers

The nucleotide sequences of the env V3 sequences from SIV-infected mangabeys were deposited in GenBank (accession numbers EU025832–EU025847).

Results

Severe, generalized loss of CD4+ T cells following SIVsmm inoculation in sooty mangabeys

Six mangabeys were i.v. inoculated with SIVsmm obtained from the plasma of a naturally infected mangabey in the Yerkes Primate Center colony (16). Preinoculation CD4+ T cell levels ranged between 1200 and 2000 CD4+ T cells/µl of blood (Fig. 1A) (16), comparable to uninfected mangabeys (11, 12, 14, 23). During acute infection, peak plasma viral loads (10^7–10^8 copies viral RNA/ml of plasma) coincided with a minor decline in CD4+ T cell levels to new, lower CD4 set-points that remained in the CD4-healthy range (500–1200 CD4+ T cells/µl of blood) (Fig. 1A) (16), similar to the majority of naturally SIV-infected mangabeys (11, 12, 14, 23). In four SIV-infected mangabeys, these new CD4 set-points remained relatively stable throughout the 6 years of follow up. However, beginning at 37 (SM1) and 51 (SM2) wpi, a further, more dramatic, CD4+ T cell decline over a 3- to 6-mo period was observed in two of the SIV-infected mangabeys (Fig. 1A). These animals developed an extreme CD4+ T cell depletion (5–80 CD4 cells/µl of blood) that has been maintained over the past 5.5 years in the absence of any clinical signs of AIDS (Fig. 1A). The authenticity of this CD4 depletion was determined using multiple flow cytometric analyses. First, to ascertain that the CD4 protein had not been altered such that our CD4 Ab no longer recognized its epitope, we verified these findings with five different monoclonal Abs that recognize the CD4 protein. Second, we assessed changes in the levels of non-CD4+ T cell subsets (CD3+CD8− CD4+ or CD3−CD8+CD4+) to determine whether the CD4 protein was being masked or internalized in the CD4+ T cells. Indeed, during the CD4 depletion to levels below 100 cells/µl of blood, we did not observe any offsetting increase in non-CD4+ T cell subsets in SM1 and SM2 (with the exception of a transient increase in CD8+CD4− T cells in SM2) (Fig. 2). Therefore, these data demonstrate that the peripheral CD4+ T cells were indeed depleted to below 100 cells/µl of blood over a 3- to 6-mo period in both SM1 and SM2.

Importantly, CD4+ T cell depletion was not limited to the peripheral blood, but was also observed in lymph nodes, lungs, and gut-associated lymphoid tissues (rectal biopsy) (Fig. 1B). Indeed, CD4 depletion in the lungs and gut resulted in <0.6% total CD4+ T cells within the CD3+ T cell population at these sites (uninfected mangabeys average 30% CD4+ T cells at these mucosal sites). This level of mucosal CD4+ T cells in SIV+ mangabeys was similar to or even lower than that previously observed to correlate with disease progression in the pathogenic SIV/macaque model (24). These results fit a model in which the onset of AIDS results from the cumulative effect of both CD4 depletion below a critical threshold as well as chronic, generalized immune activation. A thorough assessment of peripheral CD4+ T cell levels in the naturally SIV-infected mangabeys housed at the Yerkes Primate Center identified three additional asymptomatic CD4-low mangabeys, indicating this phenotype was not simply the result of the i.v. route of inoculation used to infect SM1 and SM2. Interestingly, the plasma viral load in SM1 and SM2 decreases concomitantly with the loss of CD4+ T cells, suggesting CD4+ T cells are the primary cell type replicating the virus (Fig. 1A). In situ analysis of lymph...
nodes verified this observation as a predominance of SIV⁺CD3⁺ T cells were observed, although the frequency of infected cells was low in the CD4-low mangabeys (Fig. 3A). However, CD4⁺ T cells were not the only infected cells, because the macrophage marker HAM56 was able to identify a low-frequency SIV⁺ macrophage present in the lymph nodes (Fig. 3B).

CD4-low mangabeys maintain a clinically healthy immune system despite severe CD4⁺ T cell loss

Previous studies of SIV-infected mangabeys have implicated the maintenance of healthy CD4⁺ T cell levels as one important component for preventing the onset of simian AIDS in this species (11, 14, 15). However, these two CD4-low mangabeys exhibited evidence suggestive of a preserved and clinically healthy immune system despite generalized loss of CD4⁺ T cells. During pathogenic infections of macaques or humans, there is a loss of clearly defined follicles and germinal centers and the accumulation of collagen in the lymph nodes (25, 26). However, these two CD4-low mangabeys maintained normal lymph node architecture with visible follicles and germinal centers characteristic of a healthy lymph node (Fig. 4, A and B). Indeed, clearly defined B and T cell zones could be observed following immunofluorescent staining with anti-CD20 and anti-CD3 (Fig. 4, C and D). Previous studies have determined collagen deposition is negligible in the lymph node of SIV⁺ mangabeys during chronic infection, when compared with SIV⁺ macaques (27). Furthermore, staining of the reticulin network identified an intact continuous network around the germinal centers in the lymph node of CD4-healthy and CD4-low SIV⁺ mangabeys (Fig. 4, E and F). This preserved lymphoid architecture was associated with SIV Env-specific Ab responses (Table I), as well as SIV-specific CD8⁺ cytotoxic T cell levels.

### Table I. SIV Env-specific Ab titers

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Time Point</th>
<th>End Point Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM1</td>
<td>29</td>
<td>204,800</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>102,400</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>102,400</td>
</tr>
<tr>
<td>SM2</td>
<td>29</td>
<td>51,200</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>51,200</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>51,200</td>
</tr>
<tr>
<td>SM3</td>
<td>29</td>
<td>102,400</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>102,400</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>102,400</td>
</tr>
<tr>
<td>SM4</td>
<td>29</td>
<td>409,600</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>409,600</td>
</tr>
<tr>
<td></td>
<td>113</td>
<td>409,600</td>
</tr>
<tr>
<td>SM5</td>
<td>29</td>
<td>204,800</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>204,800</td>
</tr>
<tr>
<td></td>
<td>113</td>
<td>204,800</td>
</tr>
<tr>
<td>SM6</td>
<td>29</td>
<td>204,800</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>204,800</td>
</tr>
<tr>
<td></td>
<td>113</td>
<td>204,800</td>
</tr>
</tbody>
</table>
Numerous immune cell subsets, including γδ T cells, have altered function during a HIV/SIV infection (28–34). The γδ T cells are important because they form a link between innate and adaptive immune responses and may play important roles in Ag presentation (35). An assessment of total CD3+ T cells in the peripheral blood determined that γδ T cells represented a significantly larger percentage in mangabeys (11.8%) when compared with humans (3.8%) (Fig. 6A). In HIV-infected humans, as well as SIV-infected macaques, γδ T cells demonstrate an impaired proliferative response to bacterial Ags (31, 32). In contrast, γδ T cells from the two CD4-low mangabeys, as well as the CD4-healthy mangabeys, maintained their ability to proliferate in response to antigenic stimulation with the bacterial Ags IPP and LPS as well as Con A (Fig. 6B and C). It is interesting to speculate that in the absence

**FIGURE 5.** SIV-specific CD8+ T cell responses were assessed at 279 wpi in the two CD4-low mangabeys using SIVsmm peptide-specific stimulation and intracellular cytokine staining (18). A. Flow cytometry analyses of CD8+ T cells expressing IFN-γ following Gag peptide stimulation in two CD4-low mangabeys. Unstimulated cells were used to determine the level of background IFN-γ expression. B. Summary of the percentage of CD8+ T cells expressing IFN-γ following stimulation with gag, env, and pol peptide pools, as well as following staphylococcal enterotoxin B (SEB) and PMA and ionomycin stimulation. SIV-specific CD8+ T cell responses in the CD4-low and CD4healthy mangabeys were similar to published responses in other CD4-healthy mangabeys (18).

**FIGURE 6.** A, PBMC isolated from SIV− mangabeys (n = 14) and uninfected humans (n = 14) were stained for CD3 and Pan-γδ T cell receptors. Within the CD3+ T cell population, the percentage of peripheral blood γδ T cells was significantly greater in uninfected mangabeys (11.8%) compared with humans (3.8%) (*, p < 0.0001). B, PBMC from SIV− and SIV+ mangabeys at 100 wpi were stained with CFSE before stimulation. Histogram plots representing the number of CD3+/Pan-γδ+ cells (y-axis) vs the CFSE staining (x-axis) from a representative SIV-infected CD4-healthy mangabey, SM3. CFSE staining (black) was assessed in γδ T cells from SIV− and SIV+ mangabeys following stimulation with medium alone (M), HSP65, IPP, LPS, or Con A. C, Similar numbers of γδ T cells from both SIV− (left panel) and SIV-infected mangabeys (right panel) proliferated in response to medium alone, HSP, and Con A. A significant increase in the percentage of γδ T cells proliferating in response to IPP and LPS was observed between the SIV− and SIV+ mangabeys. (CD4-low SIV+ mangabeys are indicated by the arrows) (Student’s t test at 95% confidence interval).

**FIGURE 7.** A. Longitudinal analysis of the percentage of proliferating (Ki-67+) CD8+ T cells in CD4-low (black) and CD4-healthy (gray) SIV+ mangabeys indicating similar rates of CD8+ T cell proliferation in all mangabeys. B. Assessment of longitudinal plasma markers of inflammation by Luminex array indicated levels of IFN-γ and IL-1Ra were increased during acute infection, but declined to baseline levels during chronic infection in all mangabeys. Cytokine/chemokine levels fluctuated in a similar manner in the CD4-low (black) and CD4-healthy (gray) mangabeys.
A. Coreceptor usage was assessed at three time points: during acute (4 wk), second CD4 decline (43 or 71 wpi), and chronic (195 wk) infection. Results are normalized to the percentage of CD4/CCR5 coreceptor usage set at 100%. At 195 wpi, representative clones from two CD4-healthy mangabeys predominately used the CCR5 coreceptor (all CD4-healthy mangabeys used CCR5 exclusively at all time points tested). During acute infection (4 wpi), SM1 and SM2 predominately used CCR5; however, during the second CD4 decline at 43 and 71 wpi, respectively, a broadening in coreceptor usage occurred to include CXCR4, CCR8, and STRL33. At 195 wpi, expanded coreceptor usage was retained by the viruses isolated from SM1 and SM2. Error bars, Average value ± SD of triplicate wells for each clone tested.

B. Representative sequence alignments demonstrate an increase in the number of basic amino acid residues at 195 wpi was only observed in SM1 and SM2 (boxed clones), which corresponded to expanded coreceptor usage. No evidence for increased numbers of basic amino acids was observed for CD4-healthy mangabeys.

C. Percentage of CD3+CD4+ T cells expressing the CCR5 and CXCR4 coreceptors in a SIV+ mangabey was assessed using flow cytometry. More than 90% of CD4+ T cells expressed CXCR4 on their surface while <20% of CD4+ T cells expressed both CCR5 and CXCR4.
of chronic immune activation, the CD4-low mangabeys may preserve the functionality of numerous non-CD4+ immune cells that may be important in preventing disease progression during the SIV infection.

Absence of immune activation in CD4-low mangabeys

HIV-infected patients, as well as SIV-infected macaques, experience high levels of immune activation that were sustained throughout infection (36, 37). Indeed, the level of immune activation, particularly on CD8+ T cells, is a better predictor of the rate of disease progression than viral load or CD4+ T cell levels in HIV-infected patients (36). In contrast, it is well described that SIV-infected mangabeys exhibit relatively low levels of T cell proliferation or activation (11, 12, 23). Therefore, we sought to determine whether the loss of CD4+ T cells in SM1 and SM2 was associated with an increase in immune activation. An assessment of immune activation during acute infection identified a transient increase in the percentage of proliferating (Ki-67+) CD8+ T cells (Fig. 7A) that was associated with an increase in the plasma levels of the proinflammatory markers IFN-γ and IL-1Ra in all six mangabeys (Fig. 7B). During the chronic phase of the infection, the levels of Ki-67 in the CD8+ T cells, as well as levels of these proinflammatory markers (IFN-γ and IL-1Ra), remained low in the CD4-low mangabeys, similar to the CD4-healthy mangabeys (Fig. 7, A and B). Comparison of Ki-67 levels in CD4+ T cells was problematic due to the extremely low CD4+ T cell levels; however, we have previously determined that Ki-67+ CD4+ T cell levels in the CD4-low mangabeys was similar to that of the CD4-healthy mangabeys at times before the dramatic CD4 decline in SM1 and SM2 (16). These results indicate that SIV-infected mangabeys maintained similar levels of immune activation irrespective of their CD4+ T cell levels, suggesting that the severe CD4+ T cell depletion in SM1 and SM2 is not induced by, nor induces, a state of increased immune activation in these CD4-low SIV+ mangabeys.

Severe CD4+ T cell loss associated with expanded coreceptor usage

To explore the possibility that the severe, generalized CD4+ T cell depletion of these two animals is virally mediated, we conducted an analysis of coreceptor usage by SIV env genes isolated from mangabey plasma at time points before, during, and after the CD4 depletion. To infect a target cell, HIV and SIV require the interaction of the viral envelope glycoprotein (env) with the CD4 molecule and a coreceptor; CCR5 (R5) and CXCR4 (X4) are most commonly studied although other coreceptors can also be used (1). Using a cell-cell fusion assay, we observed that viruses encoding R5-tropic envs were isolated in each mangabey at 4 wpi (Fig. 8A), as previously described for other SIV isolates (38). During the second decline in CD4+ T cells in SM1 (week 43) and SM2 (week 71), we observed an expansion of coreceptor usage such that envs isolated from these time points had a substantially increased ability to use CXCR4, CCR8, and STRL33 for cell entry (Fig. 8A). Furthermore, at 195 wpi, the CD4-healthy mangabeys continued to replicate viruses that only used the CCR5 coreceptor (Fig. 8A). In contrast, the env protein isolated from the CD4-low mangabeys at 195 wpi had a substantially increased ability to use CXCR4 and, to a lesser degree, CCR8 (Fig. 8A), indicating that the virus had maintained its multivirulent phenotype. The coreceptors GPR1 and GPR15 (data not shown) were used at a low efficiency throughout infection in both CD4-low and CD4-healthy mangabeys, while STRL33 and CCR2 usage increased slightly in the CD4-low mangabeys (Fig. 8A). These data indicate that the increase in CXCR4 and CCR8 usage was associated with specific adaptations acquired by the envelope protein in SM1 and SM2 (Fig. 8A). Expanded coreceptor usage allowed these multivirulent viruses to infect a high percentage of CD4+ T cells because CXCR4 is expressed on a majority of CD4+ T cells, whereas CCR5 is only expressed on a minor percentage of CD4+ T cells (Fig. 8C and Ref. 39). Previous observations in patients infected with X4 or dual-tropic (R5/X4) HIV strains have shown that expansion of coreceptor usage is associated with an increase in basic amino acid residues in the V3 region of the envelope gene (40). We compared the sequences of SIV strains isolated from the CD4-low mangabeys at 4 and 195 wpi and found an increase in the number of basic residues in the V3 region following CD4 decline in SM1 and SM2 (Fig. 8B). The CD4-healthy mangabeys did not exhibit any increase in V3 region basic amino acids at 195 wpi (Fig. 8B). Taken together, these results identify the expansion of coreceptor usage as a major difference between the CD4-low (SM1, SM2) and CD4-healthy experimentally SIV-infected mangabeys and offer a mechanistic explanation for the generalized CD4+ T cell depletion observed in these animals. It is also interesting to note that emergence of an X4 or dual-tropic R5/X4 virus in HIV-infected individuals is typically associated with a poor disease prognosis (1), while expanded coreceptor usage in our CD4-low mangabeys has not resulted in any signs of clinical AIDS after >5.5 years.

Discussion

The ability of HIV to infect and kill CD4+ T cells has established a clear role for this important immune cell subset in HIV pathogenesis. However, numerous studies have identified dysfunction in other immune cell subsets, including γδ T cells, NK cells, dendritic cells, macrophages and B cells, as well as an increase in generalized immune activation during HIV infection (28–34). Because of the complex nature of HIV infection in humans, it has been difficult to determine the individual contribution of CD4+ T cell depletion, chronic immune activation, or dysfunction of other non-CD4+ T cells in disease pathogenesis. However, findings here indicate that an almost complete depletion of CD4+ T cells induced by a primate lentivirus infection is not sufficient to induce AIDS in a natural host. One possible explanation for the absence of disease signs in the SIV-infected CD4-low mangabeys is that this species has evolved to survive with very few CD4+ T cells. This hypothesis would postulate that 99% of the CD4+ T cells present in the blood, lymph node, and mucosa of uninfected mangabeys can in fact serve as a “buffer” in the event of a CD4-depleting SIV infection. An alternative hypothesis is that the low levels of immune activation observed in the CD4-low SIV-infected mangabeys are critical to maintaining overall immune system function and that CD4+ T cell levels, while important, are in fact less critical to preventing the onset of AIDS. This is evident by the maintenance of proper lymphoid architecture (Fig. 4), which likely enables other immune cell subsets to function properly. It is tempting to speculate that in the absence of chronic immune activation, SIV-infected mangabeys are able to maintain the function of numerous non-CD4+ immune cells, such as γδ T cells (Fig. 6), thus avoiding the onset of opportunistic infections, lymphomas, and AIDS. Moreover, the presence of SIV-specific Abs and CD8+ cytotoxic lymphocytes at times following the CD4 decline provides evidence of a maintained functional adaptive immune response in this CD4-low environment (Fig. 5 and Table 1). It is also possible that non-CD4+ immune cells, such as γδ T cells (Fig. 6) or CD3+ CD4/CD8 double-negative T cells (Fig. 2), may be undertaking some of the critical roles typically performed by CD4+ T cells. Future experiments to assess the phenotypic and functional properties of double-negative T cells in mangabeys may be useful in assessing the role of this T cell population. The maintained, or
possibly expanded, function of other immune cells, such as γδ T cells, CD3−/CD4/CD8 double-negative T cells, monocytes/dendritic cells, or NK cells, is likely important in preventing opportunistic infections and tumors in the two CD4-low mangabeys, since the CD4+ T cell depletion is equal to or greater than that associated with disease in SIV-infected macaques (24).

In this study, we have identified an association between CD4+ T cell depletion and an expansion in SIV coreceptor use in mangabeys that exhibit a CD4-low phenotype, a phenomenon typically associated with high levels of immune activation (36, 37) and disease progression in HIV+ patients (1–4). In macaques dually infected with R5-SHIVSF162P3 and X4-SHIVSF33A, the X4 SHIV replication was more sensitive to the presence of CD8+ T cell-mediated antiviral immune response as demonstrated by the increase in the relative proportion of X4 SHIV viremia following CD8+ T cell depletion (10). The appearance of the multiruptive viruses in SM1 and SM2 at time points during the CD4 decline (43 and 71 wpi, respectively) is suggestive that the env gene evolved to use additional coreceptors as opposed to the emergence of preexisting dual-tropic variants from the inoculum. Additionally, preliminary results following passage of the multiruptive SIVsmm from the CD4-low mangabey, SM2, to three additional mangabeys has resulted in rapid, generalized CD4+ T cell depletion within 14 days in the blood (Fig. 9) and mucosal-associated lymphoid tissues. These low CD4 levels have been maintained below 80 CD4+ T cells per mm3 blood thus far through 154 days postinfection in all three mangabeys, providing further evidence that the CD4 depletion is indeed virally mediated. Multiple lines of evidence indicate that the expansion of coreceptor usage induced the CD4 depletion observed in the CD4-low mangabeys, including: 1) no change in immune activation parameters before or during the second CD4 decline; 2) the ability to detect R5/X4/R8 multiruptive virus during the period of the second CD4 decline; and 3) the ability to deplete CD4+ T cells following administration of the CD4-low virus to three additional mangabeys. In summary, our results suggest that the depletion of CD4+ T cells is virally mediated, but is not sufficient to induce clinical signs of immunodeficiency in the SIV/mangabey model.

CD4+ T cells are generally the major site of HIV/SIV replication, although replication has also been observed in a number of other cell types (41–45). In this study, the ability of SIVs replicating in the CD4-low mangabeys to use an expanded repertoire of coreceptors could potentially impact the types of cells in which the virus was replicating. Two pieces of evidence in our study indicate that CD4+ T cells are a major site of SIV replication in the CD4-low mangabeys, including: 1) the observed decrease in viral load following the CD4 T cell depletion and 2) the identification of SIV RNA+CD3+ T cells in lymph nodes. However, SIV RNA+macrophages could also be detected at a low frequency (Fig. 3). The observation that macrophages were infected during CD4-depleting SIV infections has been demonstrated previously during chimeric SHIV infection which uses both CCR5 and CXCR4 coreceptors (46). The exact contribution of the SIV+ monocytes/macrophages in maintaining the plasma viral loads (104–106 virions/ml of plasma) in the CD4-low mangabeys is not known, although it is likely that they represent an increased percentage of SIV production in the CD4-low mangabeys compared with the CD4-healthly mangabeys.

The data presented here support a model in which the major immunologic abnormality occurring during a pathogenic infection is likely the combination of: 1) A generalized loss of CD4+ T cells in secondary and mucosal-associated lymphoid tissues and 2) chronic immune activation, which may be the key factor in mediating the dysfunction of numerous immune cell subsets including CD8+ T cells, NK cells, dendritic cells, B cells, γδ T cells, and macrophages (28–34). Several mechanisms may be operative in inducing chronic immune activation, including the translocation of bacterial Ags (47), impaired function of T regulatory cells (48, 49), nef-mediated immune activation (50), and/or generalized cytokine dysregulation. In light of these new data from the SIV mangabey model, it becomes important to consider multifaceted therapeutic strategies that can decrease viral loads while reducing the global immune activation and enhancing the number/function of multiple immune cell subsets during chronic HIV infection.

Acknowledgments

We acknowledge the excellent animal care and veterinary staff at the Yerkes National Primate Center, F. Scott, A. Mobley, L. Carbalaj, P. Arca, J. Engram, S. Elmhert, B. Cervasi, Z. Ambrose, and Y. X. Xu for technical assistance; Louis Picker and Robert Doms for helpful comments and discussions; Dr. H.-Y. Wang for pathologic assessment of lymph nodes from the SIV+ CD4-low and CD4-healthy mangabeys; Elizabeth A. Strobert, Harold McClure, and Jim Else for assistance and advice regarding mangabey experimentation; and A. Leone, A. Durudas, G. Lehman, and K. Mir for helpful comments. Confocal microscopy was performed at the University of Texas Southwestern (RR017740).

Disclosures

The authors have no financial conflict of interest.

References

3056 VIRALLY-INDUCED CD4 DEPLETION IN A NATURAL HOST

viruses expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. J. Virol. 70: 6922–6928.


