Acute and Chronic T Cell Dynamics in the Livers of Simian Immunodeficiency Virus-Infected Macaques

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The mucosal immune system, particularly the gastrointestinal tract, is critically involved in the pathogenesis of human immunodeficiency virus (HIV) infection. Since the liver drains most of the substances coming from the intestinal tract, it may also play a role in the pathogenesis of HIV infection. Here we examined the percentages and absolute numbers of T cell subsets in the liver in normal and simian immunodeficiency virus (SIV)-infected macaques. Most of the T cells in the liver were CD8⁺ memory cells, and most of these had an effector memory (CD95⁺ CD28⁻) phenotype. CD4⁺ T cells constituted approximately 20% of the liver T cell population, but the vast majority of these were also memory (CD95⁺) CCR5⁺ cells, suggesting they were potential targets for viral infection. After SIV infection, CD4⁺ T cells were markedly reduced, and increased proliferation and absolute numbers of CD8⁺ T cells were detected in the liver. These data suggest that the liver is a major source of antigenic stimulation for promoting CD8⁺ T cells and possibly a source for early CD4⁺ T cell infection and destruction.

Regardless of the route of transmission, the mucosal immune system in general and the gastrointestinal system in particular (11) are central to the pathogenesis of human immunodeficiency virus (HIV) infection, with most critical events, e.g., transmission, viral amplification, and CD4⁺ T cell destruction (7, 15, 25), occurring in the intestinal tract. The liver receives all the venous blood from the intestine, including nutrients and toxins from the intestinal contents, and thus, it is conceivable that the liver also plays a role in the immunology and pathogenesis of simian immunodeficiency virus (SIV)/HIV infection. The liver has also been identified as a major source or reservoir of SIV-specific CD8⁺ T cells in SIV-infected macaques (20). Moreover, evidence suggests that the liver is a major site of rapid viral “clearance” in SIV-infected macaques (29).

Secondary or predisposing infections in the liver are also a major problem in HIV patients. Hepatitis associated with HIV-1 is being seen with increasing frequency as this virus becomes more prevalent in diverse human populations. Up to three-quarters of patients with AIDS have serious liver abnormalities (13, 21). Secondary infections with hepatitis B and/or C viruses are a major cause of morbidity and mortality in this population (3). In the United States, 150,000 to 300,000 persons are coinfecting with HIV and hepatitis C virus (HCV), representing 15 to 30% of all HIV-infected persons (22). Finally, HIV infection is often accompanied by periportal lymphocytic infiltration and bile duct proliferation in the liver (1, 9, 14). These pathogenic manifestations can be of mild or moderate severity. Despite the fact that many studies examined liver pathology during AIDS development (12), little is known about the relationship between HIV infection and the effect on hepatic lymphocytes.

It has been suggested that the liver is a major site for the trapping and destruction of activated T lymphocytes, and the liver has been referred to as a “graveyard” for senescent or dying T lymphocytes (6). Activated CD8⁺ T lymphocytes, primed in response to an antigenic challenge, enter the blood and circulate through the tissues. A subset of CD8⁺ T cells undergoes apoptosis, and among these cells, an unusually large population is trapped in the liver. Such trapping of activated, virus-specific CD8⁺ T lymphocytes has been described in SIV and influenza infection (2, 20). How-

ever, it is unknown whether the large numbers of virus-specific CD8⁺ T cells described in the liver are truly senescent, dying cells that have migrated from other tissues or whether they are recently primed cells resulting from SIV/HIV exposures in the liver.

The aim of this study was to examine and compare the kinetics of lymphocytes in the liver and peripheral blood throughout SIV infection by comparing the absolute numbers and phenotypes of CD4⁺ and CD8⁺ T lymphocytes. We also quantified absolute numbers of naïve central and effector memory lymphocytes per mm² of liver by using immunohistochemistry (IHC) and quantitative image analysis. Understanding the impact of SIV infection on the phenotype of CD4⁺ and CD8⁺ T lymphocytes in liver during various stages may provide useful information for understanding the immunology of the liver and the mechanisms of immunosuppression that may predispose the liver to opportunistic or comorbid infections.

MATERIALS AND METHODS

Animals, virus, and 5-bromo-2'-deoxyuridine (BrdU) labeling. A total of 50 male and female rhesus macaques (*Macaca mulatta*) between 3 and 20 years of age that were housed at the Tulane National Primate Research Center were used to examine lymphocytes in liver and peripheral blood. Macaques euthanized at early time points (21 days or fewer) were intravenously inoculated with 100 50% tissue culture infective doses (TCID₅₀) of SIVmac251. Chronically infected animals were drawn from pathogenesis studies in which animals were inoculated either intravenously (IV), intravaginally (IVAG), or intrarectally (IR) with SIVmac251. For immunohistochemistry and quantitative image analysis of CD3⁺ cells in liver, macaques were divided into the following groups: uninfected controls ($n = 8$); acute infection (7 to 21 days postinoculation [DPI]; $n = 18$), subdivided into 7 to 10 DPI ($n = 9$), 13 DPI ($n = 5$), and 21 DPI ($n = 4$); chronic asymptomatic infection ($n = 7$); and AIDS ($n = 17$). All animals

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FIG 1 Immunohistochemistry for CD3⁺ (dark brown) in liver from a normal uninfected macaque (A) and a macaque with chronic SIV infection (B). Note the clusters of T cells which are in the portal area in panel B. (C) Absolute numbers of CD3⁺ T cells per mm² of liver in uninfected macaques (controls) and macaques in various stages of SIV infection, as determined by immunohistochemistry for CD3. Note that the significant increases in CD3⁺ T cells per mm² are detected after SIV infection and in macaques with AIDS. Asterisks indicate significant differences from the control values ($P < 0.05$).

were monitored for general health by monthly physical examinations, complete blood count (CBC) serum chemistries (protein, albumin, globulin, alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP], electrolytes, blood urea nitrogen [BUN], glucose, creatinine [Cr]), and plasma SIV *gag* RNA levels. Serum chemistries were determined using a Cobas Mira chemistry analyzer (Roche, Rotkreutz, Switzerland) at the Clinical Chemistry Laboratory at the Tulane Regional Primate Research Center (TRPRC). Clinical variables monitored included body weight, general physical condition, presence of lymphadenopathy, splenomegaly, presence of rashes or skin abnormalities, condition of oral and conjunctival mucosas, diarrhea, and signs of opportunistic infections.

For flow cytometry, data from 22 macaques were divided into three groups: uninfected controls ($n = 8$), acute infection (7 to 21 DPI; $n = 7$), and AIDS ($n = 7$). Animals in acute infection were humanely euthanized on 7, 10, 13, or 21 DPI. Animals with AIDS were humanely euthanized between 414 and 1,071 DPI. No significant difference in the age of the animals at euthanasia was present between the groups. For *in vivo* BrdU labeling, BrdU (Sigma) was dissolved in saline, filter sterilized, and intraperitoneally inoculated into macaques at 60 mg/kg of body weight 24 h prior to euthanasia and tissue collection. All animals were maintained in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International, and all studies were approved by the Tulane Institutional Animal Care and Use Committee.

Tissue collection and analysis. Whole-blood (WB) samples were stained using a whole-blood lysis protocol as described previously (28). Liver cell suspensions were prepared by mincing approximately 3 g liver tissue into 2- to 3-mm pieces and incubating with 1 mM EDTA in Hanks balanced salt solution for 30 min, followed by 1 h in RPMI medium containing 20 U of collagenase per ml while rapidly shaking at 37°C. Liver samples were further disrupted, and single-cell suspensions were prepared by gently pipetting 5 to 10 times with a 16-g feeding needle. Cell suspensions were adjusted to 10^7 /ml, and 100-ml aliquots (10^6 cells) were stained for 30 min at 4°C with appropriately diluted concentrations of monoclonal antibodies CD3-AL700 (Invitrogen), CD8-PE-TxR (Caltag Laboratories), CD4-Qdot655 (NIH), CD28-APC, CD95-PE-Cy5, CD20-APC-Cy7 (BD Biosciences), CCR5-PE, BrdU-APC, and AC3-FITC for flow cytometry. For intracellular staining, surface-stained cells were washed in phosphate-buffered saline (PBS)/bovine serum albumin (BSA), fixed, and permeabilized with BD Cytotfix/Cytoperm buffer, followed by staining for BrdU, according to the manufacturer's instructions,

including 1 h of incubation with DNase, followed by washing with BD Perm/Wash buffer and staining with fluorescent anti-BrdU for 30 min at room temperature. Cells were washed again with BD Perm/Wash buffer, and samples were resuspended in BD stabilizing fixative buffer and acquired on a FACS Aria flow cytometer (Becton Dickinson) within 24 h of fixation. Data were analyzed with FlowJo software (Tree Star Inc.). At least 20,000 lymphocytes were collected, and data were analyzed by gating through lymphocytes and then through cells of interest.

Quantitation of lymphocytes in the liver and peripheral blood. Sections of formalin-fixed, paraffin-embedded liver tissues were stained for CD3 (T lymphocytes) by immunohistochemistry as previously described (3a). Briefly, tissue sections were deparaffinized in xylene and then rehydrated in alcohol gradients and finally water. Antigen retrieval was done by steam ($>95^\circ\text{C}$) in $1\times$ citrate buffer (pH 6.0) for 20 min, and then cooled slides were washed with $1\times$ Tris-buffered saline (TBS) solution. A protein block with Dako serum-free protein block (Carpenteria, CA) was performed, followed by a tissue peroxidase block using a peroxidase blocking reagent (Dako). After washing in TBS, slides were incubated with rabbit anti-human CD3 antibody (Dako) appropriately diluted in protein block for 60 min, followed by a TBS wash and amplification with a Mach 3 biotin-free alkaline phosphatase system (Biocare, Concord, CA) per the manufacturer's directions. CD3 lymphocytes were detected by Ferangi Blue (Biocare) or 3,3'-diaminobenzidine. Tissues were imaged using a $20\times$ objective ($200\times$) on a Leica DM LB microscope (Leica, Buffalo Grove, IL) with a Spot Insight camera utilizing Spot imaging software (Diagnostic Instruments Inc., Sterling Heights, MI). Images of 10 non-contiguous random fields, each of which was $280\ \mu\text{m}^2$, were captured from each liver section, and the numbers of CD3⁺ cells within each image were counted manually and reported as mean cells/mm² (Fig. 1).

Absolute numbers were determined for the lymphocytes in the liver by using a combination of the CD3 immunohistochemistry described above and flow cytometry. Flow cytometry percentages of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ lymphocytes within the liver lymphocyte gate (Fig. 2A) were multiplied by the mean CD3 absolute lymphocyte counts from the IHC analysis of liver tissue for each individual animal to obtain absolute numbers of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ lymphocytes, respectively (Fig. 2B to D). Next, absolute numbers of T lymphocytes were multiplied by the percentages of CD3⁺-naïve, memory, activated, or chemokine receptor-expressing CD4⁺ or CD8⁺ lymphocytes. The resultant product was the absolute number of lymphocyte subsets in liver per mm².

FIG 2 Gating strategy to detect T cell subsets by flow cytometry. (A) Cells were gated through lymphocytes and then CD3⁺ T cells, and percentages of CD4⁺ and CD8⁺ T cells were expressed as percentages of total T cells. SSC-A, side scatter; FSC-A, forward scatter. To calculate absolute numbers of T cell subsets in the liver (B to G), CD3⁺ cells were counted on immunohistochemically stained sections of liver and T cell subsets were calculated by multiplying absolute CD3⁺ T cell numbers in the liver with the subset of interest, as determined by flow cytometry. (B to I) Changes in absolute numbers and percentages of CD4⁺ and CD8⁺ T cells in the liver and blood are shown for macaques with acute infection and macaques with AIDS. Asterisks indicate significant differences from the control values ($P < 0.05$).

Absolute numbers of lymphocytes were determined by flow cytometry and the CBC lymphocyte numbers determined on EDTA anticoagulated blood collected prior to sacrifice. Percentages of CD4⁺ and CD8⁺ lymphocytes within the WB lymphocyte gate were multiplied by absolute lymphocyte counts from the CBC analysis for each individual animal to obtain absolute numbers of CD4⁺ and CD8⁺ lymphocytes, respectively.

Statistics. Statistical analysis was performed with a nonparametric Mann-Whitney U test and with a Kruskal-Wallis analysis of variance test

using GraphPad Prism software (GraphPad Software, San Diego, CA). A P value of <0.05 was considered significant. All data are expressed as means $\pm$ standard errors of the mean (SEM).

RESULTS

Increased numbers of T lymphocytes in the liver during SIV infection. In the livers of all 42 infected and 8 uninfected macaques (Table 1), CD3⁺ lymphocytes were quantified. Using a nonparametric Mann-Whitney U test, absolute numbers of T lymphocytes were significantly higher in some groups of infected macaques, with mean values of 224 ± 15.7 CD3⁺ cells/mm² for the 21-DPI group and 257 ± 40.6 CD3⁺ cells/mm² for the AIDS group versus 139 ± 12.5 CD3⁺ cells/mm² for the uninfected group (P values of 0.01 and 0.004, respectively) (Fig. 1). The Kruskal-Wallis analysis of variance test also revealed significant differences between groups. Normally, uninfected animals had very few lymphocytes in the liver (Fig. 1A), but in SIV-infected macaques, clusters of lymphocytes were often observed in portal areas (Fig. 1B), similar to what has previously been described in HIV-infected patients (1, 9, 14). Although there was a positive trend in acutely infected animals, numbers of CD3⁺ lymphocytes/mm² in other infected groups (8, 16, or 13 DPI or chronic asymptomatic) did not show statistically significant differences from that of controls (Fig. 1).

Changes in CD4⁺ and CD8⁺ T lymphocytes in liver and peripheral blood. Cell subsets were determined in the liver by gating first through lymphocytes and then through CD3⁺ T cells (Fig. 2A). Percentages and absolute numbers of CD4⁺ T lymphocytes in the liver decreased significantly in AIDS macaques, with a mean value of 21 CD4⁺ T cells/mm² of liver tissue for the control group and 7 CD4⁺ T cells/mm² for the AIDS group ($P = 0.006$) (Fig. 2B and C). Percentages and absolute numbers of CD4⁺ T lymphocytes in acute infection remained similar to those of controls (19 versus 21 cells/mm² of liver, respectively). Absolute numbers of CD8⁺ T lymphocytes slightly decreased in acute infection (7 to 21 DPI) but rebounded to higher levels in AIDS macaques compared to that of controls (Fig. 2F). The CD4/CD8 ratio in the liver was 1:3 in controls and remained the same in acute infection (data not shown). However, in the AIDS-infected group, this increased to 1:14, which may be due to significant decreases in numbers of CD4⁺ cells and increases in numbers of CD8⁺ cells (data not shown). In the peripheral blood, the absolute number of CD4⁺ T lymphocytes decreased significantly in the AIDS group (Fig. 2D), with mean values of 650 CD4⁺ T cells/ μ l blood for the controls and 80 CD4⁺ T cells/ μ l blood for the AIDS group ($P \leq 0.002$). Percentages of CD4⁺ T cells also significantly decreased in acute and AIDS stages (Fig. 2E). In contrast, numbers of CD8⁺ T lymphocytes remained fairly stable in liver (Fig. 2F) and blood (Fig. 2H), but percentages of CD8 cells increased in the liver in AIDS (Fig. 2G) and in both acute and chronic infection in blood (Fig. 2I).

Naïve and memory CD4⁺ and CD8⁺ cell populations in liver and peripheral blood. Numbers of naïve (CD28⁺ CD95⁻) CD4⁺ T cells remained stable (~ 3 cells/mm²) in the liver throughout infection (Fig. 3A). However, there was a significant depletion of effector memory (CD28⁻ CD95⁺) CD4⁺ T cells in macaques with AIDS from 11 (control) to 2 (AIDS) cells/mm² (Fig. 3B), as well as a significant decrease in central memory (CD28⁺ CD95⁺) CD4⁺ T cells in the AIDS group from 8 (control) to 3 (AIDS) cells/mm² (Fig. 3C). However, no correlations were observed between the

TABLE 1 Rhesus macaques used in this study

Disease phase and animal ID	Sex	Age at necropsy (yr)	DPI at necropsy ^a	SIVmac251 inoculation route ^b	Specific disease phase and necropsy diagnosis ^c
Control					
AG71	Female	11			No significant lesions found
BB01	Female	13			No significant lesions found
BE84	Female	11			No significant lesions found
CC10	Female	15			No significant lesions found
GN58	Female	13			No significant lesions found
GN70	Female	10			No significant lesions found
GN74	Female	13			No significant lesions found
N483	Female	16			No significant lesions found
Acute					
HI52	Female	5	7	IV	No significant lesions found
AV63	Female	4	8	IV	Lymphoid hyperplasia
BA57	Female	14	8	IV	Lymphoid hyperplasia
HI53	Female	7	8	IV	Lymphoid hyperplasia
T108	Female	13	8	IV	Lymphoid hyperplasia
HI58	Female	7	12	IV	Lymphoid hyperplasia
HI63	Female	7	12	IV	Lymphoid hyperplasia
M992	Female	16	13	IV	Lymphoid hyperplasia
AV85	Female	8	21	IV	Lymphoid hyperplasia
CB74	Female	3	21	IV	Lymphoid hyperplasia
BI58	Male	3	21	IV	Lymphoid hyperplasia
BN37	Male	3	21	IV	Lymphoid hyperplasia
BV13	Male	3	8	IV	Lymphoid hyperplasia
AV91	Male	14	10	IV	Lymphoid hyperplasia
L880	Male	11	10	IV	Lymphoid hyperplasia
C419	Male	20	10	IV	No significant lesions found
T139	Male	7	13	IV	Lymphoid hyperplasia
BA17	Male	8	13	IV	Lymphoid hyperplasia
Chronic					
N998	Female	10	180	IVAG	ASY; lymphoid hyperplasia
R908	Female	9	181	IVAG	ASY; lymphoid hyperplasia
I533	Female	11	646	IV	AIDS; pneumocystic pneumonia
R544	Female	9	414	IVAG	AIDS; meningoencephalitis
AP53	Female	6	63	IV	AIDS; septicemia, bacterial pneumonia
HG45	Female	14	148	IV	AIDS; pneumocystic pneumonia
HG58	Female	11	283	IVAG	AIDS; lymphoid hyperplasia, encephalitis
HG49	Female	11	145	IVAG	ASY; lymphoid hyperplasia
HG56	Female	10	152	IVAG	ASY; lymphoma, stomach
FA14	Female	9	1,068	IV	AIDS; pneumocystic pneumonia
HI68	Female	10	155	IVAG	AIDS; CMV
BD78	Male	4	205	IV	AIDS; vasculitis, pulmonary thrombosis
BE65	Male	6	1,071	IV	AIDS; mycobacterium avium pneumonia
T196	Male	7	230	IV	AIDS; pneumocystic pneumonia
DJ18	Male	7	173	IV	AIDS; CMV, pneumonia
FE53	Male	4	140	IR	AID; hepatitis, gastroenterocolitis
DE09	Male	4	76	IR	ASY; lymphoid hyperplasia
FK47	Male	5	98	IR	ASY; lymphoid hyperplasia
FD49	Male	5	110	IR	ASY; lymphoid hyperplasia
CF35	Male	5	91	IR	ASY; lymphoid hyperplasia
BV74	Male	5	63	IR	ASY; lymphoid hyperplasia
DI28	Male	3	80	IR	ASY; lymphoid hyperplasia
DL31	Male	3	128	IR	ASY; lymphoid hyperplasia
DB53	Male	5	77	IR	ASY; lymphoid hyperplasia

^a DPI, days postinoculation.^b IV, intravenous; IVAG, intravaginal; IR, intrarectal.^c The specific disease phase is given for the animals with chronic infection in order to distinguish between asymptomatic animals and those with AIDS. ASY, asymptomatic; CMV, cytomegalovirus.

FIG 3 Absolute numbers of CD4⁺ lymphocyte subsets in the liver (A to C) and blood (D to F), as determined by combined flow cytometry and either immunohistochemistry or CBCs (see text). Absolute numbers of naïve (A and D) (CD28⁺ CD95⁻), effector memory (B and E) (CD95⁺ CD28⁻), and central memory (C and F) (CD95⁺ CD28⁺) cells in the liver and blood. Asterisks indicate significant differences from the control values ($P < 0.05$).

numbers of CD4⁺ T cells in the liver and the plasma viral loads in infected macaques (data not shown).

In the peripheral blood, there was a significant decrease in the numbers of naïve CD4⁺ T cells, with mean values of 160 cells/ μ l blood for the AIDS group and 280 cells/ μ l blood for the controls ($P = 0.006$), as well as a modest decrease in the absolute number of naïve CD4⁺ T cells in the acute group (Fig. 3D). The absolute numbers of effector memory (Fig. 2E) and central memory (Fig. 2F) CD4⁺ T cells in the peripheral blood also decreased.

Naïve CD8⁺ T cells remained stable (~ 8 cells/mm²) in the liver throughout the course of the study (Fig. 4A), but absolute numbers of effector memory CD8⁺ T cells in the liver were modestly decreased in acute and AIDS macaques, although this did not reach statistical significance (Fig. 4B). However, central memory CD8⁺ T cells in the livers of AIDS macaques were significantly increased from 22 (control) to 53 cells/mm², and there was a modest increase in the absolute numbers of central memory CD8⁺ T

FIG 4 Absolute numbers of CD8⁺ lymphocyte subsets in the liver (A to C) and blood (D to F), as determined by combined flow cytometry and either immunohistochemistry or CBCs (see text). Absolute numbers of naïve (A and D) (CD28⁺ CD95⁻), effector memory (B and E) (CD95⁺ CD28⁻), and central memory (C and F) (CD95⁺ CD28⁺) cells in the liver and blood. Asterisks indicate significant differences from the control values ($P < 0.05$).

FIG 5 Absolute numbers and percentages of CD4⁺ CCR5⁺ (A to D) and CD8⁺ CCR5⁺ (E to H) T cells in the liver and blood, as determined by combined flow cytometry and either immunohistochemistry or CBCs (see text). Asterisks indicate significant differences from the control values ($P < 0.05$).

cells in acutely infected macaques (Fig. 4C). In the blood, absolute numbers of naïve CD8⁺ T cells decreased significantly, with means of 40 (acute), 30 (AIDS), and 15 (control) cells/ μ l blood ($P \leq 0.02$ and $P \leq 0.001$ for acute infection and AIDS, respectively) (Fig. 4D). A modest increase in the absolute numbers of central memory CD8⁺ T cells in the peripheral blood of acutely infected macaques was also observed, but it did not reach statistical significance (Fig. 4E). A similar trend was observed for effector memory CD8⁺ T cells in the peripheral blood (Fig. 4F).

CD4⁺ and CD8⁺ T lymphocytes expressing CCR5 in liver and peripheral blood. Since CCR5 is the major coreceptor for HIV and a major marker for activated memory cells, we examined CCR5 expression on CD4⁺ cells in liver for comparison with expression on CD4⁺ cells in blood. Absolute numbers (Fig. 5A) and percentages (Fig. 5B) of CD4⁺ CCR5⁺ T lymphocytes decreased significantly in the livers of acutely infected macaques and AIDS-infected animals ($P \leq 0.01$) (Fig. 5A). Absolute numbers of CD8⁺ CCR5⁺ T lymphocytes decreased in acute infection (7 to 21 DPI)

FIG 6 Absolute numbers and percentages of proliferating (BrdU⁺) CD4⁺ and CD8⁺ T cell subsets in the liver and blood are shown for CD4⁺ T cells (A to D) and CD8⁺ T cells (E to H). Asterisks indicate significant differences from the control values ($P < 0.05$).

but rebounded to slightly higher levels in AIDS-infected animals compared to that in controls (Fig. 5E), but the changes were not statistically significant. Similarly, no significant changes were detected in percentages of CD8⁺ CCR5⁺ T cells (Fig. 5F).

In the blood, absolute numbers of CD4⁺ CCR5⁺ T lymphocytes significantly decreased in both the acute and AIDS groups (as previously described [26]), with a mean of 240 cells/ μ l blood for the controls versus 8 cells/ μ l blood for the acute group and 4 cells/ μ l blood for the AIDS group ($P \leq 0.006$ and $P \leq 0.001$, respectively) (Fig. 5C). However, percentages of CD4⁺ CCR5⁺ and CD8⁺ CCR5⁺ lymphocytes remained fairly stable throughout the course of the study (Fig. 5D and H).

Proliferation and apoptosis of T lymphocytes in liver and peripheral blood. In the livers of SIV-infected macaques, there were no significant changes in numbers or percentages of proliferating CD4⁺ T cells (Fig. 6A and B). Absolute numbers of proliferating cells were, however, reduced in blood in acute infection (Fig. 6C). Percentages and numbers of CD8⁺ T cells in S-phase

division (BrdU⁺) in acutely infected macaques were higher than those in the controls (Fig. 6E and F). Similar trends in increased rates of proliferation were observed for CD8 cells in blood (Fig. 6G and H).

Numbers of apoptotic (activated caspase-3 [AC3⁺]) CD4⁺ T cells were increased in liver in acute infection but not significantly (Fig. 7A). However, higher percentages of CD4⁺ T cells were committed to apoptosis in the livers of both acute- and AIDS-stage macaques ($P \leq 0.009$ and $P \leq 0.005$, respectively) (Fig. 7B). In blood, there were no significant changes in absolute numbers of CD4⁺ AC3⁺ cells (Fig. 7C), but there were significantly higher percentages in macaques in the AIDS group (Fig. 7D). Similarly, there were significantly higher percentages and numbers of AC3⁺ CD8⁺ T cells in livers of SIV-infected macaques (Fig. 7E and F). In the peripheral blood, CD8⁺ T cells did not show any significant differences in AC3⁺ expression after SIV infection (Fig. 7G and H).

Liver function enzymes and clinical chemistries. Average basal circulating levels of markers of liver function, including ALT, ALP, and AST, were monitored monthly in all animals throughout the study but were not outside normal ranges, even in chronic, asymptomatic SIV infection (ALT, 36.4 ± 4.2 ; AST, 52.7 ± 9.8 ; ALP, 355 ± 28.4 IU/liter) or in animals euthanized with AIDS (who were not affected by chronic SIV infection) (ALT, 35.8 ± 6.5 ; AST, 47.9 ± 4.7 ; ALP, 204.3 ± 38.7 IU/liter). BUN, creatinine, and BUN/Cr ratios were also within normal limits (data not shown). Chronic SIV-infected animals did have increased globulin levels, resulting in increased albumin/globulin ratios (data not shown), and all of these results are consistent with those of previous reports of SIV infection (18).

DISCUSSION

Although not typically classified as an immunologic organ, the liver contains substantial numbers of T cells, macrophages (Kupffer cells), and other immune cells. Further, the liver drains most of the blood from the intestine, which is the major site of CD4⁺ T cell depletion and viral amplification in early SIV/HIV infection (5, 17, 25). Normally, the liver filters potential pathogens and inflammatory mediators from the portal blood, carefully maintaining a balance between immune tolerance and immune responses, which may be altered in SIV/HIV pathogenesis.

Studies have shown that microbial translocation from the gastrointestinal tract plays a major role in chronic immune activation, a characteristic feature of progressive HIV disease (4). However, while the degree of microbial translocation correlates directly with disease progression, it is notably mild or absent during acute infection (4), in which gut CD4 T cells are first depleted and then mucosal damage is detected (19, 25). Therefore, a temporal disconnect exists between the acute loss of CD4 T cells caused by HIV/SIV infection, mucosal damage, and the onset of systemic microbial translocation. Recently, however, elevated microbial translocation in macaques with chronic SIV infection was associated with intestinal epithelial damage and decreased phagocytic ability of gut epithelial macrophages, results which did not occur until late acute phase or in chronically infected macaques (8). Moreover, this study showed elevated microbial lipopolysaccharide (LPS) levels within the livers of chronically infected animals (8), but immunologic changes in the livers of acutely infected animals were not examined.

Liver T cells are located within the sinusoids and in periportal

FIG 7 Absolute numbers and percentages of apoptotic/activated caspase-3 (AC3⁺) T cell subsets in the liver and blood are shown for CD4⁺ T cells (A to D) and CD8⁺ T cells (E to H). Note that there are significant increases in percentages of apoptotic/AC3⁺ CD4⁺ T cells (B) and both absolute numbers and percentages of CD8⁺ T cells (E and F) in the liver. Absolute numbers of apoptotic CD4⁺ T cells were increased in the liver in acute infection (A) but not significantly. Similar trends were noted in blood, but significant differences were only detected for apoptotic CD4⁺ T cells in macaques with AIDS (D). Asterisks indicate significant differences from the control values ($P < 0.05$).

areas of SIV-infected macaques and HIV-infected patients (1, 9, 14) (Fig. 1). However, and unlike in other tissues, T cells in normal liver are predominantly CD8⁺, with markedly fewer CD4⁺ T cells than in lymphoid tissues or blood (23). This suggests that there are differential homing pathways for T cells in the liver compared to those in peripheral blood, lymph nodes, or spleen, in which there is usually a 1:1 or 2:1 ratio of CD4 to CD8 T cells (16, 20, 27). Here we show that following SIV infection, absolute numbers of CD3⁺ T lymphocytes increased significantly in the livers of SIV-infected

macaques at 21 DPI and in animals with AIDS, as demonstrated by morphometric analysis of immunohistochemically stained sections (Fig. 1). However, this was accompanied by a dramatic loss of effector CD4⁺ T cells and, in later stages of infection, marked increases in total CD8⁺ T cells, indicating selective loss of CD4⁺ T cells in the liver in acute infection, much like that previously described in the intestinal tract (28). Despite losses of CD4⁺ T cells, absolute numbers and percentages of CD8⁺ T lymphocytes in liver increased in animals with AIDS (Fig. 2F and G). Percentages

of CD8⁺ lymphocytes in peripheral blood also increased in animals with AIDS (Fig. 2). In normal macaques, the CD4/CD8 ratio in the livers of uninfected macaques was 1:4, and this remained the same in acute infection. However, in animals with AIDS, it decreased to 1:14, reflecting a massive decline in absolute numbers of CD4⁺ T cells and a concurrent increase in CD8⁺ T cells (data not shown).

Since the liver drains the gut, we had hypothesized that large numbers of infected CD4⁺ T cells or liver macrophages (Kupffer cells) would be detected in the liver, especially early in SIV infection, preceding the loss of liver CD4⁺ T cells. However, none of the animals in acute infection had detectable infected cells, and only one animal with AIDS (and marked lymphocytic infiltration) showed any detectable infected cells in the liver. Further, we did not find a correlation between levels of hepatic CD4⁺ T cell loss and plasma viremia (data not shown). Thus, we could not definitively prove the mechanism of hepatic CD4⁺ T cell loss in SIV infection. However, we did demonstrate increased levels of CD4⁺ T cell apoptosis (AC3) in the livers of infected animals, a process which may be the major mechanism for CD4⁺ T cell loss, at least in this tissue. The marked loss of liver CD4⁺ T cells in the absence of detectable infected cells suggests that these cells are lost through apoptosis triggered by viral particles or antigens yet not replicating virus.

In blood, the CD4/CD8 ratio was 1.5:1 in controls and inverted in AIDS. There were no significant differences in the percentages of CD4⁺ BrdU⁺ lymphocytes in the liver during the acute and AIDS stages compared to that in controls. This suggests the loss of CD4⁺ T cells in the liver is not accompanied by significant attempts to reconstitute CD4⁺ T cells. However, if proliferating cells are being infected and eliminated in tissues at matching rates, this may not be adequately reflected in the sampling and assay methods employed (24). Further, we did detect significantly higher percentages of CD4⁺ T lymphocytes committed to apoptosis (AC3⁺) during the acute and AIDS phases of SIV infection in the liver. Significantly higher percentages of AC3⁺ CD4⁺ lymphocytes were also found in the blood of macaques in the AIDS group. Combined, these data indicate that CD4⁺ T cells are being eliminated in the liver and are not capable of full restoration, whereas CD8⁺ T cells are proliferating and expanding in the liver after SIV infection.

It has been suggested that large numbers of CD8⁺ T lymphocytes that accumulate in the liver in the setting of various systemic viral infections are trapped, "end-stage" cells that will eventually be eliminated by apoptosis (2, 10). However, we observed significantly higher percentages of CD8⁺ BrdU⁺ lymphocytes in liver during the acute and AIDS phases of SIV infection (Fig. 6F), suggesting that the early loss of CD8⁺ T cells is compensated by a marked increase in local CD8⁺ T cell proliferation. Thus, at least the proliferating CD8⁺ T cells in the liver are not senescent, end-stage cells, as they are capable of DNA synthesis and regeneration. However, there was a significantly higher percentage (5 to 8%) of CD8⁺ lymphocytes in liver that were committed to apoptosis (AC3⁺) during the acute and AIDS phases of SIV infection (Fig. 6D). Nonetheless, these results suggest that at least some of the liver-associated CD8⁺ T cells in SIV-infected macaques are viable, antigen-responsive cells, a finding which is consistent with earlier work by Schmitz et al., who showed higher percentages of virus-specific CD8⁺ T cells in this tissue (20). No significant differences were detected in proliferation rates of CD8⁺ cells in peripheral

blood. It is also interesting that, unlike for CD8⁺ T cells, we did not detect increased rates of CD4⁺ T cell proliferation in the liver, even though we did detect increased rates of CD4⁺ T cell apoptosis (AC3⁺). Thus, we hypothesize that liver CD4⁺ T cells are terminally differentiated cells which may not be capable of supporting active viral replication but instead are more prone to immediate apoptosis upon exposure to SIV or SIV antigens.

Combined, these data indicate that the liver is a site of both potential viral target cells (CD4⁺ CCR5⁺) and fully functional (or reserve) CD8⁺ T cells, as the latter seem to expand and proliferate after SIV infection. Although we did not examine virus-specific CD8⁺ T cells here, our previous studies have shown that a greater proportion (7 to 29%) of liver CD8⁺ T cells are specific for SIV *gag* compared to fewer than 6% of peripheral blood mononuclear cells (PBMCs) (20). However, we were unable to detect significant levels of virus-infected cells in the liver (data not shown) to explain the large presence of virus-specific cytotoxic T lymphocytes (CTLs). Since the liver drains the intestinal tract, in which abundant viral replication is occurring, it is likely that abundant viral antigens or even replication-defective viral particles are passed to the liver throughout infection, a process which may result in antigen priming and expansion of CTLs. Prior reports have shown that the liver is the major site of viral clearance, even when intravenously inoculated with large amounts of virus (29). Alternatively, some level of viral replication may occur in the liver, but the greater numbers and efficiency of virus-specific CD8⁺ T cells generated in this tissue may mask the actual presence of infected cells. Regardless, the data presented show that the normal composition of T cells in the liver is markedly altered in early SIV infection, a change which could contribute to coinfection with a variety of pathogens and opportunistic infections that target the liver in HIV patients.

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