

# S-phase entry leads to cell death in circulating T cells from HIV-infected persons

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**Abstract:** Central memory T cells are thought to play a critical role in memory T cell homeostasis by undergoing self-renewal and by maturing into effector T cells that mediate immunity at tissue sites. Circulating T cells in S phase of the cell cycle are found at increased frequencies during HIV infection and are predominantly composed of cells with a central memory phenotype. Here, we tested the hypothesis that CD4 and CD8 S-phase T cells have different capacities to complete cell cycle and survive. S-phase T cells in peripheral blood from HIV-infected donors were identified by incubating whole blood with BrdU *ex vivo*. Upon *in vitro* cultivation, S-phase T cells were more likely to die than to complete mitotic division. Intrinsic differences were observed between CD4 and CD8 S-phase T cells during incubation. Higher frequencies of CD4+ S-phase T cell underwent apoptosis after incubation in medium alone or after TCR stimulation, and CD4+ S-phase T cells were less readily induced to proliferate after incubation with IL-2 than were CD8+ S-phase T cells. CD4+ and CD8+ S-phase T cells expressed low levels of Bcl-2, which could contribute to their heightened susceptibility to cell death. Intrinsic differences in the proliferation and survival of CD4+ and CD8+ S-phase T cells could influence the homeostatic maintenance of these T cell subsets in HIV disease. *J. Leukoc. Biol.* 83: 1382–1387; 2008.

**Key Words:** T lymphocytes · apoptosis · proliferation

## INTRODUCTION

Immune activation in HIV infection predicts CD4+ T cell decline and disease progression, independent of plasma HIV RNA levels [1–3]. Immune activation also is related to disease outcome in SIV-infected monkeys [4]; heightened levels of immune activation are associated with disease progression in susceptible animals (rhesus macaques), and low levels of immune activation are associated with a lack of disease progression in resistant animals (sooty mangabeys). Despite the recognized relationship between immune activation and CD4+ T cell loss in persistent lentivirus infection, there is little direct evidence that can explain why chronic immune activation,

which clearly affects CD4+ and CD8+ T cells in HIV infection, doesn't lead to similar depletion of both T cell subsets.

One compelling explanation for differences in CD4 and CD8 T cell homeostasis in HIV infection is derived from recent studies in SIV-infected macaques. These studies identify a critical role for central memory T cells in maintaining memory T cell homeostasis [5]. Central memory T cells are thought to be long-lived cells that self-renew during homeostatic proliferation, and some of these cells can differentiate into effector cells, representing a mechanism for reconstituting the effector memory pool that exists at tissue sites [5]. During HIV/SIV infection, the pool of mucosal effector CD4+ T cells is massively depleted early in infection [6, 7], whereas central memory T cells are relatively preserved. Over time, however, central memory T cells also are depleted, and in the SIV-infected rhesus, this is associated with direct viral infection of these cells [5]. Thus, susceptibility of CD4+ central memory T cells to HIV infection may limit self-renewal of these cells as well as limit the reconstitution of effector memory cells at tissue sites and consequently, underlie disease progression.

Another factor that could influence differential survival of CD4+ and CD8+ T cells during chronic immune activation relates to the intrinsic properties of these cells. For example, in mouse studies, CD8+ T cells appear to have a greater capacity to expand than do CD4+ T cells after *in vivo* bacterial or viral infection [8, 9], and memory CD8+ T cells are better sustained over time than are memory CD4+ T cells [9]. Furthermore, CD4+ T cells display an increased dependence on thymic function [10] and lymph node architecture [11] for homeostasis than do CD8+ T cells. Thus, besides differences in susceptibility to HIV infection, intrinsic properties of CD4+ and CD8+ T cells might preferentially predispose CD4+ T cells to depletion in conditions of chronic immune activation or T cell depletion.

In these studies, we have investigated the relative survival of circulating T cells that spontaneously enter S phase in the peripheral blood of HIV-infected persons as a model to explore T cell homeostasis in HIV disease. These S-phase T cells have been identified *in vivo* after systemic administration of BrdU

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Received September 21, 2007; revised February 12, 2008; accepted March 4, 2008.

doi: 10.1189/jlb.0907643

[12] and also *ex vivo* by their ability to incorporate BrdU during brief exposure [13, 14]. Our previous studies have partially characterized the cells that incorporate BrdU *ex vivo* as having predominantly a “central memory” phenotype, expressing CD45RO and lymph node homing receptors [14]. Interestingly, the cells that spontaneously enter S phase in circulation of HIV-infected persons are not enriched for markers of recent TCR stimulation (CD69 or CD25), and we have suggested that these cells may have been activated by bystander mechanisms, perhaps in the lymph node microenvironment. Here, we find that CD4+ and CD8+ S-phase T cells, which are presumably activated to undergo cell-cycle progression *in vivo*, have different capacities to complete mitotic division and to survive *in vitro* under different experimental conditions.

## MATERIALS AND METHODS

### Donors

Peripheral blood samples were obtained from 28 HIV-infected persons for these studies. The numbers of subjects involved in different experiments are indicated in figure legends. As our previous studies indicated that viremia was associated with increased frequencies of S-phase T cells in peripheral blood, we primarily included persons without virologic control (median plasma HIV RNA=41,250 copies/ml; range 151–241,123 copies/ml) in these experiments. The median CD4 cell count was 351, ranging from 5 to 1131 cells/ $\mu$ l. The median age was 41 years, ranging from 34 to 52 years. Most subjects (79%) were not receiving antiretroviral therapy at the time of the study.

### BrdU label and cell culture

Whole blood was collected in heparin-coated tubes and incubated with BrdU (10  $\mu$ M, BD PharMingen, San Diego, CA, USA) for 1 h at 37°C to label cells in S phase. PBMC were prepared from the whole blood by isolating cells over a Ficoll-Histopaque (Sigma Chemical Co., St. Louis, MO, USA) cushion and washing 2 $\times$  with PBS. For cellular proliferation studies, PBMC were incubated with PBS containing 10  $\mu$ M CFSE (Molecular Probes, Eugene, OR, USA) and 0.3% BSA for 10 min at 37°C. Cells were then washed 3 $\times$  with RPMI, 10% FBS solution. PBMC or CFSE-labeled PBMC were incubated in medium alone (RPMI, 10% FBS, 10 mM L-glutamine, and antibiotics) or in medium supplemented with recombinant (r)IL-2 (360 U/ml, Chiron, Emeryville, CA, USA) or anti-CD3 antibody (100 ng/ml, BD PharMingen).

### Flow cytometry

Cellular proliferation was measured by CFSE dye dilution, and proliferation indices and division indices were obtained using FlowJo analytical software (Tree Star, Inc., Ashland, OR, USA). PBMC without CFSE label were used to evaluate apoptosis and bcl-2 expression among S-phase T cells. Annexin V reagent was used to evaluate cellular apoptosis according to the instructions in the BD PharMingen Annexin V staining kit. To further assess the incorporation of BrdU within these cells, the staining procedure outlined in the BrdU kit (BD PharMingen) was followed with the exception that the buffers were supplemented with 1 $\times$  Annexin V buffer. The inclusion of Annexin V buffer in these subsequent steps was needed to preserve the calcium-dependent attachment of Annexin V to phosphatidylserine (PS) during further incubation and washing.

Additional fluorochrome-labeled Igs (anti-CD4, anti-CD8, and anti-bcl-2) were purchased from BD PharMingen. Flow cytometric analyses were performed using a FACSCalibur or the Becton Dickinson LSR II flow cytometer.

### Statistics

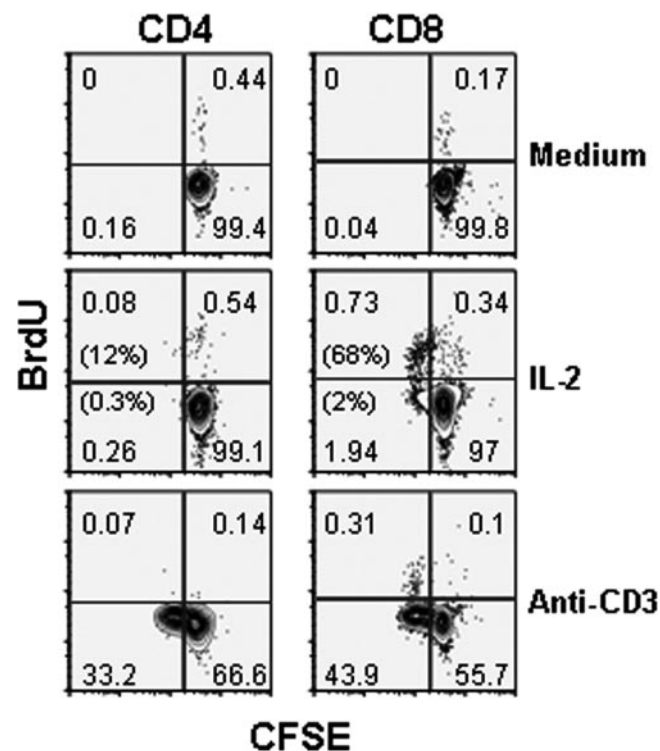
Student's *t*-tests or nonparametric tests were used to determine statistical differences between mean or median values and were used according to the distribution of the data. Spearman's correlations were used to assess relationships between continuous variables.

## RESULTS

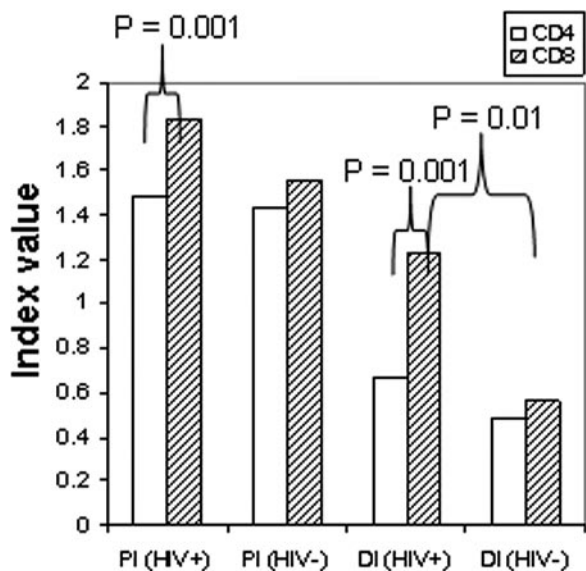
### Differences in the mitotic division of peripheral CD4+ and CD8+ S-phase T cells during *in vitro* culture

S-phase T cells in circulation were identified by incubating whole blood with BrdU for 1 h at 37°C [14]. PBMC were then isolated, further labeled with CFSE-tracking dye, and incubated *in vitro* for 1 or 3 days. In addition, some cells were incubated with rIL-2 (360 U/ml) or with anti-CD3 antibody (100 ng/ml). Despite entering S phase in whole blood, S-phase T cells rarely completed mitotic division after overnight culture or even after 3 days of incubation (Fig. 1). The failure of S-phase T cells to spontaneously divide during incubation in medium alone was observed in cells from HIV+ subjects and in cells from healthy controls (mean $\pm$ SD of percent CFSE low cells for HIV+ donors at 3 days=2.9% $\pm$ 4.5 and 3.3% $\pm$ 5 for S-phase CD4 and CD8 cells, respectively; and for healthy control donors=3.3%+3.5% and 4.4%+3.2% for CD4+ and CD8+ S-phase T cells, respectively).

In the presence of IL-2, however, CD8+ BrdU+ T cells proliferated and became enriched proportionally within the CD8+ T cell subset (Figs. 1 and 2). The IL-2 induction of mitotic division in S-phase T cells was dose-dependent as



**Fig. 1.** Differential requirements for proliferation of CD4 and CD8 S-phase T cells. Whole blood from a HIV-infected person was incubated with BrdU for 1 h at 37°C. PBMC were isolated and labeled with CFSE-tracking dye. Cells were incubated in medium alone (RPMI, 10% FBS, L-glutamine, and antibiotics) or in medium supplemented with rIL-2 (360 U/ml) or anti-CD3 antibody (100 ng/ml). Cells were assessed by flow cytometry for expression of CD4, CD8, BrdU, and CFSE after 3 days of incubation. For IL-2-treated cells, the numbers in parentheses represent the percentages of BrdU+ or BrdU- cells that diluted tracking dye.



**Fig. 2.** IL-2 induces S-phase T cell proliferation. Cells from HIV+ and HIV- donors were labeled with BrdU and then CFSE as described in Figure 1. Cells were incubated for 3 days with IL-2 (360 U/ml) before being assessed for cellular proliferation by flow cytometry. The average number of divisions among CFSElow S-phase cells, proliferation index (PI), or the average number of cell divisions among all S-phase cells, division index (DI), was determined with FlowJo software. Statistically significant differences are shown. Comparison between CD4+ and CD8+ S-phase cells within the same subject population was performed with paired Wilcoxon signed ranks test, and differences between subject categories were determined with Kruskal-Wallis ( $P < 0.001$ ) and Mann-Whitney tests;  $n = 16$  for HIV+ donors, and  $n = 7$  for healthy donors.

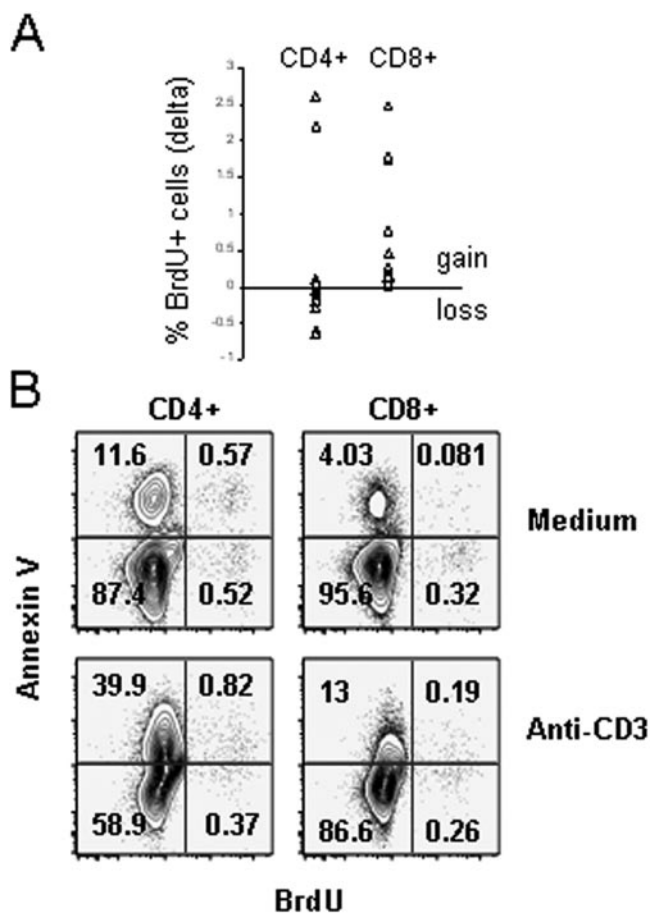
demonstrated in cells from two HIV-infected subjects. The percentage of BrdU+ CD8+ cells with diluted tracking dye diminished from 36% to 8.9% in cells from one HIV+ donor and from 10.3% to 1.3% in cells from a second HIV+ donor when cells were incubated for 3 days with 360 U/ml and 36 U/ml rIL-2, respectively. CD4+ T cells also survived better in the presence of IL-2, but cellular division was significantly less than that observed among CD8+ BrdU+ T cells in the presence of this cytokine (Fig. 2). Cell division indices were significantly greater among CD8+ S-phase T cells compared with CD4+ S-phase T cells within the samples from HIV+ subjects (Fig. 2). Interestingly, the division index (average number of cell divisions of BrdU+ cells) was significantly greater among the CD8+ S-phase cells from HIV-infected persons compared with CD8+ S-phase T cells from healthy controls (Fig. 2). Overall, these observations suggest that the CD8+ S-phase T cells from HIV-infected persons may be hyper-responsive to IL-2 stimulation.

After 3 days of in vitro culture, it was also clear that the proliferation responses to IL-2 were enhanced among the BrdU+ S-phase T cells compared with the BrdU- T cell subset. The mean percentage ( $n=16$ ) of BrdU+ T cells with reduced CFSE label was 49.3% and 27.2% for CD8+ and CD4+ cells, respectively. In contrast, the mean percentage of BrdU- T cells that diluted CFSE-tracking dye was only 2.7% and 1.5% for CD8+ and CD4+ cells, respectively;  $P$  values  $< 0.001$ . These observations provide evidence that S-phase T cells identified by this method are functionally distinct from

other T cells in peripheral blood and suggest that the activation signals promoting cell entry in vivo are not sufficient to permit completion of cell cycle ex vivo in the absence of additional stimulation.

### Heightened susceptibility of CD4+ S-phase T cells to apoptosis

Following stimulation with anti-CD3 antibody, the proportion of BrdU+ cells within the CD4+ T cell subset was diminished (Figs. 1 and 3A). In contrast, CD8+ S-phase T cells that were activated with anti-CD3 antibody were able to dilute tracking dye and represented a higher proportion of total CD8+ T cells at the end of 3 days (Figs. 1 and 3A). We considered the possibility that the lack of CD4+ T cell accumulation in anti-CD3-stimulated cells may have resulted from heightened cell death among these S-phase cells compared with the S-phase CD8+ T cells. Analyses of cell-surface PS expression indicated that CD4+ S-phase T cells were more likely to bind Annexin V after stimulation with anti-CD3 antibody than were



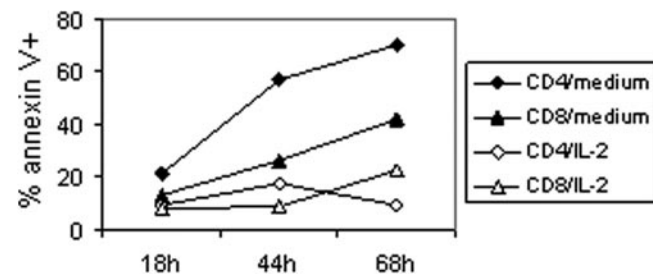
**Fig. 3.** Poor recovery and increased apoptosis among CD4 S-phase T cells after activation with anti-CD3 antibody. (A) Results from 16 HIV+ donors showing the difference in the percentage of CD4 or CD8 S-phase T cells derived from subtracting the percentage of S-phase T cells in medium alone from the percentage of S-phase T cells in cells cultured with anti-CD3 antibody. (B) A representative experiment demonstrating flow cytometric analyses of Annexin V binding among CD4 and CD8 S-phase (BrdU+) T cells after 2 days of incubation in medium alone or in medium treated with anti-CD3 antibody. The donor was HIV+ with a CD4 cell count of 847 cells/ $\mu$ l and a plasma HIV RNA level of 37,600 copies/ml.

CD8+ S-phase T cells (mean percent PS+ cells after anti-CD3 stimulation=74.1 and 35.3 among CD4+ and CD8+ S-phase T cells, respectively;  $n=5$ ,  $P=0.004$ ; representative dot-plot in Fig. 3B). CD4+ S-phase T cells incubated in medium alone for 18 h also tended to undergo apoptosis at higher frequencies than CD8+ S-phase T cells, although the differences did not reach statistical significance until later time-points (Fig. 4;  $P=0.08$  at 18 h). Thus, CD4+ S-phase T cells were more likely to become apoptotic and die spontaneously or after TCR engagement than were CD8+ S-phase T cells.

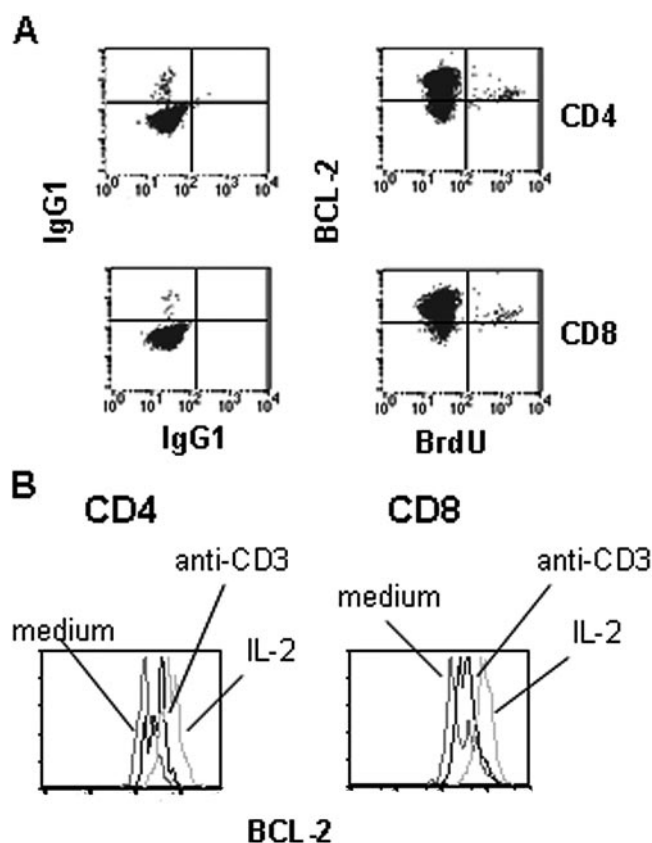
We further evaluated PS surface expression among cells incubated in medium alone or in medium supplemented with rIL-2 over a 3-day period. The mean percentages of Annexin V-binding S-phase T cells were increased compared with Annexin V-binding non-S-phase T cells among the CD4+ (21.5% vs. 9.6%) and CD8+ (12.9% vs. 2.3%) subsets after overnight incubation in medium alone. The percentages of S-phase T cells that expressed surface PS (Annexin V+) were plotted over three different time-points, and the time required for 40% of the S-phase T cells to express surface PS was estimated to be 32 h for CD4+ S-phase T cells and 67 h for CD8+ S-phase T cells (Fig. 4). Thus, without intervention, S-phase CD4+ T cells are expected to die more rapidly than S-phase CD8+ T cells. The addition of IL-2 to the cultures diminished the percentages of cells binding Annexin V, even at time-points prior to detectable cell division, indicating that in addition to promoting mitosis, exogenous IL-2 protected S-phase T cells from apoptosis.

### Reduced Bcl-2 expression among S-phase T cells

Susceptibility to spontaneous T cell apoptosis in vitro has been associated with low expression of the antiapoptotic protein, Bcl-2. Therefore, we measured the levels of Bcl-2 expression in peripheral S-phase T cells ex vivo and after in vitro culture under different experimental conditions. Freshly isolated CD4+ and CD8+ S-phase T cells expressed relatively low levels of Bcl-2 protein compared with most non-S-phase T cells (Fig. 5A). Incubation of PBMC in vitro with IL-2 or anti-CD3



**Fig. 4.** CD4 S-phase T cells die more rapidly than CD8+ S-phase T cells in vitro. PBMC derived from BrdU-labeled whole blood were incubated for indicated periods in medium alone or in medium with rIL-2 (360 U/ml) and assessed for the percentages of BrdU+ CD4+ and BrdU+ CD8+ T cells that bound Annexin V. The mean percentage of Annexin V+ S-phase T cells is shown after 18 h ( $n=10$ ), 44 h ( $n=5$ ), and 68 h ( $n=5$ ) incubation. Apoptosis of CD4+ S-phase cells was significantly higher than apoptosis of CD8+ S-phase T cells at 44 h and 68 h ( $P<0.05$ ; Wilcoxon signed ranks test) among cells incubated in medium alone.



**Fig. 5.** Low Bcl-2 expression in S-phase T cells and induction with IL-2 or anti-CD3 antibody stimulation. (A) Whole blood from a HIV-infected person was incubated 1 h with BrdU ex vivo, RBC were lysed with FACS Lyse solution, and cells were stained for surface expression of CD4 and CD8 as well as the intracellular expression of Bcl-2 and BrdU. The results shown are representative of four experiments with different HIV+ donors. (B) PBMC from ex vivo BrdU-labeled whole blood was incubated for 3 days in medium alone or in medium treated with rIL-2 or anti-CD3 antibody. Results are representative of three experiments.

antibody resulted in the induction of Bcl-2 expression among peripheral S-phase T cells (Fig. 5B), with IL-2 consistently inducing more Bcl-2 protein expression than did anti-CD3 stimulation. There were no obvious differences in the degree to which Bcl-2 was induced by either reagent when the CD4+ and CD8+ S-phase T cell subsets were compared. These results demonstrate that although CD4+ and CD8+ S-phase T cells express low levels of Bcl-2 upon isolation and can be induced similarly to express Bcl-2 with activation, the CD4+ S-phase T cells appear to be more susceptible to spontaneous apoptosis and less readily rescued by TCR stimulation.

### DISCUSSION

In contrast to CD4+ T cells, which are progressively depleted during HIV infection, CD8+ T cells maintain their numbers at least until the later stages of disease. The progressive decline in CD4+ T cells during the chronic phase of HIV disease has been related to the plasma HIV RNA levels [15] and HIV-1 coreceptor use [16], suggesting that lytic viral infection might be a key determinant in pathogenesis. In contrast, recent

longitudinal analyses indicate that the variability in plasma HIV RNA levels and variability in rates of CD4+ T cell decline are only weakly related in chronic HIV infection [17], and variability in levels of HIV RNA in plasma account for only half of the measurable risk for disease progression [18]. Furthermore, SIV-infected sooty mangabeys and African green monkeys resist disease progression despite high-level viremia [19–21]. Thus, although lytic HIV infection may result in death of some CD4+ T cells, this mechanism alone may not be sufficient to account for all CD4+ T cell losses in HIV infection.

Recent studies indicate that perturbations in central memory T cells may underlie HIV/SIV disease progression [5]. Here, we find that circulating S-phase T cells, which we have previously defined as primarily central memory cells, have a high propensity for apoptosis and limited capacity to complete cell division without intervention. Conceivably, such intervention could occur *in vivo* and stem from cytokines such as IL-2, IL-15, or IL-7, which promote T cell survival and cell-cycle progression. Interestingly, our studies indicate that circulating CD8 and CD4 S-phase T cells respond differently to IL-2 stimulation, with CD8 S-phase T cells having a greater capacity to complete mitosis after IL-2 stimulation than CD4 S-phase T cells, particularly in the context of HIV infection. Thus, proliferating CD8 central memory T cells may have a better capacity to expand in HIV infection than their CD4+ T cell counterparts. The diminished effect of IL-2 on CD4+ S-phase T cells may in part stem from direct infection of CD4 cells by HIV; however, it is notable that the major difference in IL-2 responsiveness among cells from HIV-infected persons is not associated with a failure of CD4+ S-phase T cells to respond to IL-2 as much as it reflects an exaggerated response by CD8+ T cells. Further studies to explore the intrinsic properties of CD8 S-phase T cells in HIV disease may help uncover the mechanisms that result in this “hyper-responsiveness” of CD8+ S-phase T cells in HIV infection.

In contrast to the enhanced survival that occurs with IL-2 intervention, CD4+ S-phase T cells that are incubated in medium alone or in the presence of a TCR trigger are highly prone to apoptosis. This heightened susceptibility to cell death may predispose these cells to depletion *in vivo*. CD8 S-phase T cells are also prone to apoptosis without intervention, although their survival is moderately better than CD4 S-phase T cells at least *in vitro*. Moreover, the CD8 S-phase T cells appear to expand after TCR stimulation, whereas CD4+ S-phase T cells undergo apoptosis with limited cell division. Although previous studies have shown that S-phase entry after cytokine treatment of murine CD4+ T cell clones or CD8+ T cell lines made these cells more susceptible to activation-induced cell death (proapoptotic [22]), our work characterizes for the first time the consequences of this type of activation in primary human lymphocytes naturally undergoing S-phase entry in peripheral blood. Moreover, our work demonstrates the unexpected finding that CD4+ S-phase T cells are more susceptible to this type of cell death than are CD8 S-phase cells. Conceivably, receipt of these additional cytokine or cognate peptide signals *in vivo* promotes relative preservation and survival of the activated CD8+ T cells and may help

to explain why circulating CD8+ T cells tend to have shorter telomeres than CD4+ T cells in HIV disease [23, 24].

We have additionally noted here that circulating S-phase T cells express relatively low levels of the antiapoptotic molecule, Bcl-2. This finding is consistent with the high levels of spontaneous apoptosis that we observe in S-phase T cells from circulation; however, as the expression of Bcl-2 is not readily distinguishable between the CD4+ and CD8+ S-phase subsets, it is unlikely that differences in Bcl-2 can explain the differences in susceptibility of CD4 and CD8 S-phase T cells to spontaneous or activation-induced apoptosis. Further studies will be required to understand these mechanisms.

The frequencies of S-phase T cells in peripheral blood of HIV-infected persons are directly related to plasma HIV RNA [13, 14] and are diminished with initiation of highly active antiretroviral therapy [13]. Although the S-phase T cells express markers of activation, they do not show signs of recent TCR stimulation [14], suggesting that these cells are being induced to proliferate by bystander activation or by homeostatic mechanisms (independent of direct foreign antigen stimulation). The heightened proliferation of T cells in HIV infection is not sufficient to reconstitute CD4+ T cell loss and in circumstances of dysregulated cell-cycle entry, may even contribute to CD4 decline. Importantly, among healthy individuals, few T cells in circulation are found in S-phase, and even in chronic SIV infection, T cell proliferation occurs primarily in lymph nodes, and activated T cells in blood are largely cells that are not in the cell cycle [25, 26]. It is likely that the T cells progressing through the cell cycle while in circulation represent a subset of dysregulated T cells that may not be in a microenvironment conducive to their survival. We have described previously a similar circumstance for CD4+ T cells from HIV-infected persons that spontaneously enter cell cycle after *in vitro* cultivation, although those cells were not necessarily in S-phase upon isolation from the blood [27]. Together, these observations suggest that continuous, dysregulated cell-cycle entry *in vivo*, which our previous studies suggested was proportionally greater among CD4+ T cells, may contribute to T cell loss in HIV infection. We propose that these S-phase T cells, predominantly of the central memory phenotype, are induced to enter cell cycle in lymphoid tissues. This would predict that T cells activated by bystander mechanisms to die are concentrated in lymphoid tissues in HIV infection, and this appears to be the case [28]. Overall, our studies uncover several intrinsic differences between actively dividing CD4 and CD8 T cells and suggest that these differences could contribute to the relative survival and sustainability of these cellular subsets in chronic HIV disease.

## ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health AI-36219, the Center for AIDS Research, Case Western Reserve University. We thank Dr. Robert Asaad for obtaining patient samples.

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