Evidence for Increased T Cell Turnover and Decreased Thymic Output in HIV Infection


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Evidence for Increased T Cell Turnover and Decreased
Thymic Output in HIV Infection¹


The effects of HIV infection upon the thymus and peripheral T cell turnover have been implicated in the pathogenesis of AIDS. In this study, we investigated whether decreased thymic output, increased T cell proliferation, or both can occur in HIV infection. We measured peripheral blood levels of TCR rearrangement excision circles (TREC) and parameters of cell proliferation, including Ki67 expression and ex vivo bromodeoxyuridine incorporation in 22 individuals with early untreated HIV disease and in 15 HIV-infected individuals undergoing temporary interruption of therapy. We found an inverse association between increased T cell proliferation with rapid viral recrudescence and a decrease in TREC levels. However, during early HIV infection, we found that CD45RO−CD27high (naive) CD4⁺ T cell proliferation did not increase, despite a loss of TREC within naive CD4⁺ T cells. A possible explanation for this is that decreased thymic output occurs in HIV-infected humans. This suggests that the loss of TREC during HIV infection can arise from a combination of increased T cell proliferation and decreased thymic output, and that both mechanisms can contribute to the perturbations in T cell homeostasis that underlie the pathogenesis of AIDS. The Journal of Immunology, 2001, 167: 6663−6668.

T cell depletion of CD4⁺ in HIV infection can arise as a result of increased destruction and/or reduced production of T cells through a number of mechanisms, none of which are mutually exclusive, and for each of which there is experimental evidence (1−3). T cells can be destroyed by direct or indirect virus-induced mechanisms, or through Ag-specific CTL-mediated lysis (4−9). Reduced T cell production can result from diminished peripheral expansion of pre-existing T cells or inhibition of de novo generation of naive T cells from thymocytes or hematopoietic progenitor cells (8, 10−18). Sequestration of cells in lymphoid tissues may also affect peripheral T cell numbers (19−21). The recovery of T cell numbers during highly active antiretroviral therapy (HAART)³ can occur through a number of different mechanisms, which may result in the reconstitution of qualitatively different immune function. Peripheral expansion or redistribution of pre-existing T cells (17−20, 22) will result in a T cell repertoire that reflects that already marred by HIV infection, whereas de novo generation of new naive T cells from the thymus (23−27) will constitute a more diverse T cell repertoire (28, 29).

In vivo bromodeoxyuridine (BrdU) incorporation studies in SIV-infected monkeys showed increased turnover in all T cell populations, with memory T cells affected more than naive (7, 30). Studies using expression of the Ki67 nuclear Ag as a marker of cell proliferation indicated that total T cell turnover increased in naive and memory subsets during infection (31). This suggested that CD4⁺ T cell loss was due to interference of the virus with “T cell renewal capacity” rather than with peripheral production, and that redistribution accounted for increased CD4⁺ T cell numbers during treatment (31). However, a more recent study (32) also using Ki67 showed that turnover rate, but not proliferation, increased in CD4⁺ T cells, suggesting their increased death and decreased renewal (32). In vivo labeling with deuterated glucose confirmed some of these findings, showing that HIV infection caused a decrease in memory (but not naive) CD4⁺ and CD8⁺ T cell half-life with a compensatory increase in production of CD8⁺ but not CD4⁺ T cells (33).

The measurement of TCR rearrangement excision circles (TREC) has been used to assess thymic output in individuals with and without HIV infection (23, 26, 34−37), and after hematopoietic stem cell transplantation (29, 38, 39). In the majority of individuals with untreated HIV-infection, TREC levels were below normal, but increased after viral suppression with HAART (23, 26, 37). This was taken to indicate that the thymus, in both adults and children, is suppressed by HIV infection, but contributes to T cell

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3 Abbreviations used in this paper: HAART, highly active antiretroviral therapy; BrdU, bromodeoxyuridine; TREC, TCR rearrangement excision circles.

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reconstitution during HAART. In this study, we sought to determine whether increased T cell turnover, decreased thymic output, or both occur in HIV infection. We measured peripheral blood TREC levels and parameters of CD4+ and CD8+ T cell proliferation, including Ki67 expression and ex vivo BrdU incorporation, in 22 individuals with early untreated HIV disease (3–12 mo after infection), and in 15 successfully treated HIV-infected individuals who underwent temporary interruption of therapy.

Materials and Methods

**Human subjects**

Twenty-two patients with early HIV infection (3–12 mo after seroconversion) were seen at the University of Texas Southwestern Medical Center and had not been on antiretroviral drugs at the time of blood draw. CD4+ T cell counts were 220-1080 cells/μl (mean 602) and viral loads were <400 to >7.5 × 10^8 RNA copies/ml. Fifteen patients were asymptomatic HIV-infected adults with baseline CD4+ T cell counts of ≈ 350 cells/μl who had been on continuous HAART for a minimum period of 1 year, with viral loads consistently below the limits of detection for at least that period of time (40). On day 0, patients discontinued all antiretroviral drugs, and were naive for Thymic output.

**Measurement of TREC in MACS-sorted cells**

Quantification of TREC in sorted CD4+ and CD8+ T cells was performed by quantitative PCR with an ABI7700 system (PerkinElmer/Cetus, Norwalk, CT) as previously described (29). PBMC were separated into CD4+ and CD8+ cells using MACS microbeads (Miltenyi Biotec, Auburn, CA). Cells were lysed in proteinase K (Boehringer Mannheim, Indianapolis, IN) and PCR was performed on 5 μl of cell lysate (50,000 cells). A standard curve was plotted, and TREC values for samples were calculated by the ABI7700 software. Samples were analyzed in duplicate. TREC levels are expressed as TREC per microgram of DNA (1 μg of genomic DNA is equivalent to 150,000 cells). Cell lysees have been checked for consistency of DNA content using β-actin and CCR5 control PCR; inter assay variability was found to be less than 13% of mean for the same sample in 20 different assays (data not shown).

**Ex vivo BrdU uptake analysis**

Blood samples were incubated with 100 μM BrdU for 4 h at 37°C. Cell surface staining was performed using Abs to CD3, CD45RO, CD4, and/or CD8 (BD Biosciences, San Jose, CA). Cells were treated with OptiLyse (ImmunoTech, Westbrook, ME) for 10 min at room temperature, then with 1% paraformaldehyde and 1% Tween 20 in PBS for 15 min at 37°C. Cell fixation/walk, CT) as previously described (29). PBMC were separated into CD4+ and CD8+ T cells using MACS microbeads (Miltenyi Biotec, Auburn, CA). Cells were lysed in proteinase K (Boehringer Mannheim, Indianapolis, IN) and PCR was performed on 5 μl of cell lysate (50,000 cells). A standard curve was plotted, and TREC values for samples were calculated by the ABI7700 software. Samples were analyzed in duplicate. TREC levels are expressed as TREC per microgram of DNA (1 μg of genomic DNA is equivalent to 150,000 cells). Cell lysees have been checked for consistency of DNA content using β-actin and CCR5 control PCR; inter assay variability was found to be less than 13% of mean for the same sample in 20 different assays (data not shown).

**Naive T cell Ki67 analysis and FACS sorting**

Analysis was performed by surface staining cells for either CD4/CD45RO/CD27 or CD8/CD45RO/CD27 (BD Biosciences), followed by fixation/permeabilization and intracellular staining for Ki67 (BD Biosciences). Cells were analyzed by four-color flow cytometry using a FACS Calibur. Ki67 expression was measured in both CD45RO+CD27+ (naive) and CD45RO− (memory/effector) CD4+ and CD8+ T cells using Paint-a-Gate cluster analysis. For FACS sorting of naive and memory T cells, cells were stained as above (but without Ki67), and were sorted for TREC analysis using a FACSort (BD Biosciences).

**Statistical analyses**

Two-tailed Mann-Whitney U test and Spearman’s rank correlation coefficients were performed using SAS and Prism software (SAS Institute, Cary, NC). Analysis of covariance was used to assess differences in CD4+ and CD8+ T cell TREC between HIV-infected individuals and the healthy controls, adjusting for age. A p value of <0.05 was considered significant.

**Results**

**TREC levels measure thymic output**

TREC frequency in total and in naive T cells has been shown to decrease with age, after thymectomy, and in HIV infection (23, 26, 34–36, 41). However, a recent mathematical model has suggested that this decrease in TREC solely reflects a theoretical increase in the naive T cell division rate, and not decreased thymic output (42). Therefore, we sought to test this experimentally by measuring changes in T cell division with age using Ki67 expression as a surrogate marker of cell proliferation. Fig. 1 shows that Ki67 expression does not increase in either CD4+ or CD8+ CD45RO−CD27+ (naive) T cells in healthy individuals aged between 23 and 88 years of age (during which period the most rapid drop in naive T cell TREC is seen; Refs. 23 and 26). Furthermore, it has recently been shown that although TREC decrease after thymectomy, there is no increase in CD27+ (naive) or CD45RO− memory T cell Ki67 expression (41). The fact that Ki67 may be raised in nonproliferating or activated cells (3, 43) does not affect this analysis because the aim in this study was to exclude any increases in proliferation. Thus, a decrease in TREC levels can indeed reflect a decrease in thymic output.

**TREC levels and BrdU uptake after interruption of therapy**

It has recently been shown that after total thymectomy, TREC levels begin to fall after only 3 mo (41). Therefore, in HIV infection, any decrease in TREC related to decreased thymic output would not be expected to be observed until at least 3 mo after seroconversion. Consequently, decreases in TREC before 3 mo of HIV infection would reflect primarily increased T cell proliferation.

To examine this, we initially studied T cell TREC levels in 15 HIV-infected individuals who had been successfully treated with HAART, who had undetectable viral loads, and then underwent interruption of therapy (40, 44). As previously reported, recrudescence of viral replication occurred in all the patients within 28 days of interruption of therapy. This allowed us to longitudinally assess changes in TREC and proliferation during a rapid rise in HIV levels—a situation reminiscent of acute HIV infection. As viral load rose, both CD4+ and CD8+ T cell TREC levels decreased concomitantly. We then performed ex vivo BrdU incorporation to determine whether this fall in TREC was, in part, secondary to increased T cell proliferation. The correlation between the S-phase BrdU fraction and viral load has been previously described (44). In
the interval between interruption and resumption of therapy, there was a significant negative correlation between the change in the percentage of BrdU"CD4" T cells and the change in CD4" T cell TREC levels \( r = -0.6, p = 0.02, \) and 95% confidence interval = \(-0.86 \) to \(-0.09; \) Fig. 2). Thus, as CD4" T cell proliferation increased with a concomitant increase in viral load (40), TREC levels decreased. The change in the percentage of BrdU"CD8" T cells also varied inversely with the change in CD8" T cell TREC levels. However, this relationship was not statistically significant for this sample size \( r = -0.3, p = 0.3, \) and 95% confidence interval = \(-0.72 \) to \(-0.31, \) and a larger study sample would be required to establish a statistically significant relationship. It is a possibility that preferential redistribution of TREC-containing cells out of the peripheral circulation could cause the decrease in TREC. Of course, these data do not rule out a concomitant decrease in thymic output; it is simply not possible to differentiate the effects of thymic output and T cell proliferation. However, bearing in mind the delayed effects of thymectomy on TREC levels (41), the rapid fall in TREC during an acute rise in HIV load more likely reflects increased T cell proliferation than decreased thymic output.

**TREC levels and Ki67 expression in early HIV infection**

Inhibition of thymic function should become detectable by 3 mo after HIV infection. To differentiate experimentally between thymic inhibition and increased peripheral T cell proliferation, it is necessary to measure an independent marker of T cell proliferation in naive and memory T cells, and to measure or calculate the TREC content of the naive T cell pool. If naive T cell proliferation is constant, then changes in TREC levels reflect changes in the supply of naive T cells. Therefore, we measured TREC levels and Ki67 expression in naive and memory CD4" and CD8" T cell subsets in a separate group of 22 patients with early (3–12 mo after seroconversion) untreated HIV infection. Ki67 expression was used, rather than BrdU incorporation, because these were cryopreserved samples. It should be stressed, however, that the number of Ki67" cells and the S-phase fraction (BrdU" cells after a 4-h in vitro pulse) are not equivalent measures of T cell activation, and many more cells express Ki67 than are actually in S-phase at any particular instant in time. The longevity of Ki67 expression after mitosis remains unclear.

Fig. 3 shows that both CD4" and CD8" T cell TREC were significantly lower than in uninfected age-matched controls \( p = 0.008 \) and 0.001, respectively. For measurement of Ki67 expression in naive and memory T cells, subsets were very carefully defined so that the naive subset would contain few cells outside the CD27"CD45RO" population. Fig. 4 shows that the percentage of Ki67"CD4" and Ki67"CD8"CD45RO" (memory) T cells was significantly increased in HIV-infected individuals compared with uninfected individuals (5.7-fold and 6.9-fold, respectively; \( p < 0.0001 \) for both). We also confirmed that the percentage of Ki67"CD45RO" (memory) T cells was significantly higher than that of naive T cells within the infected and uninfected groups (CD4, 17.4-fold and \( p < 0.0001; \) CD8, 5.2-fold and \( p = 0.0003; \) CD4, 1.9-fold and \( p = 0.0031; \) CD8, 2.7-fold and \( p = 0.0017, \) infected and uninfected, respectively). However, although the percentage of Ki67"CD8"CD45RO"CD27high (naive) T cells was
Naive T cell Ki67 expression is shown in Fig. 5. These T cells may have recently been naive cells that are transitioning to activated cells and that will proliferate. Thus, accurate measurement of activation and proliferation in naive CD4⁺ T cells requires rigorous phenotypic definition of this population by flow cytometry.

**Naive T cell TREC levels in early HIV infection**

Therefore, to determine whether thymic output was decreased in early HIV infection in the context of unchanged naive CD4⁺ T cell proliferation (and also to exclude memory T cell expansions as a cause of decreased total T cell TREC), we calculated naive T cell TREC from the total measured TREC and the percentage of naive T cells determined by flow cytometry. Our calculation assumed that the contribution of TREC from memory T cells was negligible. This is a valid assumption, as we have measured TREC in highly FACS-purified CD4⁺ T cell populations from 12 individuals and have found that CD45RO⁻ (memory) T cells have, on average, only 2% of the TREC content of CD45RO⁺CD27⁻ (naive) T cells in the same individual. Furthermore, as an example of the concordance between calculated and actual measured naive T cell TREC, we FACS sorted CD45RO⁺CD27⁻ (naive) T cells in one individual, measured TREC directly, and compared this result with TREC calculated from unsorted T cells and the naive T cell percentage, as shown in Tables I and II.

We found that both CD4⁺ and CD8⁺ naive T cell TREC in early HIV infection were significantly lower than in uninfected age-matched controls (p = 0.006 and 0.001, respectively; Fig. 6a). Because the percentage of Ki67⁺ CD8⁺ CD45RO⁻ CD27⁻ (naive) T cells was increased in HIV-infected individuals, the reduced naive CD8⁺ TREC levels could have been solely or partly due to increased naive CD8⁺ T cell proliferation. However, the percentage of Ki67⁺ CD4⁺ CD45RO⁻ CD27⁻ (naive) T cells was not increased, and therefore, the decrease in TREC within CD45RO⁺CD27⁻ (naive) CD4⁺ T cells could not have been due to increased proliferation of cells in this compartment. This suggests that the decreased naive CD4⁺ T cell TREC levels caused by HIV infection should be reversible when virus replication is suppressed. To confirm this, we longitudinally followed, after initiation of HAART, the CD45RO⁺CD27⁻ (naive) CD4⁺ T cell TREC levels of five patients who had low TREC before therapy. After initiation of HAART, which reduced viral loads to undetectable levels, CD45RO⁺CD27⁻ (naive) CD4⁺ T cell TREC rose to normal levels, suggesting that changes in thymic output were responsible for the changes in TREC within the naive CD4⁺ T cells before and after HAART (Fig. 6b).

**Discussion**

There is a considerable body of evidence that HIV can infect the thymus, both in vitro and in vivo, and compromise its function (10–14, 16, 46, 47). Recent studies of CD4⁺ T cell depletion and reconstitution in HIV infection have tended to appreciate the multifactorial nature of immune homeostasis—the composition of naive and memory/effector pools in blood and lymphoid tissue, the role of the thymus, and the effect of clinical stage of the disease.

### Table I. TREC levels per 10⁵ cells in CD45RO⁻CD27⁺ (naive) and CD45RO⁺ (memory) T cell subsets measured in sorted T cells in 12 individuals

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tr>
<td>CD45RO⁻CD27⁺</td>
<td>3,271</td>
<td>9,106</td>
<td>24,072</td>
<td>4,224</td>
<td>356</td>
<td>5,960</td>
<td>906</td>
<td>8,256</td>
<td>418</td>
<td>496</td>
<td>1,427</td>
<td>2,209</td>
</tr>
<tr>
<td>CD45RO⁺</td>
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<td>0</td>
<td>0</td>
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in therapy, the rapid fall we observed in TREC levels clearly reflects a rebound of viral replication after interruption of increased T cell turnover, occur in HIV infection. In individuals aimed to determine whether decreased thymic output, as well as unlikely that the fall in TREC reflects a marked decrease in thymic output, as it has recently been shown that TREC levels only begin to decrease 3 mo after total thymectomy in HIV-uninfected individuals.

The measurement of peripheral blood TREC provides insight into both thymic function and T cell proliferation. If naïve T cell TREC levels decrease in the absence of an increase in proliferation, it may be concluded that there is a decrease in the supply of new TREC+ cells, most likely from the thymus. In this study, we aimed to determine whether decreased thymic output, as well as increased T cell turnover, occur in HIV infection. In individuals experiencing a rebound of viral replication after interruption of therapy, the rapid fall we observed in TREC levels clearly reflects the increase in T cell proliferation. Although not impossible, it is unlikely that the fall in TREC reflects a marked decrease in thymic output, as it has recently been shown that TREC levels only begin to decrease 3 mo after total thymectomy in HIV-uninfected individuals.

Therefore, we reasoned that if HIV infection suppressed thymic output, we would be able to detect this effect at least 3 mo after infection. Indeed, our results from the analysis of individuals with early HIV infection 3–12 mo after seroconversion suggest that decreased thymic output begins to affect T cell homeostasis by this stage of the disease. TREC levels were decreased in both CD4+ and CD8+ T cell subsets, and were also significantly decreased when the CD45RO−CD27high (naive) T cell TREC were calculated. As both CD45RO+ (memory) and CD45RO−CD27high (naive) CD8+ T cell populations had higher Ki67 expression in HIV-infected individuals, it was not possible to distinguish between the effects of increased proliferation and reduced thymic output for CD8+ T cells. However, the percentage of Ki67+CD4+CD45RO−CD27high (naive) T cells did not increase. The finding that TREC within naïve CD4+ T cells were significantly lower in infected individuals in the context of unaltered naïve CD4+ T cell proliferation suggests that the input of TREC+ naïve CD4+ T cells into the peripheral naïve T cell pool from a “source” has decreased. The thymus is the most likely source for such cells.

However, some studies have found that the percentage of Ki67+CD4+ naïve T cells increased in HIV-infected individuals (1, 31). The discrepancy between our data and these studies could be due to a number of reasons; for example, our subjects had a higher mean CD4 counts, and some Ki67+ cells might be nondividing (3, 43). However, as we have shown, it is more likely that the incorporation of transitional T cells, cells that were naïve and have now become activated, into the phenotypically defined naïve T cell subset accounts for the apparent increase in CD4+ naïve T cell activation and/or proliferation in such studies.

Thus, our data show that by 3 mo after HIV infection, a decrease in TREC within CD45RO−CD27high (naive) CD4+ T cells is clearly detectable in the absence of an increase in CD45RO−CD27high (naive) CD4+ T cell proliferation, which suggests a decrease in thymic output. It is possible that the decrease in TREC could be due to members of the naïve pool entering cell cycle, losing TREC due to dilution, and then reverting to a naïve phenotype. However, there is no evidence in humans for activated or memory CD4+ T cells reverting to a CD45RO−CD27high phenotype. The effect of HIV on thymic function was further confirmed with the observation that in those individuals with low TREC, CD45RO−CD27high (naive) CD4+ T cell TREC increased with suppression of virus on HAART. This suggests that the thymus can recover from its suppression during HIV infection. It is important to note that an increase in naïve T cell TREC can only occur in the context of active thymic output. Therefore, even if decreases in CD4+ T cell TREC occurred due to proliferation and the thymus remained unaffected by HIV, the increase in naïve T cell TREC during HAART indicates that the thymus contributes to immune reconstitution. An appreciation of the relative roles of the thymus and peripheral T cell pool in immune reconstitution in HIV infection may provide a framework for the rational design of interventions that accelerate and improve the nature of T cell reconstitution in HIV-infected individuals.

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References


