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Dynamics of T Cells and TCR Excision Circles Differ After Treatment of Acute and Chronic HIV Infection\(^1,2\)

Sharon R. Lewin,\(^3\)† Ruy M. Ribeiro,‡ Gilbert R. Kaufmann,§ Don Smith,§ John Zaunders,¶ Matthew Law,§ Ajantha Solomon,* Paul U. Cameron,* David Cooper,§ and Alan S. Perelson‡

We quantified T cell proliferation and thymic function in primary HIV infection (PHI; \(n = 19\)) and chronic HIV infection (CHI; \(n = 14\)) by measuring Ki67 staining and TCR excision circle (TREC) number. After antiretroviral therapy of PHI there is a profound decrease in the number and percentage of Ki67\(^*\) T cells (<6% Ki67\(^*\)) with no significant increase in TREC per million cells and a transient increase in TREC per milliliter. In contrast, after antiretroviral therapy of CHI there is a reduction in the percentage but little change in the total number of Ki67\(^*\)CD4\(^+\) T cells associated with increases in both TREC per million cells and TREC per milliliter. Using a mathematical model that accounts for proliferation, death, and redistribution of T cells, we find that redistribution is consistent with the TREC changes observed during treatment of PHI and that an increase in thymic output is needed to explain the increase in TREC during treatment of CHI. Consideration of TREC per milliliter shows that changes in proliferation alone cannot explain the changes in TREC. In addition, although increased proliferation of memory cells in HIV infection has been established, we find no difference in TREC per million CD45RA\(^-\) T cells between healthy and infected individuals (\(p = 0.154 \text{ for } CD4^+; p = 0.383 \text{ for } CD8^+\)). Finally, although the number of TREC per million cells is always much lower in memory T cells than in naive T cells, in the setting of HIV infection, given that memory cells make up a larger proportion of total T cells, we find that 50% of TREC per milliliter in CD4\(^+\) T cells is harbored in the CD45RA\(^-\) “memory” subset of our infected subjects. The Journal of Immunology, 2002, 169: 4657–4666.

The role of the thymus in T cell homeostasis is critical to our understanding of T cell depletion, T cell turnover, and immune reconstitution. A fraction of recent thymic emigrants carries episomal TCR excision circles (TREC)\(^5\) that do not replicate with cell division (3, 4). The number of TREC per million cells is dependent on thymic output, T cell proliferation, and the rate of death of both TREC-positive and -negative T cells. Hazenberg et al. (5) demonstrated an inverse relationship between the number of TREC per million cells and cell division in the naive T cell population from individuals with chronic HIV infection (CHI), suggesting that the reduction of TREC per million cells in HIV infection is a consequence of increased T cell proliferation and is not due to a reduction in thymic output. As suggested by Hazenberg et al. (5), interpretation of these results is complex because T cell proliferation, T cell death, and thymic function are all altered by HIV infection (6–8) and, in addition, each of these processes will also affect the quantification of TREC (4, 9).

To help unravel these competing processes we quantified TREC in two ways. First, we measured TREC per million T cells (CD4\(^+\) and CD4\(^-\)) and then, to account for changes in T cell number due to either HIV infection or therapy, we calculated TREC per milliliter of blood. Proliferation, for example, is thought to decrease TREC by dilution (5). This is clearly true if one measures TREC per million CD4\(^+\) T cells, because division will not increase the number of TREC but will increase the number of cells. However, if one measures TREC per milliliter there will be no change because the number of TREC remains unchanged during cell division. If death occurs simultaneously with proliferation to maintain cell numbers, then TREC per milliliter will decrease as TREC\(^+\)

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\(^*\)Abbreviations used in this paper: TREC, TCR excision circle; PHI, primary HIV infection; CHI, chronic HIV infection; \(T_n\), T lymphocytes in blood; \(T_r\), T lymphocytes in lymphoid and other tissues; WB, Western blot; HAART, highly active antiretroviral therapy.
cells die. Thus, when multiple processes occur, interpretation may be difficult and precise methods of analysis are needed.

Therefore, we measured the total number and the percentage of proliferating T cells and the number of TREC per million cells in sorted CD4+ and CD4− T lymphocytes, and we calculated the number of TREC per milliliter of blood, in the setting of primary HIV infection (PHI) and CHI before and after effective antiretroviral therapy. We also developed a mathematical model that can account for changes in TREC due to input from a thymic source, cell proliferation, cell death, and lymphocyte redistribution. We then used this model to interpret the effects of antiretroviral therapy.

Materials and Methods

Patients

Patients were defined as having PHI if they presented with signs or symptoms of acute retroviral syndrome, had a negative or evolving Western blot (WB) pattern with detectable HIV RNA and proviral DNA, or had a positive WB pattern and a negative ELISA/WB result within the previous 6 mo. Patients were defined as having CHI if they had known HIV infection for >6 mo, with consistently detectable HIV RNA during this time. No patient had received prior antiretroviral therapy. Blood samples were obtained from both PHI (n = 19) and CHI (n = 14) patients recruited into Institutional Review Board–approved clinical trials of two nucleoside reverse transcriptase inhibitors (didanosine, lamivudine, stavudine, or stavudine plus a protease inhibitor (nelfinavir or indinavir).

Cell sorting and real-time PCR for TREC quantification

Concentrations of CD4, CD8, and naive T cells (CD45RA+62L+) were determined by flow cytometry of whole blood, as previously described (10). PBMC were prepared from blood by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) centrifugation and freezing. After thawing, PBMC were incubated with fluorochrome-labeled mAbs, CD3-PE and CD4-FITC, for 30 min on ice (BD Biosciences, San Jose, CA) and sorted into CD3+ CD4− and CD3− CD4+ populations using a cell sorter (MoFlo CLS; Cytomation, Fort Collins, CO). A tight lymphocyte gate in the forward scatter-side scatter diagram was used. At least 2 × 10^6 cells were sorted for each population. DNA was extracted from the purified cell populations using direct cell lysis (3). Sorting into CD3+ CD4+ and CD3− CD8− cells was not possible due to limitations in the number of fluorochromes available. Therefore, for the CD8 T cell studies there is an assumption that CD8 T cells are the major cells in the CD4+ T cell fraction. The percentage of CD3+ CD4− T cells in the CD3− CD4+ fraction has been reported to be 5% and to be unaltered by HIV infection, CD4 count, or antiretroviral therapy (11).

Quantification of TREC per million PBMC, CD4+ cells, or CD4− T cells was performed using real-time PCR and molecular beacons as previously described (4). Results for TREC are expressed per million cells or per microliter. If TREC are expressed per million cells or per microliter, they are multiplied by 10^6 (cells per microliter) × 10^3 = (TREC per million cells) × (cells per microliter) × 10^−3).

Intracellular Ki67 staining

The fraction of dividing T cells was assessed by the combination of surface staining with CD3-PE and CD4-PerCP and intracellular staining with Ki67-FITC (DAKO, Carpinteria, CA) as previously described (12). All assays were performed on frozen cells. Direct comparison of fresh and frozen PBMC from both uninfected (n = 4) and HIV-infected (n = 4) donors revealed no effect of freezing on the percentage of positive Ki67 cells (data not shown).

Model

To interpret the data obtained in this study, we developed a mathematical model (Fig. 1) that is a generalization of the model presented by Hazenberg et al. (5). The model includes T lymphocytes in blood (T_B), and in lymphoid and other tissues (T_L). These populations are then divided into TREC+ (T_B1, T_L1) and TREC− (T_B2, T_L2) subpopulations. This division is convenient for our study but does not necessarily reflect other common subsets. For instance, the TREC− cells will be a mixture of naive and memory cells. The blood compartment receives an input of T cells from the thymus (σ), of which a fraction (f) contains TREC. Here we ignore potential extrathymic sources of new TREC+ T cells. Although these sources may exist (13), they are likely negligible (3, 13, 14).

Cells in the blood die at rate d if they lack TREC, and at a smaller rate d_L if they contain TREC, reflecting the higher number of naive cells in the TREC− population (5, 9, 15). The TREC− cells proliferate at rate p per cell, whereas the TREC+ cells proliferate at rate p_L per cell, which we assume is less than p, due to the increased content of naive cells. We assume proliferation is restricted to the lymphoid tissue, although our results remain the same if proliferation occurs at the same rate in blood and tissue. We note that, because TREC do not appear to replicate or degrade during cell division (3, 16), proliferation of TREC− cells does not change the number of these cells but increases the number of TREC+ cells, with one TREC− daughter cell being created at each division of a TREC+ cell. If, over large periods of time, TREC degrade at rate δ_L, then we could include this in the model by adding a term decreasing the number of TREC− cells at rate δ_L and similarly increasing the number of TREC+ T cells at the same rate. Here, as in other models (5), we make δ_L = 0.

Because measurements of TREC-containing cells are made in blood, we also kept track in the model of the effects of cell trafficking. We assumed that TREC+ lymphocytes migrate from blood to tissue at rate v_B, and from tissue back into blood at rate v_LT, whereas the corresponding rates for TREC− T cells are v_LT and v_B. Finally, we assume that T cells die at the same rate in tissue and in blood. Using these assumptions, the model in Fig. 1 can be converted into the following system of differential equations for either the CD4+ or CD4− T cell populations:

\[
\frac{dT_B}{dt} = (1-f)\sigma - dT_B - v_B T_B + v_LT_L
\]

\[
\frac{dT_B}{dt} = f\sigma - dLT_B - v_BT_B + v_LT_L
\]

\[
\frac{dT_L}{dt} = \left(p - dLT_L + p_LT_B + v_BT_B - v_LT_L\right)
\]

\[
\frac{dT_L}{dt} = -dLT_L - v_BT_B + v_LT_B
\]

In the experiment, we measured TREC per milliliter and TREC per million cells in sorted CD4+ and CD4− T cells obtained from blood. The first quantity is simply 10^6 × T_B, because T cell concentrations are usually measured as cells per microliter. TREC per million cells in blood is calculated as 10^6 × T_B/(T_B + T_L).

We used the model to determine steady state levels of TREC− and to analyze how antiretroviral treatment, which leads to changes in the parameters, would change TREC per milliliter and TREC per million cells in blood. Solution of the model indicates that the steady state levels of TREC per milliliter and TREC per million cells in blood are, respectively,

\[
\text{TREC per milliliter} = \frac{(d_L + v_L)\sigma}{d_L(d_L + v_LT_L + v_LT_B)} \times 10^3,
\]

\[
\text{TREC per million cells} = \frac{f(d_L + v_L)\sigma}{(d_L + v_L)} \times 10^6
\]

\[
\frac{f(d_L + v_L)\sigma}{(d_L - p v_B + v_L) - p v_B}
\]
Note that Eq. 2 shows that the proliferation rates, \( p \) and \( p_1 \), do not affect the steady state level of TREC per milliliter, a point of some importance. Similarly, inspection of Eq. 3 demonstrates that the steady state of TREC per million cells is not affected by the total thymic output \( \sigma \), or only by the fraction of the output that is TREC\(^+\). We have also considered a variant of Eq. 1 in which T cell proliferation is density dependent, i.e., the term

\[
pT_{\text{T}}^{-} + p_1 T_{\text{T}}^{-}
\]

is replaced by

\[
pT_{\text{T}}^{-} \left( 1 - \frac{T_{\text{T}}^{-} + T_{\text{T}}^{+}}{T_{\text{T}}^{-}} \right) + p_1 T_{\text{T}}^{-} \left( 1 - \frac{T_{\text{T}}^{-} + T_{\text{T}}^{+}}{T_{\text{T}}^{-}} \right)
\]

In this case, the expression for the steady state of TREC per milliliter does not change, but the expression for TREC per million cells becomes more complex (data not shown) and now does depend on \( \sigma \).

To study the effect of antiretroviral therapy, we calculate the derivatives of the steady state expressions for TREC per milliliter and TREC per million cells with respect to each relevant parameter. If the derivative is positive, then increases in the parameter lead to increases in the steady state TREC value. If the derivative is negative, then increases in the parameter lead to decreases in the steady state TREC value. For example, to see the effect on TREC per milliliter caused by a decrease in the death rate of TREC\(^+\) cells \( (d_T) \) during therapy, we differentiate Eq. 2 with respect to \( d_T \). We obtain

\[
-\frac{d_T (v_{LT} + v_{LT} + v_{LT} + v_{LT})}{d_T (v_{LT} + v_{LT} + v_{LT} + v_{LT})} \times 10^3,
\]

which is always negative, for any value of \( d_T, v_{LT}, v_{LT}, v_{LT}, \) and \( \sigma \). Thus, we conclude that an increase in \( d_T \) will cause a decrease in TREC per milliliter, or equivalently that any decreases in the death rate of TREC\(^+\) cells lead to increases in TREC per milliliter in blood. We note that this technique is general and obviates the need to use specific parameter values to assess the impact of parameter changes on TREC per milliliter and TREC per million cells.

To analyze the influence of more than one parameter changing simultaneously, as might be expected during treatment, we make two additional assumptions. First, that TREC per million cells is similar in blood and tissue, as reported in rats (17) and humans (3, 18). Second, that the fraction of T cells in blood remains constant. This assumption has been extensively used in the HIV literature, usually by assuming that 2% of lymphocytes are in blood. These assumptions simplify the expression for the steady state level of TREC per million cells (data not shown) and allow the analysis of the effects of simultaneous parameter changes. The simplified expression depends only on the parameters \( p, p_1, d, d_T, \) and \( f \).

For simplicity of analysis, we assume that migration rates are the same for TREC\(^+\) cells and TREC\(^-\) cells, i.e., \( v_{BT} = v_{BT} \) and \( v_{LT} = v_{LT} \). There is experimental evidence that supports this assumption, at least in murine models (17, 19–21), where it is argued that differential accumulation of naive and memory cells in different tissues are due, for example, to different proliferation rates in diverse compartments, as we assume in our model, rather than different migration rates. In any case, even if this simplification is not fully tenable in humans, our results (see Table I) remain unchanged as long as therapy induces similar changes in the migration patterns of TREC\(^+\) and TREC\(^-\) cells (e.g., treatment reduces both \( v_{BT} \) and \( v_{BT} \) by the same amount).

**Statistics**

Comparison of acute and chronic infection was made using the Wilcoxon rank sum test. Comparison of the results under therapy vs baseline was done using a paired, one-tailed Wilcoxon signed rank test. Correlations between continuous variables were assessed using the Pearson correlation coefficient. Significance was determined as \( p < 0.05 \).

**Results**

**Analysis of the model**

The model in Fig. 1 (see Materials and Methods) was used to explore the expected effects of antiretroviral therapy on the steady state values of TREC per milliliter and TREC per million cells. We present these results in Table I. For example, reduction in the death rate of TREC\(^+\) cells \( (d_T) \) leads, as expected, to an increase in TREC per million cells and TREC per milliliter. However, therapy reduces the death rate of TREC\(^-\) lymphocytes as well \( (d) \), with the opposite effect: it reduces TREC per million cells and has no effect on TREC per milliliter. So the net effect of reduced death rates varies depending on how therapy affects \( d \) relative to \( d_T \). Examining the effects of therapy on extrathymic T cells shows that a decrease in the proliferation rate of mature T cells after effective antiretroviral therapy (22) leads to different outcomes in the two measures of TREC content, increasing TREC per million cells but leaving the number of TREC per milliliter unchanged (Table I).

In contrast, changes in recirculation rates, which lead to release of lymphocytes trapped in the lymph nodes to the blood, increase TREC per milliliter but do not change TREC per million cells, assuming that TREC per million cells is the same in lymph nodes and blood, as reported in rats (17) and humans (3, 18). Finally, if HIV-1 infection affects thymic function, then treatment may lead to an increase in thymic output. Increases in \( \sigma \) lead to an increase in TREC per milliliter but will lead to an increase in the steady state value of TREC per million cells only if T cell proliferation is

<table>
<thead>
<tr>
<th>Parameter Change</th>
<th>TREC per Million Cells</th>
<th>TREC per Milliliter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pretherapy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-infected</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Post-therapy expected effect</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death of TREC(^+) cells</td>
<td>( d_T \downarrow )</td>
<td>( \uparrow )</td>
</tr>
<tr>
<td>Death of TREC(^-) cells</td>
<td>( d \downarrow )</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>Proliferation</td>
<td>( p \downarrow, p_1 \downarrow )</td>
<td>( = )</td>
</tr>
<tr>
<td>Redistribution lymph nodes—blood</td>
<td>( v_{BT} \downarrow, v_{LT} \downarrow )</td>
<td>( = )</td>
</tr>
<tr>
<td>Thymic output</td>
<td>( \sigma \uparrow )</td>
<td>( = ) or ( \uparrow )</td>
</tr>
<tr>
<td>Fraction TREC(^+) cells</td>
<td>( f \uparrow )</td>
<td>( \uparrow )</td>
</tr>
<tr>
<td><strong>Observation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH1</td>
<td></td>
<td>Transient ( \uparrow )</td>
</tr>
<tr>
<td>CD4(^+)</td>
<td></td>
<td>Transient ( \uparrow )</td>
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<tr>
<td>CD4(^-)</td>
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<td>CD4(^+)</td>
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<tr>
<td>CD4(^-)</td>
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</tbody>
</table>

\(^a\) Predictions are based on the model described in Materials and Methods, where \( \sigma \) = thymic output; \( p \) = proliferation rate of TREC\(^+\) cells; \( p_1 \) = proliferation rate of TREC\(^-\) cells; \( d_T \) = death rate of TREC\(^+\) cells; \( d \) = death rate of TREC\(^-\) cells; \( v_{BT} \) = rate of T cell redistribution from blood to lymphoid tissue; \( v_{BT} \) = rate of T cell redistribution from lymphoid tissue to blood.

\(^b\) Increase (\( \uparrow \)) occurs only in models with density-dependent T cell proliferation.
density dependent (see Materials and Methods). Another possible effect of treatment-induced recovery of the thymus is to increase the fraction of TREC\(^+\) cells leaving the thymus (\(j\)) due to reduced thymic proliferation of maturing T cells. If this occurs, then both TREC per million cells and TREC per milliliter increase (Table I).

**TREC in CD45RA\(^+\) and CD45RA\(^-\) T cells in healthy and HIV-1-infected individuals**

In a subset of individuals with HIV-1 infection (PHI, \(n = 2\); CHI, \(n = 6\)) and age-matched HIV-uninfected individuals (\(n = 4\)), we quantified TREC per million cells in separated CD4\(^+\) and CD4\(^-\) T cells, further sorted into CD45RA\(^+\) and CD45RA\(^-\) subsets. The number of TREC per million cells was significantly higher in CD45RA\(^+\) cells than in CD45RA\(^-\) T cells in both healthy and infected individuals, as previously reported for naive and memory T cells (3, 4, 9). When we compared TREC per million CD45RA\(^-\) cells between infected and uninfected individuals, we found no statistical difference (\(p = 0.154\) for CD4\(^+\) and \(p = 0.383\) for CD4\(^-\); Fig. 2). This may be a consequence of the small sample size or, more likely, it may be explained by an increase in the input of TREC-containing cells into the CD45RA\(^+\) pool of HIV-infected individuals, due to lymphocyte activation or transient acquisition by a CD45RA\(^+\) T cell of a memory-like phenotype (CD45RA\(^-\)) after homeostasis-driven proliferation (23, 24). The data shown here suggest that this added input balances the decrease in TREC due to increased proliferation and death rates in the CD45RA\(^-\) lymphocyte population of HIV-infected individuals.

We then analyzed the fraction of TREC per million cells in CD45RA\(^+\) T cells calculated as TREC\(^+\) CD45RA\(^+\) /TREC\(^+\) CD45RA\(^-\) + TREC\(^+\) CD45RA\(^-\). We found that in uninfected individuals this fraction is 7 and 4% in CD4\(^+\) and CD4\(^-\) T cells, respectively, in agreement with the reported range of 2–10% (4, 9). In contrast, the percentage of TREC per million cells in CD45RA\(^-\) T cells was significantly higher in infected patients when compared with uninfected patients, with 19% (\(p = 0.03\)) and 23% (\(p = 0.04\)) in the HIV-infected CD4\(^+\) CD45RA\(^-\) and CD4\(^-\) CD45RA\(^-\) fractions, respectively. Again, this higher percentage of TREC\(^+\) CD45RA\(^-\) T cells in HIV-infected individuals may be a consequence of high rates of immune activation.

We calculated TREC per milliliter, as described in Materials and Methods, for CD4\(^+\) CD45RA\(^+\) and CD4\(^-\) CD45RA\(^-\) T cells separately. Even though the number of TREC per million cells is larger in CD45RA\(^+\) T cells, there are many fewer CD45RA\(^-\) T cells than CD45RA\(^+\) T cells in these HIV-1-infected individuals; thus, in terms of TREC per milliliter, only 50% are in CD45RA\(^+\) T cells (95% confidence interval, 31.2–68.7%) (Table II). For this reason we believe that the TREC contribution of the CD45RA\(^-\) population should not be neglected in the setting of HIV-1 infection.

In three of the eight HIV-infected individuals we then analyzed the changes in TREC per million cells in CD45RA\(^-\) and CD45RA\(^+\) cells after 8, 24, and 48 wk of antiretroviral therapy. There was an increase in total CD4\(^+\) T cells and undetectable viral load for most of this period in all three individuals. In the three individuals there was a trend for an increase of TREC per million CD45RA\(^+\) cells. In the CD45RA\(^+\) population, TREC per million cells did not change significantly despite the significant reduction in proliferation that occurs in this lymphocyte pool after highly active antiretroviral therapy (HAART) (data not shown). This is an indication that the input of TREC-containing cells into the CD45RA\(^+\) cell population is also reduced after initiation of treatment, possibly due to the reduction in the activation state of the immune system after treatment (2, 25).

**Baseline comparison between CHI and PHI individuals**

Baseline characteristics of individuals with PHI and CHI before treatment are shown in Table III. Table III and previous work (3, 4) show that HIV infection leads to a decrease in TREC-containing cells. Individuals with CHI, in relation to individuals with PHI, have significantly lower numbers of TREC, measured as TREC per million cells or as TREC per milliliter, in both CD4\(^+\) and CD4\(^-\) T cell compartments (\(p < 0.001\)). In PHI, TREC per million CD4\(^+\) T cells is strongly correlated with TREC per million CD4\(^-\) T cells (\(r^2 = 0.74; p < 0.0001\)), but this is not observed in CHI (\(r^2 < 0.1; p = 0.5\)).

There is no significant difference in the percentage of Ki67\(^+\) CD4\(^+\) T cells between the PHI and CHI groups before therapy (\(p = 0.086\)). This suggests that the growth fraction of CD4\(^+\) T cells is similar in both PHI and CHI. However, there is a higher total number of Ki67\(^+\) T cells (per microliter) in both CD4\(^+\) and CD4\(^-\) populations in PHI than in CHI (\(p < 0.005\)). We note that the PHI group in this study represents patients with true PHI (see Materials and Methods) before they reach a set point in viral load and who have significantly higher viral loads than the individuals with CHI (see Table III).

In individuals with PHI there was no correlation between baseline CD4\(^+\) T cell count and the percentage of Ki67\(^+\) CD4\(^-\) T cells or the absolute number of Ki67\(^+\) CD4\(^-\) T cells (\(p = 0.47\) and \(p = 0.10\), respectively). However, such a correlation has been seen in CHI (15, 26). In the CD4\(^-\) fraction of individuals with PHI, we found a strong correlation between baseline CD4\(^-\) numbers and both the percentage of Ki67\(^+\) CD4\(^-\) and absolute numbers of Ki67\(^+\) CD4\(^-\) T cells (\(p = 0.012\) and \(p < 0.001\), respectively), again in contrast to previous findings in CHI (26).

**Effect of antiretroviral therapy on T cell turnover and TREC**

Over the first 8 wk of effective antiretroviral therapy there was an increase in both the total CD4\(^+\) and naive CD4\(^+\) T cell populations in PHI and CHI. Higher cell counts in relation to baseline were
maintained throughout the follow-up period. The percentage of CD4² naïve T cells in relation to the total pool of CD4⁺ cells remained constant throughout the period of therapy in both study groups. This indicates that the recovery of these populations (total CD4⁺ and naïve CD4⁺) proceeds at the same rate during HAART. We also observed a sustained increase over baseline of the number of CD8⁻ naïve cells after treatment of PHI.

In general, the percentage of Ki67⁺ CD4⁺ and Ki67⁺ CD4⁻ T cells declined after antiretroviral therapy of individuals with PHI and CHI, in agreement with previous studies (5, 15) (Fig. 3). However, when we measured the total number of cells expressing Ki67 per microliter of blood, the results were quite different for PHI and CHI (Fig. 3, C and D, respectively). In treated PHI, there was a sustained and significant reduction in the total number of Ki67⁺ cells in relation to baseline for both CD4⁺ and CD4⁻ populations, for all of the posttherapy follow-up. In contrast, in CHI the absolute number of Ki67⁺ CD4⁺ T cells was not reduced and we did not detect any significant difference between the number of Ki67⁺ CD4⁻ T cells at baseline and at 8, 24, and 48 wk of antiretroviral therapy. This result differs from that obtained by Hazenberg et al. (5), where a significant increase in total numbers in the number of Ki67⁺ CD4⁻ cells was observed already after 10 wk of HAART, even though a transient increase was observed again at 24 wk. Most likely, our individuals with CHI are a more homogeneous group at a more advanced stage of HIV infection, as measured by CD4⁻ T cell counts (median CD4⁻ cells: 180 cells per microliter in this study and 280 cells per microliter in Ref. 5).

We measured the changes in TREC per milliliter and TREC per million cells during therapy (Fig. 4). In the CD4⁺ T cell population there was a clear difference between PHI and CHI. In individuals with PHI there was only a transient increase in TREC per milliliter after 8 wk of therapy (p = 0.028, one-sided test). This significant increase in relation to baseline rapidly disappeared and was not observed at 24 and 48 wk (Fig. 4C). In contrast, for CHI there was a significant increase in CD4⁺ TREC per milliliter at all time points (p < 0.02, paired one-sided test) in relation to baseline and there was either a significant increase or a trend for an increase in the number of TREC per million CD4⁺ T cells (p = 0.091 at 8 wk, p = 0.046 at 24 wk, and p = 0.055 at 48 wk) (Fig. 4D).

In the CD4⁻ T cell population the results obtained were similar to the CD4⁺ T cell population (Fig. 4). After treatment of CHI there was a sustained increase in TREC per milliliter and TREC per million cells from baseline (with p < 0.02 for all comparisons except TREC per million CD4⁻ cells at 8 wk (p = 0.076)). However, after treatment of PHI there was only a transient trend for increased TREC per milliliter at 8 wk, which did not reach significance (p = 0.060), whereas the number of TREC per million CD4⁻ cells increased slowly and the difference in relation to baseline was significant only at 48 wk (p = 0.046).

We next looked for correlations between T cell proliferation and TREC content in both acute and chronic infection. Before treatment for both CD4⁺ and CD4⁻ T cells there was no correlation between TREC per million cells or TREC per milliliter and the percentage of Ki67⁺ or total Ki67⁺ T cells for either PHI or CHI. After effective antiretroviral therapy of PHI and CHI, there was a significant and consistent reduction in the percentage of Ki67⁺ cells. However, we could not detect any significant correlation between reduction in the percentage of Ki67⁺ and changes in

<p>| Table II. Comparison of TREC per milliliter in CD45RA⁺ and CD45RA⁻ CD4⁺ T cells in eight HIV-infected subjects (two PHI and six CHI) |
|---------------------------------------------------------------|-----------------------------|---------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Subject</th>
<th>TREC/10⁶ CD4⁺ Cells</th>
<th>CD4/µl</th>
<th>TREC/ml in CD4⁺ Cells</th>
<th>% TREC in CD45 RA⁻</th>
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<tr>
<td>1</td>
<td>6,631</td>
<td>2,766</td>
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<td>8</td>
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<td>2,944</td>
<td>391</td>
<td>66</td>
</tr>
<tr>
<td>SE</td>
<td>6,335</td>
<td>580</td>
<td>72</td>
<td>15</td>
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</tbody>
</table>

*Means and ranges of the measured quantities for the two groups under study. The p value, computed by the Wilcoxon rank sum test, denotes the significance of differences between the two groups.*
TREC per million cells, as suggested before (15). In Fig. 5A, we show some clear examples (PHI 1-3, CHI 1) of individuals with a profound reduction in their CD4/CD1100 proliferation rates, without an increase in their TREC per million values, particularly during the first 8 wk of treatment. In fact, analysis of changes in single individuals showed considerable variation in the posttherapy evolution of TREC per million cells and TREC per milliliter (Fig. 5). The patient-to-patient variability in TREC after treatment of PHI and CHI is consistent with our previous work (4) and reinforces the idea that the observed reduction in proliferation rates was not the driving force behind the measured changes in TREC.

**Discussion**

We found that TREC per million CD45RA T cells of individuals with PHI and CHI before therapy was decreased relative to healthy donors, as previously reported (3–5, 9). However, in the CD45RA subset there was no significant difference in TREC per million cells between age-matched healthy and HIV-infected adult subjects (Fig. 2). The preservation of TREC in CD45RA T cells in HIV infection may be a reflection of the higher degree of immune activation and consequent transition from a CD45RA to a CD45RA state in both CD4+ and CD4 T cells (27). Alternatively, as described in mouse models of lymphopenia, naive T cells may acquire a memory-like phenotype during homeostasis-driven proliferation (23, 24) without loss of TREC.

Furthermore, our analysis of TREC per milliliter in CD45RA+ and CD45RA− T cells shows that, in the setting of HIV-1 infection, only ~50% of TREC are in "naive" CD45RA+CD4+ cells. We calculated TREC per milliliter from TREC per million cells by adjusting for the absolute numbers of CD45RA+ and CD45RA− T cells per milliliter of blood. When we performed this calculation (Table II) we found equivalent amounts of TREC per milliliter in CD45RA+CD4+ and CD45RA−CD4+ T cells. Therefore, even though the number of TREC per million cells is larger in CD45RA+ than in CD45RA− T cells, because there are more CD45RA+ than CD45RA− T cells in HIV infection, this will increase TREC per milliliter for CD45RA− T cells. This would not necessarily be the case in HIV-infected children or young adults.

Several processes affect TREC in a cell population. TREC measured in blood can increase due to the input of TREC+ naive cells from the thymus or input of TREC+ cells from an extrathymic source. Two processes lead to a decrease in TREC: dilution of

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**FIGURE 3.** Changes in proliferation measured as the percentage of Ki67+ cells (upper panels) and total Ki67+ cells per microliter (lower panels) during 48 wk of effective antiretroviral treatment of PHI (A) and CHI (B). The edges of the boxes are the 25th and 75th percentiles, the horizontal line in the box is the median, the symbols indicate the means, and the whiskers represent the 10th and 90th percentiles. Changes in Ki67+ cells per microliter, measured as paired differences (within individuals) between the week shown and baseline (corresponding to the horizontal line at 0), are shown after effective treatment of PHI (C) and CHI (D). The p values shown at the bottom of the panel are for the paired differences with baseline.
TREC due to proliferation (without TREC replication) and loss of TREC due to the death of TREC\(^+\) cells. These processes differently affect TREC per milliliter and TREC per million cells (Table I), because TREC per milliliter depends only on TREC\(^+\) cells, whereas the number of TREC per million cells depends on both TREC\(^+\) and TREC\(^-\) cells. To account for the changes in TREC per milliliter and TREC per million cells we developed a model that incorporates T cell proliferation, death, redistribution, and thymic production.

During antiretroviral therapy of PHI, we observed a transient increase in TREC per milliliter and unchanged TREC per million cells. According to our model, these observations can be explained by lymphocyte redistribution from lymphoid tissue to blood (Table I). Treatment reduces the amount of virus in lymph nodes (28–30) and hence promotes the release of T cells back into the circulation. The concomitant input of TREC\(^+\) cells into the blood increases TREC per milliliter, but, because TREC per million cells seem to be identical in blood and lymph nodes (3, 17, 18), redistribution...
leaves the number of TREC per million cells in the blood unchanged. Redistribution has been implicated in the initial rise of T cell counts posttherapy but not in the continuing rises seen with months of therapy (31–34). Thus, redistribution could also explain the transient nature of the increase in TREC per milliliter, because redistribution would not constitute a permanent source of TREC cells into the blood. Other effects do not offer a convincing explanation for the transient increase in TREC per milliliter. For example, increases in the thymic output of TREC+ cells lead to continuing increases in TREC per milliliter and TREC per million cells, which we do not observe. However, we cannot rule out a small increase in thymic output due to therapy. During PHI, thymic output only contributes a small fraction of the TREC already present in blood (5), and thus a small increase due to therapy may be difficult to detect. Surprisingly, proliferation seems not to be a major influence on the changes in TREC in CD4+ and CD4− T cells in PHI, because despite a consistent and pronounced decrease of markers of proliferation, we do not observe a consistent increase in TREC per million cells (Fig. 5A). This result is consistent with a recent report on early HIV infection that suggested that the decrease in CD4+ T cell TREC per million during HIV infection could not be the result of increased turnover of naive CD4+ T cells (9). In addition, decreases in proliferation should not affect TREC per milliliter (see below).

In CHI, after initiation of treatment, we observe a sustained increase in both TREC per million cells and TREC per milliliter. In the model, this can only be a reflection of increased thymic production of TREC+ cells (α or f) or a preferential reduction of the death rate of TREC-containing cells (Table I). The TREC per million cells exiting the thymus is larger than the TREC per million cells of the blood; thus, new thymic cells contribute not only to increases in TREC per milliliter but also to increases in TREC per million cells. Because TREC only disappear when cells die, reduction in the death rate of TREC+ cells due to therapy results in increases in TREC per milliliter if there is an unchanged or increased source of TREC from the thymus. However, reduction of the death rate of TREC+ cells can lead to the observed increase in TREC per million cells only if there is a smaller reduction in the death rate of TREC+ cells. We do not know of any biological reason or observation for the preferential reduction of the death rate of TREC+ cells, and thus reduction of the death rate per se is unlikely to explain the observed changes in TREC with therapy of CHI. Reductions in proliferation have been proposed as responsible for the increase in TREC per million cells (9, 15). However, proliferation does not affect TREC per milliliter (Eq. 2), and thus reductions in proliferation cannot account for the observed increase in TREC per milliliter.

In summary, only the increased output of TREC+ T cells from the thymus explains the observed changes in TREC with treatment of CHI. Moreover, we find a very significant correlation between the changes in TREC per milliliter and the changes in CD4+ naive cells per microliter posttherapy (p = 0.001, with a random effects model), suggesting that the increase in naive cells is mainly due to recent thymic emigrants (35) rather than to expansion of existing naive cells.

Our results are partly in contrast with a recent finding of a positive correlation between the increase in TREC per million cells and the fold decrease in proliferating naive T cells 6 mo after effective therapy for HIV infection (15). Another study also describes an inverse correlation between TREC per million naive cells (CD45RA+) and naive cell division (measured by Ki67 expression in CD45RO−CD27+ cells), although these results were cross-sectional and did not include detailed treatment analysis (5). One can speculate that this inverse correlation may have been a consequence of the inclusion of cells with a phenotype between that of true naive and effector/memory cells (so called “transitional cells”) into the naive cell gate, which may artificially increase the percentage of Ki67 in the “naive” fraction, as argued before (9). In fact, when transitional cells are excluded from the analysis there is no association between TREC per million cells and Ki67 in the naive cell fraction in individuals with early HIV infection (9). We also did not find a correlation between TREC per million total CD4+ or CD4− T cells and Ki67 expression in
total CD4⁺ or CD4⁻ T cells either before treatment or following 24 wk of treatment. Whether this discrepancy is due to differences in the duration of infection at the time of treatment initiation, differences between naïve T cells and total T cells, or other factors is unknown. However, we believe caution is necessary in interpreting associations between decreases in proliferation and increases in TREC.

It is clear that changes in proliferation do not affect TREC per milliliter, because TREC are not created nor destroyed when cells divide (3) (see Model, Eq. 2, and Table I). In contrast, a reduction in proliferation rates leads to an increase in TREC per million cells, but only in the context of unchanged death rates. However, such a scenario leads to a decrease in cell counts, which is clearly not observed with HIV treatment (Fig. 5). In fact, it is well established that treatment results in reduction of both proliferation and death rates (22, 36, 37). Our model shows that if proliferation (p₁, p₂) and death (d₁, d₂) rates are reduced by the same proportion, then the equilibrium number of TREC per million cells will remain unchanged. This is because the total number of cells depends on p₁ + d₁ and p₂ + d₂, whereas the number of TREC⁺ cells depends on d₂; if all these parameters are reduced equally then both the TREC⁺ cells and the total cells increase by the same proportion and TREC per million cells remains unchanged.

Finally, we emphasize that in our patients, even though the the percentage of Ki67⁺ CD4⁺ cells correlates well with the percentage of Ki67⁺ CD4⁺ cells in both PHI and CHI (r² = 0.8, p < 0.0001 in PHI; r² = 0.4, p = 0.037 in CHI), TREC per million CD4⁺ cells correlates with TREC per million CD4⁺ cells only in PHI (r² = 0.7, p < 0.0001) and not in CHI (r² < 0.1, p = 0.5). This indicates that similar high levels of proliferation are maintained in both CD4⁺ and CD4⁻ T cells during infection, with different impacts on TREC per million cells in those two lymphocyte populations. Thus, discussions in terms of the effect of single processes on TREC should be taken with caution, because several processes—proliferation, death, thymic output, redistribution—occur simultaneously (9). Taken together our results demonstrate that, to accurately interpret TREC data, one needs to use more sophisticated theoretical models, such as the one presented here, with concomitant assessment of disease status, TREC per milliliter, TREC per million cells, T cell proliferation, and T cell death, in sorted naive and memory cells.

We have shown that perturbations in T lymphocyte dynamics differ significantly in PHI and CHI, and we demonstrate that reduction in TREC after HIV infection cannot be explained by proliferation alone. Furthermore, after effective antiretroviral therapy of PHI and CHI, we have identified different patterns of increases in TREC per milliliter and TREC per million cells. Using a mathematical model to study the likely mechanism explaining these changes, we conclude that the changes in TREC after effective therapy for PHI are due to T cell redistribution from lymphoid tissue to blood. However, after treatment for CHI, an increase in thymic output is necessary to explain TREC increases, in particular the observed increase in TREC per milliliter. Thymic contribution is an important component of immune reconstitution after treatment of CHI and should be the target of future development of new therapeutic agents.

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References


