Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection

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Recent thymic emigrants can be identified by T cell receptor excision circles (TRECs) formed during T-cell receptor rearrangement. Decreasing numbers of TRECs have been observed with aging and in human immunodeficiency virus (HIV)-1 infected individuals, suggesting for thymic impairment. Here, we show that in healthy individuals, declining thymic output will affect the TREC content only when accompanied by naive T-cell division. The rapid decline in TRECs observed during HIV-1 infection and the increase following HAART are better explained not by thymic impairment, but by changes in peripheral T-cell division rates. Our data indicate that TREC content in healthy individuals is only indirectly related to thymic output, and in HIV-1 infection is mainly affected by immune activation.

It was postulated that HIV-1 induced CD4^{+} T cell depletion could at least partially be due to interference of HIV with *de novo* production of T cells at the level of T cell progenitor function or thymic output. Several studies have focused on the influence of HIV-1 infection on thymic function, although the results were inconclusive[1,2]. Recently, a new method has been reported by Douek and colleagues[3] that may allow a more direct estimation of thymic T cell production. Formation of a productive T cell receptor (TCR)α-gene requires deletion of the TCRδ-gene, which is positioned within the TCRα-locus[4]. The TCRδ-gene is flanked by two TCRδ-deleting elements, δRec and ψJ-α, which preferentially rearrange to each other, thereby deleting the TCRδ locus[5–7]. The deleted TCRδ-gene remains present as an extrachromosomal circular excision product, the so-called signal joint T-cell receptor excision circle (Sj TREC or TREC) (refs. 8 and 9). These circles are episomal, do not replicate during mitosis and are thus diluted during cell division[4,8]. With quantitative polymerase chain reaction (PCR), TRECs can be detected and quantified, thereby offering a tool to identify recent thymic emigrants and to estimate thymic output. Using TREC measurements, it was suggested that, although thymic function declines with age, limited thymic output was still measurable at old age[3,10–12]. In HIV-1 infected individuals, low TREC contents in T cells and PBMC were reported in a large percentage of patients studied[3,10,13]. TREC content increased during highly active anti-retroviral therapy (HAART), suggestive for improvement of pre-treatment impairment of thymic function[3,10]. However, the effect of dilution of these episomal circles, resulting from repeated episodes of pathogen induced cell division during life, or from a chronically activated state of the immune system such as observed in HIV-1 infection[14–16], has not been taken into account in analyses and interpretation of these data. Here we combined measurements of TRECs and cell division rates in naive T cells of healthy and HIV-1 infected individuals with mathematical modeling, to come to a better interpretation of TREC measurements with respect to thymic output.

Sj TRECs in healthy individuals

First, to validate the assay, we measured the TREC content of CD4^{+} and CD8^{+} T cells of 49 HIV-1 negative individuals, from 0 (cord blood) to 84 years of age. The number of TREC copies declined with increasing age ($P_s < 0.001$, $R_s = -0.800$, data not shown). To study whether TRECs were diluted by cell proliferation, we cultured cord blood peripheral mononuclear cells (PBMC) from HIV-1 negative donors with interleukin (IL)7 (10 ng/ml) and phytohemagglutinin (PHA, 1 µg/ml) for 7 days.

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Table 1 Characteristics of healthy individuals and HIV-1 infected patients

	CD4+ T cells			CD8+ T cells		
	HIV⁻	HIV⁺	P	HIV⁻	HIV⁺	P
	(n = 17)	(n = 33)		(n = 14)	(n = 7)	
Age (years)	37 (24–62)	41 (24–57)	ns	40 (24–62)	42 (27–54)	ns
T cells (per μl)	880 (620–1430)	400 (160–1350)	< 0.001	405 (210–640)	1570 (770–2720)	< 0.001
Naive T cells (per μl)	494 (276–1027)	150 (22–756)	< 0.001	248 (149–354)	218 (126–440)	ns
Ki-67+ naive T cells (%)	0.4 (0.1–0.8)	3.1 (0.7–5.7)	< 0.001	0.3 (0.1–0.9)	3.4 (1.7–30.7)	< 0.001
Sj TRECs(×10⁻³ copies /CD45RA+ cell)	25.1 (5.5–221.4)	12.1 (0.08–57.1)	< 0.05	31.4 (4.2–219.1)	2.5 (0.13–7.8)	< 0.001

Depicted are median and range. Statistical significance was calculated with the Mann-Whitney U test; $P < 0.05$ was considered statistically significant.

Sequentially, the number of PBMC, the proportion of cell death and TRECs were measured. As expected, *in vitro* cell division led to dilution of TRECs (data not shown). We recently demonstrated that during HIV-1 infection peripheral T-cell division rates are differentially increased in all lymphocyte subsets, including the naive CD4+ and naive CD8+ T-cell pools[14]. This may confound the interpretation of TREC analysis when measured in total PBMC or non-separate CD4+ and CD8+ T cells. We have found that the number of TRECs in the naive CD4+ T-cell pool (FACS-sorted purified CD27+ CD45RA+ cells) is relatively high, although significant numbers of TRECs were also detected in the memory population[12] (data not shown). Because of practical limitations, TREC analysis could only be performed on purified CD45RA+ T cells and not on 'truly naive' fractions defined by co-expression of CD27. Therefore, subsequent TREC analysis was performed exclusively in purified CD45RA+ CD4+ and CD45RA+ CD8+ T-lymphocyte subsets, in parallel with assessment of peripheral cell division rates in the CD27+ CD45RA+ subsets using Ki-67 expression[17] (see Methods). Throughout the text, the term 'naive' denotes truly naive T cells, as being CD45RA+ and CD27+, otherwise the term CD45RA+ is used.

Sj TRECs during HIV-1 infection

Table 1 shows characteristics of HIV-1 infected patients and age
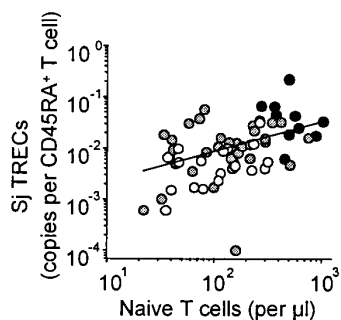

Fig. 1 Correlation between TRECs and naive T-cell numbers. In HIV-1 infected Dutch patients (●) and non-infected individuals (●), the number of TRECs per CD45RA+ CD4+ T cell and the size of this subset are correlated ($R_P = 0.427$, $P_P = 0.003$). (○) represent HIV-negative Ethiopian individuals (see text).

matched healthy individuals that were included in our study. HIV-1 infected patients had lower numbers of CD4+, and higher numbers of CD8+ T cells, compared to healthy individuals. The number of naive CD4+, but not naive CD8+ T cells, was significantly reduced. Percentages of Ki-67+ naive T cells were increased in these subsets. The percentage of Ki-67+ naive CD4+ T cells was negatively correlated with the number of naive CD4+ T lymphocytes, as reported previously[14] ($R_s = -0.843$, $P_s < 0.001$; data not shown). HIV-1 infected patients had a significantly lower TREC content of CD45RA+ CD4+ and CD45RA+ CD8+ T lymphocytes compared to healthy subjects. The number of TRECs per CD45RA+ T cell correlated with the size of the naive CD4+ T-cell pool, such that patients with high naive T cell numbers had a high CD45RA+ CD4+ T-cell TREC content ($R_P = 0.427$, $P_P = 0.003$; Fig. 1).

Effects of thymic output and cell division

The TREC content of a naive T cell population may depend to various extents on thymic production, cell death and cell division, priming of naive T cells to become memory lymphocytes and intracellular degradation of TRECs (ref. 18). We therefore developed a mathematical model to interpret our data.

In this model, N is the total number of naive (CD4+ or CD8+) T cells and T the total amount of TRECs in the naive T-cell population, $\sigma(a)$ is the age (a)-dependent source of naive T cells from the thymus, α the rate of naive T-cell division, δ the rate of cell death and priming of naive cells and c is the amount of TRECs in recent thymic emigrants (RTEs). Changes in the number of naive T cells can be described as $dN/dt = \sigma(a) + \alpha N - \delta N$ (Equation 1).

The total amount of TRECs in this population increases proportionally to thymic production, and decreases when naive T cells die, become primed or possibly because of intracellular degradation of the TRECs (δ_i), and can be expressed as $dT/dt = c\sigma(a) - \delta T - \delta_i T$ (Equation 2).

Note that the *total* amount of TRECs in the naive T-cell population does not decrease by division (α). Division only affects the *average* TREC content per naive T cell. By the 'quotient-rule' of differentiation, this average, defined as $A = T/N$, should change according to $dA/dt = \sigma(a)(c - A)/N - \delta_i A - \alpha A$ (Equation 3).

The quasi steady state is at $\hat{A} = c / [1 + N(\delta_i + \alpha) / \sigma(a)]$ (Equation 4). In the absence of division and degradation, when $\delta_i + \alpha = 0$, the steady state is $\hat{A} = c$. In other words, lower thymic output is only reflected by a reduction in TREC content of a naive cell when there is division of naive T cells ($\alpha > 0$), and/or intracellular degradation ($\delta_i > 0$). Moreover, if the number of naive T cells N in our model remains proportional to thymic production $\sigma(a)$, the $\sigma(a)$ term cancels from Equation 4. Thus, in that case the TREC content per naive T cell is essentially a measure of division and/or degradation, not of thymic output.

To validate our model, we studied quantitatively how the different parameters are expected to influence the average TREC content of a naive T cell. In the following equation thymus production is assumed to decay exponentially and division to be density dependent: $\sigma(a) = \sigma_0 \exp[-va]$ (Equation 5a), and $\alpha = \alpha_0 / (1 + N^2 / h^2)$ (Equation 5b), where σ_0 is the newborn thymus production of naive T cells, v the involution rate of the thymus, σ_0
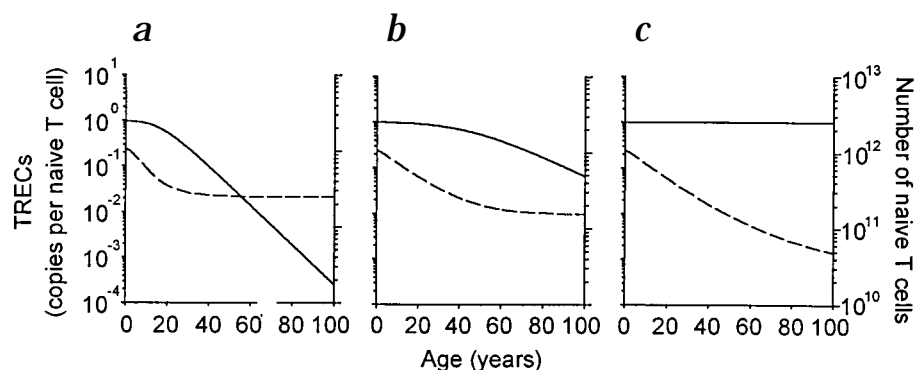
Fig. 2 The model behavior as a function of age (*a, b* and *c*). Thymic output declines exponentially, and the naive T cell population is maintained by (*a*) a modest increase in cell division (*α*), (*b*) a density dependent decrease in death rate (*δ*) and a constant cell division rate (*α* = 10^{-3}), or (*c*) a density dependent decrease in death rate (*δ*) in the absence of cell division (*α₀*=0). Solid lines represent TREC content per naive T cell, dashed lines the total number of naive T cells. *a*, $\alpha_0 = 0.1$/day, $\delta = 10^{-3}$/day and $\varepsilon = 0$. *b*, $\alpha = 10^{-3}$, $\delta_0 = 0$/day and $\varepsilon = 9.1 \times 10^{-16}$. *c*, $\alpha_0 = \delta = 0$/day, and $\varepsilon = 8.9 \times 10^{-16}$. Parameters: $c = 1$, $\delta_i = 0$, $h = 2.5 \times 10^{10}$ cells, $\sigma_0 = 10^9$ cells/day and $v = 0.1$/year.

is the maximum division rate of naive T cells (which is approached at the lowest naive T-cell counts) and *h* the naive T-cell count at which the division is half maximal. This model is in good agreement with the data obtained from healthy individuals[12] (Fig.2). In all three panels, the thymic output decreases exponentially, but the naive T-cell population is maintained by only modest homeostatic changes in either the division (*α*) or the death (*δ*) rate (see equation 5c). In the case of a density dependent increase in division rate (*α*), the average number of TRECs per naive T cell remains high until puberty, and then decreases several orders of magnitude (Fig. 2*a*). Alternatively, the lifespan of naive T lymphocytes could be prolonged with the decrease in T-cell density that is associated with age-related decline in thymic output. Therefore, we included density dependent death of naive T cells, described as $\delta = \delta_0 + \varepsilon N$ (Equation 5c) in our model. In this equation, εN allows for a density dependent increase in death rate. The average TREC content also declines orders of magnitude when there is a density dependent decrease in the death rate ($\varepsilon > 0$) and a constant naive

T-cell division rate ($\alpha = \alpha_0$, Fig. 2*b*). However, when $\alpha = 0$ and δ is density dependent, the average TREC content remains constant during life (Fig. 2*c*).

When TRECs are measured in separated CD45RA⁺ T cell populations, rather then in 'truly naive' T cells, reversion of CD45RA⁻ memory cells into this population could also affect the TREC content. Reversion can be included in our model by allowing for a 'source' of revertants, *R*, in Equation 1. Because revertant memory cells should contain little or no TRECs, Equation 2 should remain the same. The *R* term reflects the number of memory cells reverting to a naive phenotype on a daily basis. We have studied the extended model numerically and found that reversion can only have an observable effect on the TREC content when more than half of the total production of naive T cells is due to reversion (i.e., when $R > \sigma(a) + \alpha N$). Although there might be reversion of some memory cells[19–21], it seems unlikely that this occurs at such high rate in healthy people[20]. Thus, in our model it is the increase in division rate around puberty that sets the decrease in TREC content after puberty. Furthermore, CD45RA⁻ CD45RO⁺ memory cells are thought to revert to a CD45RA⁺ CD45RO⁻ phenotype only when in a resting state, that is, in the absence of their specific antigen. In HIV-1 infection, given the hyperactivation, this is probably a rare event. If a substantial reversion rate does exist, it just enhances the decreasing effect of naive T-cell division on TREC content. In any case, peripheral cell division is required (assuming no intracellular decay), and is an important factor, for decreasing TREC content.

Effects of HIV-1 infection on TREC content

HIV-1 infection could influence the TREC content of the naive T cell compartment in various ways: 1) direct infection of the thymus could lead to abolishment of thymic production (*σ*), 2) HIV-mediated killing of naive cells or an increase in the rate of priming into memory cells could lead to an increasing loss of naive T cells (*δ*), or 3) direct activation of naive T cells, or virus-induced depletion of the naive T-cell compartment, inducing a homeostatic response, could lead to a rise in naive T-cell division rates (*α*).

We investigated these effects separately, by choosing a 30-year old individual from Fig. 2 and studying the impact of the above mentioned possibilities during one year of HIV-1 infection (Fig. 3). Blocking thymus production (*σ* = 0) hardly influenced the average TREC content or naive T cell numbers (Fig. 3*a*). This is explained by the longevity of these cells. Increasing the death and priming rate *δ* 10-fold led to a decrease in the number of naive T cells and an increase in the TREC content of naive T cells (Fig. 3*b*). The latter is due to the fact that by increasing the death rate, the average naive T cell will become younger. Thus, a larger fraction of all naive T cells will be a recent thymic emigrant, and will hence have a higher average TREC content. Finally, increasing the division rate *α* 10-fold had a strong and rapid effect on the TRECs (Fig. 3*c*). Various values of *α* were tested, and even when naive T-cell division was increased only threefold compared to normal rates, the TREC content of the naive T-cell population

Table 2 Characteristics of HIV-1 negative Ethiopian individuals

	Ethiopians (n = 18)	Comparison to:	
		HIV⁻	HIV⁺
Age (years)	42 (35–45)	ns	ns
CD4 T cells (per μl)	748 (427–1354)	ns	$P < 0.001$
Naive CD4 (per μl)	112 (36–316)	$P < 0.001$	ns
Ki67⁺ naive CD4 (%)	0.9 (0.2–6.2)	$P < 0.05$	$P < 0.005$
Sj TRECs (×10⁻³copies/ CD45RA⁺ cell)	4.9	$P < 0.001$	$P < 0.01$

Depicted are median and range. Values were compared to the Dutch HIV-negative (HIV-; n = 17) and HIV-1 infected (HIV+; n = 33) subjects described in Table 1. Statistical significance was calculated with the Mann-Whitney U test; P < 0.05 was considered statistically significant.
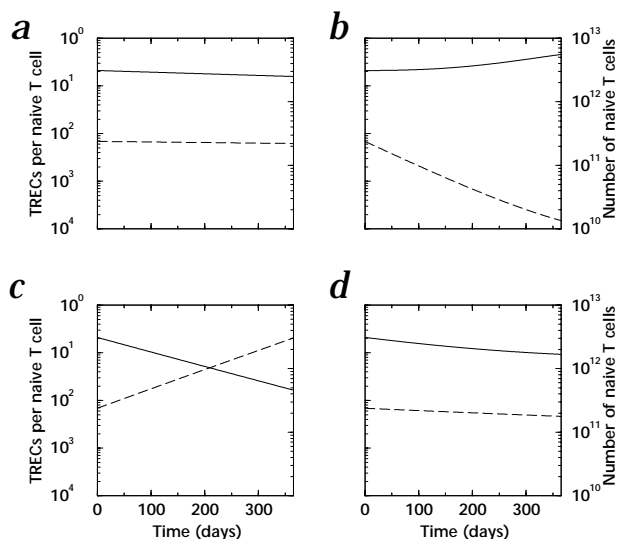
Fig. 3 The model behavior of an HIV-1 infected, 30-year old individual over a time period of one year. **a**, Blocking thymic output, that is, setting σ = 0, hardly influences the average TREC content (solid line) or naive T-cell numbers (dashed line). **b**, Increasing the death and priming rate (δ) 10-fold, leads to an increase in TREC content and a decrease in the number of naive T cells. **c**, Elevating the division rate (α) 10-fold, leads to a decrease in TREC content and an increase in naive T-cell numbers. **d**, Increasing both δ and α 5-fold leads to the observed decrease in the TREC content and in naive T cell numbers. Parameters: $\alpha = 7.9 \times 10^{-4}$/day, $c = 1$, $\delta = 10^{-3}$/day, δ_i = 0/day and $\sigma = 5 \times 10^7$cells/day.

did reduce (data not shown). To simulate HIV-1 infection more realistically, we simultaneously increased the cell division rate α and death and priming rate δ 5-fold. This led to the observed loss of TRECs and a slow decrease in naive T cell numbers (data not shown). Theoretically, increasing the intracellular degradation rate δ_i 10-fold could lead to a similar decrease in TRECs, however, the normal lifespan of a TREC is not known, and it seems unlikely that HIV-1 infection could affect TRECs in this manner.

Thus, our model shows that the decrease in TRECs during the relatively short period of HIV-1 infection (3–7 years) is most simply explained by increased division rates in the naive T-cell compartment. We have recently shown that this is mostly driven by immune activation rather than by homeostatic mechanisms[14]. This explanation is supported by our observation that the proportion of Ki-67+ naive T cells was negatively correlated with the number of TRECs per CD45RA+ T cell (R_s = -0.556, P_s = 0.001 and $R_{P=}$ -0.74, $P_{P=}$ 0.001 for CD4+ and CD8+ T cell subsets respectively, Fig. 4*a* and *b*). Furthermore, compared with the group of healthy individuals, HIV-infected patients had a significantly decreased TREC content of the CD45RA+ CD8+ T-cell pool, but did not have lower numbers of naive CD8+ T cells (Table 1). This is in agreement with our previous results, demonstrating that cell division rates in the naive CD8+ T cell compartment were increased in patients with normal numbers of naive CD8+ T cells[14]. Finally, although naive T cell division is the simplest explanation, it does not exclude the possibility that reversion of memory cells from the CD45RA- to the CD45RA+ phenotype contributes to the observed decrease in TREC content, especially for the CD8+ T cells.

Dilution of TRECs by immune activation
To determine whether a decline in TRECs could be observed independently of HIV-1 infection, we studied HIV-negative Ethiopians who have a chronically activated immune system, probably due to the increased pathogen exposure in the domestic Ethiopian environment[22]. Compared to HIV-negative Dutch individuals, Ethiopians have
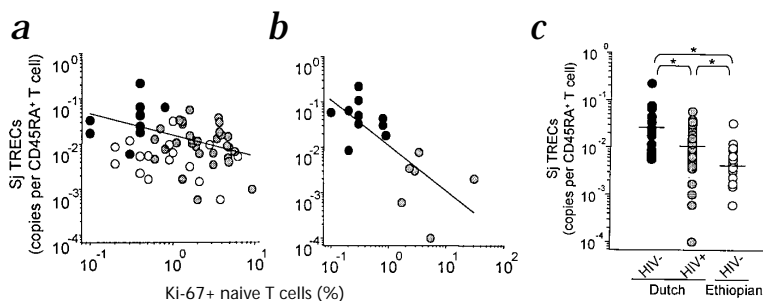
lower numbers of naive CD4+ and naive CD8+ T cells, concomitant with an expansion of the primed CD45RO+ CD27- T-cell compartment[22]. If the decline in TREC content of the CD45RA+ T-cell population in HIV-1 infection were indeed related to peripheral dilution caused by cell division, one would expect low TREC contents in T cells from this group of Ethiopian individuals. Eighteen HIV-negative Ethiopians, participating in a cohort study performed in Akaki, Ethiopia, as part of the Ethiopian–Netherlands AIDS Research Project (ENARP), were selected based on their seronegative status. Compared with HIV-negative Dutch subjects, Ethiopian individuals had reduced numbers of naive CD4+ T cells, increased Ki-67+ naive CD4+ T cells and decreased CD45RA+ T cell TREC contents (Table 2). The number of TRECs per CD45RA+ CD4+ T cell in these subjects was even lower than in the HIV-1 infected Dutch patients included in our study (Fig. 4*c*). Even though Ki-67 expression is a measure for ongoing cell division and not for the replicative history of the naive cells, it does correlate with low TREC content in these individuals (Fig. 4*a*).

Effect of highly active anti-retroviral therapy on TRECs
In the above described group of HIV-1 infected Dutch individuals, 1–2 years of HAART led to a rise in peripheral naive T-cell numbers, a steep reduction in the proportion of dividing cells in all subsets and a small increase in the TREC content (Fig. 5; data not shown for CD8+ T cells). None of these parameters reached normal concentrations ($P < 0.01$ for all time points, all parameters, compared with healthy subjects). In eight patients, we analyzed recovery of TRECs shortly after introduction of HAART in more detail. Immediate increase of TREC content in the CD45RA+ T cell pool was only observed during the first 3 months of HAART and coincided with a decline in naive CD4+ T-cell divi-

Fig. 4 *In vivo* dilution of TRECs. (**a** and **b**) In HIV-1 infected Dutch patients (◐), and in non-infected Dutch individuals (●) the number of TRECs declines with increasing percentages of (*a*) dividing naive CD4+ T cells (R_s = -0.556, P_s = 0.001) and (**b**) dividing naive CD8+ T cells ($R_{P=}$ -0.740, $P_{P=}$ 0.001). (○) represent HIV-negative Ethiopian individuals (see text). **c**, HIV-negative Ethiopian individuals have an even lower TREC content per CD45RA+ CD4+ T cell compared with HIV-negative and HIV-infected Dutch subjects (* $P < 0.05$).
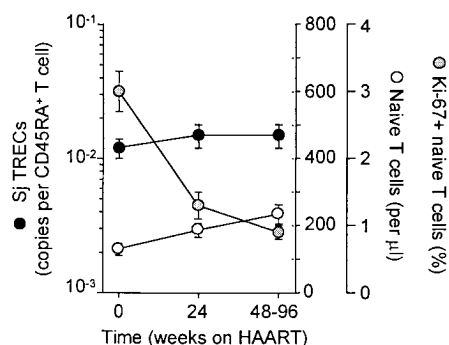
Fig. 5 Effect of HAART on TREC content. The number of naive CD4^{+} T cells (○), the percentage of Ki-67^{+} naive CD4^{+} T cells (◐) and the TREC content per CD45RA^{+} CD4^{+} T cell (●) were measured in 33 HIV-1 infected individuals during untreated HIV-1 infection, after 6 months of HAART and after 1 to 2 years of HAART. At all timepoints, the number of naive cells, Ki-67 expression in these cells and the number of TRECs were significantly different from control values (Mann Whitney U test, $P < 0.01$).

sion rates, but not with improvement of naive CD4^{+} T-cell numbers. Analysis of the naive CD8^{+} T cell pool revealed similar dynamics (data not shown).

Discussion

Elevated cell division rates could obscure the interpretation of TREC data. Because many CD45RA^{-} 'memory' T cells, particularly, may have a history of very rapid cell division in individuals with chronically activated immune systems[16], lowering the average TREC content[12], we measured Sj TRECs in purified CD45RA^{+}CD4^{+} and CD45RA^{+}CD8^{+} T-cell subsets in parallel with peripheral cell division rates in the naive populations. We compared data obtained from healthy individuals with those from subjects that were shown to have a persistently activated immune system, including HIV-1 infected patients[14,16] and a group of HIV-negative Ethiopian factory workers.

Mathematical modeling was used to describe the relation among the various parameters that affect TREC content. This showed that when in healthy individuals the net thymic output decreases, for example because of age-related involution of thymic tissue, the number of TRECs per produced naive T cell does not significantly decrease (see equation 3), as has been experimentally shown[11]. The mere fact that the TREC content of naive T cells declines with age[12], according to our model suggests increased naive peripheral T-cell division that accompanies lower thymic T-cell production.

It is now well established that HIV-1 infection leads to chronic immune activation reflected in elevated division rates in all T-cell subsets, including even the naive CD4^{+} and CD8^{+} T-cell compartment[14,16,23]. This immune activation is observed immediately following HIV-1 infection, and is most pronounced in CD8^{+} T cells[24,25]. We measured cell division in truly naive T cells that were characterized by expression of CD45RA and CD27 antigens. Contamination of this population by memory cells that reverted to a naive phenotype is thereby excluded, because expression of the CD27 antigen is lost upon prolonged antigen-specific stimulation and this loss is irreversible[26]. Naive T cells can be activated by stimulatory cytokines, independent of antigen, without losing their naive phenotype[27–29]. According to the model, naive T cell division would have a rapid and strong effect on the average TREC content. Even a two- to threefold increase in cell division rates, which is not reflected by changes in telomere lengths[30,31], can induce an observable dilution of TRECs. Indeed, TREC content is low in HIV-1 infection and acute HIV-1 infection leads to a significant reduction in the number of TRECs already after 90 days[10], an observation that our model cannot explain by an immediate fall in thymic output (Fig. 3a). It has been estimated that in adults, thymic output is in the order of 10^7 to 10^8 lym-

phocytes per day[32]. Even if HIV-1 infection would immediately and completely abolish thymic output, this alone could not lead to such a rapid fall in cellular TREC content (Fig. 3a). Elevated rates of T-cell division of naive cells are the most probable cause of the reduced TREC content we observed. Our hypothesis is further supported by the finding that non-HIV mediated persistent immune activation in healthy Ethiopians also leads to a significant loss of TRECs. However, because mixing with low-TREC revertant memory cells could also contribute to the observed reduction in TREC content, it will be important to obtain information on TREC contents of sorted naive T cells. Indeed, preliminary data from our lab obtained in a small group of HIV-1 infected patients showed lower TREC content of sorted 'truly naive' CD27^{+}CD45RA^{+}CD4^{+} T cells compared with healthy age-matched individuals ($n = 5$ and $n = 3$, medians 1.3×10^{-3} and 7.2×10^{-3} Sj TREC copies per CD27^{+}CD45RA^{+}CD4^{+} T cell, respectively).

Anti-retroviral treatment resulted in improvement of the TREC content of CD45RA^{+} T cells, which correlated with declining naive T-cell division rates, but not with increasing naive T-lymphocyte numbers. This is in agreement with recent findings of Zhang et al.[10], who found significant HAART- induced recovery of TREC content only in patients with low pre-treatment numbers of TRECs, which did not correlate with recovery of the naive T-cell pool. Rather, the increase in TREC content during HAART most probably is a composite of normalizing naive T cell peripheral death and division rates[14], some redistribution of naive T cells to the blood[33] and a continuous but low, possibly normal, thymic lymphocyte production.

Taken together, loss of TRECs in HIV-1 infection may primarily be caused by continuous hyperactivation of the immune system as reflected by increased cell division even of naive T cells. Although interference by HIV with thymic output could contribute to CD4^{+} T-cell depletion[3,30,32,34], measurements of TREC content in CD45RA^{+} T cells fail to provide experimental evidence for such thymic impairment. Alternatively, it may be that the intrinsically low thymic output in adults cannot compensate for the continuous loss of naive cells incurred by the increased priming of naive cells due to persistent immune activation, resulting in gradual depletion of CD4^{+} T cells without in fact exhaustion of thymic output[35].

Methods

Subjects. Cryopreserved peripheral blood samples from 33 HIV-1 infected patients and 35 non-infected healthy subjects were analyzed. Cryopreservation was performed using a computerized freezing device that results in optimal quality of frozen cells for functional studies[36]. Frozen blood samples were stored in liquid nitrogen. Patient blood samples were obtained from HIV-1 infected individuals participating in three separate trials: the Amsterdam cohort study on HIV-1 infection in homosexual men (n = 7), the Bristol Meyers Squibb-50 trial (n = 18) and the CHEESE study (n = 8). Samples were obtained before and during treatment with triple-therapy regimens, containing two reverse transcriptase inhibitors (nucleoside analogues) and one protease inhibitor. As controls, HIV-1 negative Dutch laboratory personnel (n = 17) and Ethiopian factory workers (n = 18) were included. HIV-1 negative individuals were age-matched with the HIV-1 infected patients. In 14 out of 17 healthy Dutch individuals, and 7 out of 33

HIV-infected subjects, enough material was available to perform measurements of TRECs in both the CD45RA+ CD4+ and CD45RA+ CD8+ T-cell compartment. Selection of HIV-negative Ethiopian factory workers was based on their numbers of peripheral blood CD4+ T lymphocytes, varying from fewer than 500 to more than 1000 cells per microliter. No correlation was found between the TREC content of CD45RA+ CD4+ T cells and the total number of CD4+ T cells in that population. It is of note that although total CD4+ T cell numbers varied in this study population, the number of naive CD4+ T cells was significantly lower compared with HIV-negative Dutch individuals, even when total CD4+ T cell numbers were comparable (Table 2).

Cell proliferation. Peripheral blood T-cell proliferation was studied by flow cytometric measurements of Ki-67 nuclear antigen expression on naive CD27+ CD45RO- CD4+ and naive CD27+ CD45RO- CD8+ T cells, as described previously[14,37,38]. Measurements of cell division rates with this method yield similar results as have been obtained previously by *in vivo* labeling of dividing cells with deuterated glucose[39]. Peripheral blood mononuclear cells were thawed and incubated with CD4- or CD8-PerCP mAb, CD45RO-PE (Becton Dickinson, San Jose, California) and biotinylated CD27 mAb (CLB, Amsterdam, The Netherlands). After washing, cells were incubated with streptavidin-APC (Becton Dickinson). Cells were then fixated and permeabilized with FACS lysing solution and FACS permeabilization buffer (Becton Dickinson), respectively. Lymphocytes were stained intracellularly with Ki-67-FITC mAb (Immunotech, Marseille, France), after which cells were fixed using Cellfix (Becton Dickinson) and analyzed on a FACSCalibur (Becton Dickinson) with Cellquest software.

Cell separation. CD45RA+ CD4+ and CD45RA+ CD8+ T cells were purified from thawed PBMC by magnetic separation over columns, using the MiniMACS multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc, Sunnyvale, California). Briefly, after 15 min incubation with 20 µl CD4 or CD8 conjugated magnetic beads per 10^7 cells, CD4+ and CD8+ T cells were isolated from PBMC by positive selection over MiniMACS separation columns. Magnetic beads were then released, cells were incubated with CD45RA-conjugated magnetic beads and passed over columns. With this technique, at least 90% purity of the fractions was achieved, such that fewer then 10% of CD4+ or CD8+ T-cell fractions were contaminated with CD8+ or CD4+ T cells, respectively, or with CD45RA- T cells. As outlined above, truly naive T cells are defined by co-expression of CD45RA and CD27, however, due to practical reasons, TREC analysis was performed in purified CD45RA+ T cells. Contamination of purified CD45RA+ T cells with CD27- CD45RA+ cells such that it will affect TREC contents cannot be excluded, especially in the CD8+ T-cell subset of HIV infected individuals[26]. It is less likely to occur in CD4+ T cells, because the population of CD4+ CD27- CD45RA+ T cells is limited in size[22].

Real-time PCR. DNA was purified from CD45RA+ CD4+ and CD45RA+ CD8+ T cell fractions using the QIAamp Blood Kit according to manufacturer's instructions (Qiagen, Hilden, Germany) . To detect Sj TRECs, a real-time quantitative PCR method was used[40]. In this PCR, annealing of primers with a site-specific probe, containing a quencher and a reporter dye, results in AmpliTaq Gold DNA Polymerase mediated cleavage of the probe and subsequent separation of the quencher from the reporter, thereby inducing fluorescence of the reporter dye. Each PCR reaction was performed in a 50-µl solution containing 100–200 ng DNA of cell suspension of interest, $1.0 \times$ TaqMan Buffer A (Perkin Elmer Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 5.0 mM MgCl₂, 200 µM dNTPs, 900 nM forward and reverse primer, 200 nM probe, and 1.25 U AmpliTaq Gold (PE Biosystems). The sequences of the primers and probe used are the following: forward primer 5′–CCATGCTGACACCTCTGGTT–3′, reverse primer 5′–TCGTGAGAACGGTGAATGAAG–3′ and the probe 5′–CACGGTGATGCATAGGCACCTGC–3′. As an internal control measurement, to normalize for input DNA, the Cα constant region that remains present on TCR genes despite rearrangement processes was amplified in every sample tested (forward primer 5′–CCTGATCCTCTTGTCCCACAG–3′; reverse primer 5′–GGATTTAGAGTCTCTCAGCTGGTACA–3′ and probe 5′–ATCCAGAACCCTGACCCTGCCG–3′). Using these primer/probe combinations (PE Biosystems), sequences of 131 bp and 70 bp, respectively, were amplified. A standard was created, by cloning the Sj fragment in the Hind III site of a pUC-19 vector, and the number of Sj copies present in a given cell population was calculated by including a dilution series of this standard in each PCR experiment. PCR was performed under the following conditions: 50 °C for 2 min followed by 95 °C for 10 min, after which 50 cycles of amplification were carried out (95 °C for 15 sec, 60 °C for 1 min). For each sample the Ct-value, defined as the minimal number of cycles necessary to exceed threshold values, was measured and applied to the standardization curve created from the dilution series described above.

Statistical analysis. Group characteristics were compared with the Mann-Whitney U test. Normality of groups was tested using the Shapiro-Wilk W Test for normality. Based on the outcome of this test, correlations were calculated using either Spearman's rank correlation coefficient (R_s) or Pearson's correlation coefficient (R_p).

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