IL-2 therapy and thymic production of naive CD4 T cells in HIV-infected patients with severe CD4 lymphopenia

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Objectives: IL-2 therapy increases memory and naive CD4 T cells in HIV-infected patients, but its effect on thymopoiesis is unknown. To investigate this effect, we quantified T-cell receptor rearrangement excision circles (TREC) in CD4 T cells from lymphopenic AIDS patients treated with highly active antiretroviral therapy and IL-2.

Methods: CD4 cell subsets were evaluated by flow cytometry using anti-CD45RO/RA, CD62L, Ki67 and CD95 monoclonal antibodies. The proportion of recent thymic emigrant had been quantified by a real-time polymerase chain reaction assay for signal joint TREC in peripheral blood mononuclear and purified CD4 T cells.

Results: At initiation of IL-2, TREC copies/μl of blood were correlated with naive T cell numbers and age. Both naive and TREC numbers/μl significantly increased over time in all patients, with a wide range of TREC increases. Higher percentages of CD4+CD45RO-negative cells positive for the Ki67 cell-cycle marker were found in patients with a low TREC increase, but remained stable under IL-2. TREC and naive cell recovery were correlated; they also correlated with the numbers of TREC and naive cells at the start of IL-2, and with age, suggesting a thymic origin for naive T-cell recovery. A mathematical model showing the linear recovery of naive cells and TREC under IL-2 also strongly suggested that a naive T-cell increase reflects thymic export and involves little net death and proliferation.

Conclusion: Although we cannot rule out a mechanism of altered proliferation or death rate, the thymus plays an important role in the long-term recovery of naive T cells under IL-2 therapy.

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Keywords: CD4 lymphopenia, HIV, IL-2, T-cell receptor rearrangement excision circles, thymus

Introduction

IL-2 regulates the turnover of mature T lymphocytes and is currently being tested as an immunotherapeutic agent to improve CD4 cell counts in HIV-infected patients [1–3]. Indeed, IL-2 in conjunction with antiretroviral therapy, allows for a sustained increase in CD4 cell counts in patients with moderate CD4 cell depletion

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[1–3]. Of interest is the fact that this CD4 cell increase involves both memory CD4+CD45RO+ and naive CD4+CD45RA+ T cells [4]. It is known, however, that IL-2 almost exclusively enhances the peripheral turnover of mature memory T cells but has no known effect on naive T-cell production [5]. These observations raise the question of the role of IL-2 in naive T-cell turnover. The CD45RA isoform is not an accurate marker of the naive cell status. Memory T cells can revert their CD45 isoform from RO to RA [6]. Whether IL-2 therapy acts on this small subset of revertant memory CD4 T cells or really enhances the production of naive T cells is not known. Although extensively debated [7–10], IL-2 is not believed to amplify thymus output, which is mostly regulated by IL-7. Finally, none or very little proliferation of naive T cells is observed in the periphery, where naive T cells proliferate and convert to effector or memory T cells when encountering their specific antigen. In severe lymphopenia, however, some naive T cells might undergo some homeostatic proliferation, during which IL-7 is also believed to play a major role [10,11].

Rearrangement of the T-cell-receptor (TCR) delta genes leads to the excision of circular DNA fragments from genomic DNA during the early stages of double positive CD4+CD8+ thymocyte development in the thymus [12]. T-cell receptor rearrangement excision circles (TREC) within peripheral blood T cells have been used as markers of naive T cell production [13,14]. The proportion of naive T cells carrying TREC declines with age and during HIV infection, and increases under potent antiretroviral therapy [12,15,16].

We have recently shown that IL-2 significantly increases the CD4 and naive CD4+CD45RA+CD62L+ cell numbers in patients with severe CD4 cell depletion failing to reconstitute their CD4 cells after antiretroviral therapy alone [17,18]. To evaluate whether this increase reflects a positive effect of IL-2 on thymus production, we quantified the TREC in purified CD4 T cells. To avoid problems of interpretation as a result of the different capacity of naive and memory T cells to proliferate and to allow an unbiased estimate of thymic output, results were expressed as TREC copies/μl and not as TREC content per cell. To assist in the interpretation of our data, a mathematical model was formulated. This model, describing the dynamics of the naive CD4 T-cell population and the TREC, allowed us to estimate thymic production and the parameters relating to the proliferation and death rates of naive T cells under IL-2 therapy.

Methods

Patients

Patients were selected from a double-arm randomized clinical trial studying the efficacy of IL-2 infusions [17,18]. Patients were selected as follows: CD4 cell counts remained between 25 and 200 cells/mm^3, despite an antiretroviral regimen containing two nucleoside analogues and a protease inhibitor for at least 6 months, which was efficient at reducing the plasma HIV-RNA load below 200 copies/ml. Patients ($n = 72$) were randomly assigned to receive adjuvant scIL2 4.5 MIU twice a day for 5 days every 6 weeks from day 0 of the study or after a delayed period of 24 weeks, in addition to their previous highly active antiretroviral therapy (HAART). After week 24, IL2 was given as 9 MIU for eight cycles of 5 days every 6 weeks or as 9 MIU for seven cycles of 5 days every 8 weeks. Blood samples were collected at day 0, weeks 6 or 12, weeks 24 or 28, weeks 50 or 52 and week 80. Patients ($n = 13$) with the highest CD4 cell counts at baseline were selected for TREC quantification on frozen cells. Eight age-matched healthy individuals were used as controls for TREC analysis.

Flow cytometry

Absolute CD4 and CD8 cells were counted in fresh blood samples according to standard procedures [19]. Lymphocyte subpopulations were analysed on fresh whole blood by three-colour flow-cytometry as previously described [19] with the following monoclonal antibodies (mAb): CD3-PE, CD4-PECY5, CD8-PECY5, CD45RA-PE, CD62L-FITC, CD45-FITC/CD14-PE, Ki67-FITC (Immunotech, Marseille-Luminy, France), CD25-FITC, HLA-DR-PE, CD95-FITC (Becton Dickinson, Strasbourg, France). Intracellular Ki67 expression in CD4 cells was analysed as previously described [19] on 20 000–100 000 CD4 cell events. The expression of Ki67-positive cells was analysed at day 0 of each IL-2 cycle.

CD4 T-cell purification

Peripheral blood mononuclear cells (PBMC) were isolated on gradient density. CD4 T cells were purified from PBMC by magnetic separation, using the MiniMACS multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc., Sunnyvale, CA, USA), with less than 5% residual CD8 cells.

Real-time polymerase chain reaction assay

DNA was purified from CD4 T cells using the DNAzol technique according to the manufacturer's instructions (Life Technologies, Gibco, Pontoise, France). Real-time polymerase chain reaction assay (Taqman, Perkin Elmer Applied Biosystems, Courtaboeuf, France) for signal joint (Sj) TREC was performed in 50 μl containing 100–400 ng DNA, 25 μl Universal Master Mix (Perkin Elmer Applied Biosystems, Courtaboeuf, France), 400 nM forward (5′–CAC ATCCCTTTCAACCATGCTGACA–3′) and reverse primers (5′–AGAACGGTGAATGAAGAGCGACA–3′) and 200 nM specific probe (5′–TGCCCACTCCT GTGCACGGTG–3′) under the following conditions:

50°C for 2 min, 95°C for 10 min, followed by 45 cycles of amplification (95°C for 15 s, 60°C for 1 min). To normalize for input DNA, an internal control measurement, a non-repeated region of the *GAPDH* gene was amplified in every sample test (forward primer 5′–CTCCCCACACACATGCACTTAC–3′, reverse primer 5′–CCTAGTCCCAGGGCTTTGA TT–3′, and the probe 5′–AAAAGAGCTAGGAAG GACAGGCAACTTGGC–3′). For each sample the number of SjTREC copies was determined using a dilution series of the pGTH310 clone containing the Sj fragment in the SacI site of a pGEM 4 vector [20].

IL-7 determination

Plasma specimens were stored at −80°C. Aliquots were thawed and analysed in duplicate using a commercial high sensitivity immunoassay, according to the manufacturer's instructions (Quantikine HS IL-7 immunoassay kit; RD Systems, Minneapolis, MN, USA).

Statistical analysis

Comparisons with baseline values were determined using the Wilcoxon non-parametric test calculated with Statview 4.02 software (Abacus, Berkeley, CA, USA). Pearson correlations were tested for their significance using a *t*-test (Minitab, State College, PA, USA), and non-parametric correlations were established through a Spearman rank correlation calculated using Statview 4.02 software (Abacus). *P* values below 0.05 were considered significant.

Mathematical analysis

We used a simplified version of a mathematical model [21] describing the dynamics of naive CD4 T cells (CD45RA+ CD62L+) and SjTREC counts to describe the data. Naive CD4 T cells are produced by the thymus at a rate of σ cells per week and have a net rate of disappearance δ_N. δ_N is defined as the net naive cell death rate (which is the death rate δ_T), plus the rate of priming into memory phenotype, minus the possible peripheral proliferation of naive cells under lymphopenic conditions [21]. SjTREC in the CD4 cell population appear at a rate proportional to the rate of thymic production of naive cells $c\sigma$, and disappear when naive cells die at rate δ_T. c is the average TREC content of a naive cell and will vary between patients [22]. Intracellular decay of the TREC is assumed to be negligible over the relatively short timescale of the study. The role of memory cells in the dynamics of TREC is omitted, because TREC levels are more than sixfold lower in memory cells than in naive cells (see section 1 of the results).

The model can be mathematically summarized as follows:

$$dN/dt = \sigma - \delta_N N \quad (1)$$

$$dT/dt = c\sigma - \delta_T T \quad (2)$$

We tested whether one can ignore the net disappearance rate of naive cells and TREC at the timescale of the study, by comparing a model in which disappearance rates are set to zero and one where it is not. Statistically, one decides on including δ_N and δ_T in the model by comparing the sum of squares of the residuals through a partial *F*-test at the $P = 0.01$ level.

The patients in this study were selected from others on the basis that their CD4 cell counts did not increase under HAART alone. It is thus very likely that their naive cell population is in a steady state under HAART before IL-2 therapy (with $N(t) = N_0$ and $T(t) = T_0$). Therefore, parameters of production and loss of cells cannot be estimated under HAART, because we only know the ratio $N_0 = \sigma/\delta_N$. For the period under IL-2, parameter estimates and their confidence intervals were computed for the model that best fitted the data for all patients [23]. Estimates are given for the naive T cells only, as too few datapoints were available for the TREC to estimate TREC recovery and rates of disappearance reliably.

Results

T-cell receptor rearrangement excision circles at baseline of IL-2 therapy in patients failing CD4 cell reconstitution under highly active antiretroviral therapy alone

We first set up our assay by measuring the TREC content in naive and memory CD4 T cell sub-populations purified from PBMC of healthy controls (26–50 years). To quantify simultaneously and precisely the number of cells and TREC copies an internal control (*GAPDH*) was amplified in every sample. The TREC content was 10-fold higher in naive CD4+CD45RA+ CD62L+ than in memory CD4+CD45RO+ T cells, as shown in Fig. 1a, yielding similar results to those previously reported [16]. A non-significant correlation between the age and the TREC content of PBMC was observed in healthy donors ($r = -0.53$; $P = 0.18$) (Fig. 1b).

TREC counts in CD4 T cells from the 13 HIV-positive patients (median age 48 years, range 32–66) who failed to reconstitute CD4 cell counts with HAART despite undetectable plasma viremia were determined at the baseline of IL-2 therapy. Patients had a median CD4 cell count of 131 cells/mm^3 (range 79–191) and a severe depletion of naive CD4+ CD45RA+CD62L+ T cells (median 30 cells/mm^3, range 6–97). The median plasma IL-7 level was 2.6 pg/ml (range 0.3–7.5), which is less than the levels observed in healthy controls (17 pg/ml, range 9.4–

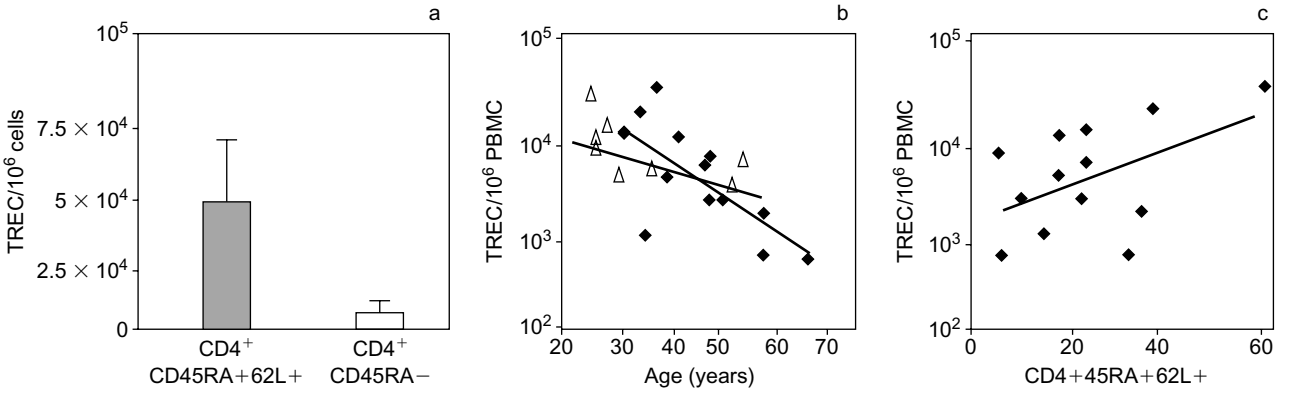

Fig. 1. Quantification of T-cell receptor rearrangement excision circles by real time polymerase chain reaction. The T-cell receptor rearrangement excision circle (TREC) content is determined by real-time polymerase chain reaction assay for signal joint, and to normalize for the input of DNA a portion of the *GADPH* gene is co-amplified in every sample test. The TREC content is 10-fold higher in purified naive T cells (CD4+CD45+CD62L+) than in memory T cells (CD4+CD5RA-negative) of healthy controls (a); the numbers of TREC are negatively correlated with the age in healthy (Δ) or HIV-1-infected patients (◆) (respectively $r = -0.53$, $P = 0.18$ and $r = -0.55$, $P = 0.049$) (b) and positively correlated with naive T-cell percentages ($r = 0.72$, $P = 0.0005$) (c) in HIV-1-infected patients.

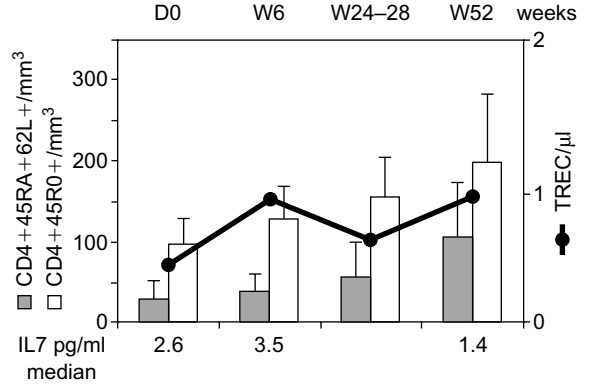

Fig. 2. Changes in signal joint T-cell receptor rearrangement excision circles, CD4 naive and memory T cells and IL-7 under IL-2. Data are median values and bars are 75th percentiles. T-cell receptor rearrangement excision circles (TREC) are quantified in the purified whole CD4 T cell population and results are expressed as numbers of TREC copies/µl. Naive (CD45RA+CD62L+) and memory (CD45RO+) CD4 T cells are determined on whole blood, and normal values are 414 ± 20 and 482 ± 69 cells/mm^3, respectively. IL-7 is determined on plasma with an immunoassay. Day 0 is the day of the first cycle of IL-2 for all patients.

24.1) (Fig. 2). The median number of TREC per 10^6 PBMC in the 13 HIV-positive patients was 3799 copies (range 567–30 429). In patients, the TREC numbers per PBMC were positively correlated ($r = 0.72$; $P < 0.001$) with the percentage of CD4+CD45 RA+CD62L+ cells (Fig. 1c), and negatively correlated ($r = -0.55$; $P = 0.049$) with age (Fig. 1b).

Effects of IL-2 therapy on naive T cells and T-cell receptor rearrangement excision circles

Nine million IU IL-2 were injected subcutaneously for 5 days every 6–8 weeks. As described in the Methods section, six patients received IL-2 at the initiation of the trial, and seven after a period of 24 weeks. No increase in naive CD4 T cells was observed in these seven patients during the first 24 weeks under HAART alone, with a mean change in CD4+CD45RA+ CD62L+ cells of 8 ± 13 cells/mm^3 over that period (Fig. 3) ($P = 0.13$). Overall, the 13 patients received a median of eight cycles of IL-2 (range eight to 11) over a period of 52 weeks. IL-2 therapy induced a significant median increase of 84 cells/mm^3 (range 14–179) naive CD45RA+CD62L+CD4+ T cells at week 52 ($P < 0.001$) (Fig. 2 and Fig. 3). The memory CD4+ CD45RO+ T cell counts showed a similar increase of 76 cells/mm^3 (range 26–205) ($P < 0.001$) (Fig. 2). The increase in naive CD4 T cells was accompanied by a significant decrease in plasma levels of IL-7 from a median concentration of 2.6 pg/ml (range 0.2–7.5) at baseline to 1.4 pg/ml (range 0.1–7.8) after 52 weeks of IL-2 therapy ($P = 0.03$) (Fig. 2).

TREC were quantified in the purified whole CD4 T cell population. Naive subpopulations could not be isolated in quantities allowing the measurement of TREC, because of the severe CD4 cell depletion at entry. To avoid problems of interpretation as a result of the different proliferation capacity of naive and memory T cells, results were expressed as numbers of TREC copies/µl and not as TREC content per cell. TREC copies/µl of blood significantly increased from a median of 0.41 copies/µl (range 0.08–4.7) at baseline of IL-2 infusions to 0.9 copies/µl (range 0.16–10.44) at week 52 ($P = 0.005$) (Fig. 2). However, wide variations from patient to patient were observed in TREC changes (Fig. 3). Indeed, a more than twofold increase was observed over the IL-2 treatment period in seven patients defined as group A and represented in Fig. 3a, whereas TREC remained approximately con-

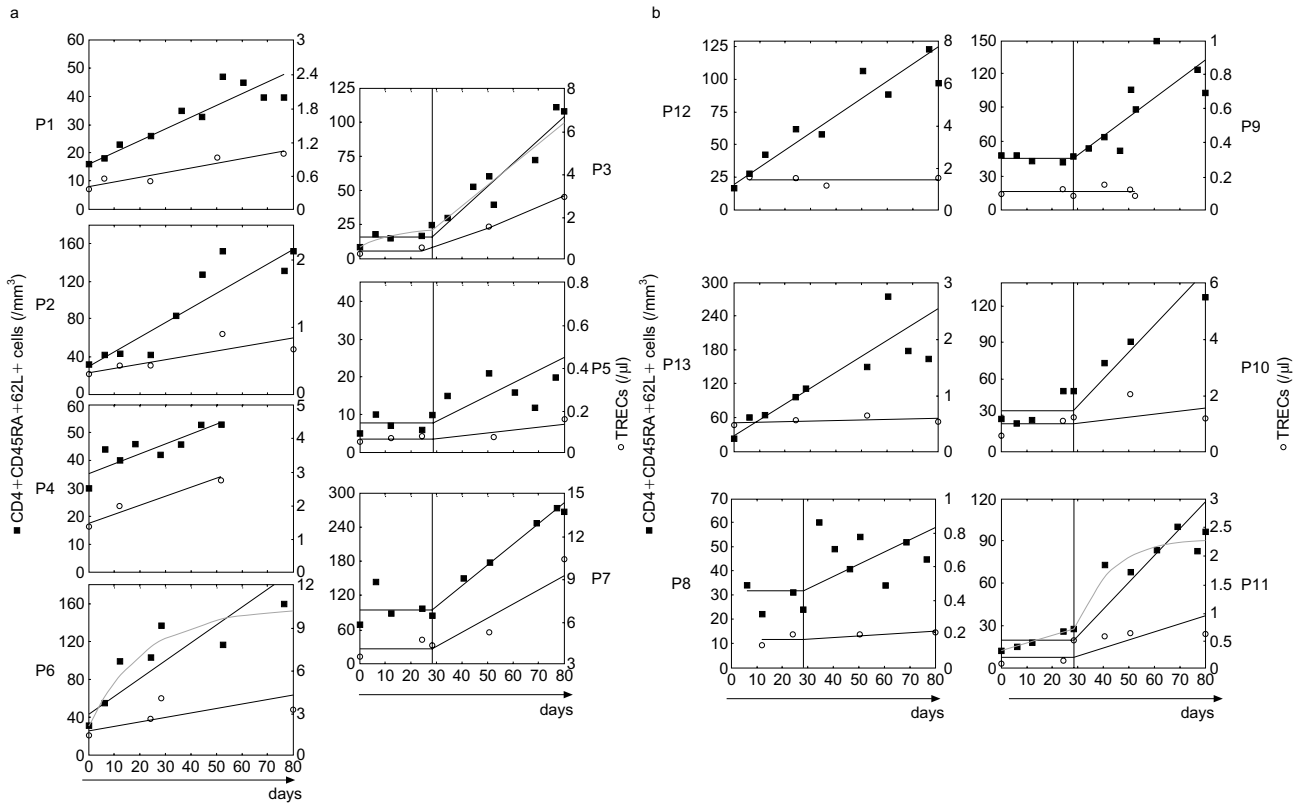

Fig. 3. Patient data with model fits. Black squares denote CD4+RA+CD62L+ T cells, open circles refer to T-cell receptor rearrangement excision circle (TREC) counts. The vertical line indicates the start of IL-2 treatment. When there is no vertical line, patients started treatment at day 0. Patients are separated into two groups; patients with a more than twofold increase in TREC over the IL-2 period (a) and patients with TREC approximately constant with a 1–1.3-fold increase over the IL-2 period (b). Fits correspond to a scenario in which naive cells and TREC are in steady state under highly active antiretroviral therapy (HAART), and increase linearly under IL-2 therapy. For all patients, except patients 6 and 11, including a net disappearance rate to describe the dynamics of naive T cells and TREC under IL-2 did not significantly improve the fit to the data. In the graphs for patients 6 and 11, the dashed line represents the fit of the model with the non-zero net disappearance rates of naive cells. For patient 3, the dashed line represents the best fitting scenario, with a non-zero death rate of naive cells under HAART.

stant, with a 1–1.3-fold increase in the six other patients defined as group B and represented in Fig. 3b. The latter results contrast with an increase in naive T cells in all patients.

Mathematical modelling of the naive and T-cell receptor rearrangement excision circle dynamics under IL-2 therapy

According to inclusion criteria, CD4 cell numbers were in steady state before the initiation of IL-2. This was verified in six out of seven patients with delayed IL-2. Overall, for the period with HAART only, the estimates for the average recovery rate of naive cells cannot be obtained, because we typically fit a steady state scenario, in agreement with the way patients were selected. Fig. 3 shows the fits for all patients. If naive CD4 T-cell recovery were caused by net proliferation ($\delta_N < 0$), the increase would be exponential. This is not the case, because the data are best described by a straight line. The data do not provide evidence for a sub-linear recovery either ($\delta_N > 0$), with the exception of patient nos. 6 and 11.

In other words, in the period under IL-2 (up to 80 weeks), the net death rate of naive cells and the net rate of disappearance of TREC can be set to zero without significantly affecting the quality of the fits. Indeed, a straight line yields a better fit than a curve. Including the net disappearance rate for naive cells in the model [δ_N in (1)], i.e. naive cell death plus priming minus proliferation, does not significantly improve the quality of the fit (*F*-test on the sum of squares obtained from the fits, at the $P = 0.01$ level) for each of the patients. Similar findings are made for the net disappearance rates of TREC [δ_T in (2)]. In conclusion, naive cell death and priming seem to be balanced by the proliferation, if any, of naive cells under IL-2. The naive cell recovery (slope of the linear regression) under IL-2 therapy would thus be of thymic origin, and we estimate that on average 0.24 cells/mm^3 a day

(SD 0.14 cells/mm^3 a day) are produced by the thymus under treatment (Table 1). Note that there are two mechanisms for this increased naive cell recovery under IL-2: either thymic production is increased or cells live longer. Our model cannot discriminate between the two scenarios, because we have no estimate of thymic production under HAART alone. Both alternatives remain compatible with the interpretation that the linear naive cell recovery is largely caused by the thymus.

Factors influencing the recovery of naive cells and T-cell receptor rearrangement excision circles under IL-2

Recovery rates of naive cells can differ by a factor of 10 between patients (Table 1), raising the question as to what factors contribute to the recovery of naive cells as computed in Table 1. Naive cell recovery correlated with both the number of naive cells and TREC at the start of IL-2 therapy (Spearman rank correlation $\rho = 0.68$, $P = 0.02$, Fig. 4a, and $\rho = 0.61$, $P = 0.04$, Fig. 4b, respectively) and correlated negatively with age ($\rho = -0.59$, $P = 0.04$, Fig. 4c). Memory CD4 T cell counts at the start of IL-2 treatment did not influence the recovery of naive cells under IL-2 ($\rho = 0.19$, $P = 0.53$). There was no correlation between the recovery of naive cells and seric IL-7 levels at the start of IL-2 therapy ($\rho = -0.17$, $P = 0.56$). Assuming that *Fas* expression is associated with the susceptibility of a cell to die, we evaluated the correlation of initial levels of *Fas* expression on naive cells and their recovery. No correlation between these two parameters was found ($\rho = -0.39$, $P = 0.18$). In addition, naive cell recovery was not correlated with recovery rates of memory CD4 T cells ($\rho = 0.28$, $P = 0.31$).

We then analysed the correlates of TREC recovery (the slope of the line fitted to the data, see Fig. 3). TREC recovery was positively correlated with the number of TREC at the start of IL-2 therapy ($\rho = 0.59$, $P = 0.05$, Fig. 4d), and negatively with age ($\rho = -0.65$, $P = 0.03$, Fig. 4e). However, it did not correlate with IL-7 levels, nor with the numbers of memory CD4 cells at the start of IL-2 therapy ($\rho = -0.26$, $P = 0.4$; $\rho = -0.03$, $P = 0.93$, respectively). TREC recovery did not correlate either with the recovery of naive or memory cells ($\rho = 0.48$, $P = 0.11$; $\rho = 0.54$, $P = 0.07$, respectively).

Overall, both naive cells and TREC recovery under IL-2 are strongly influenced by the parameters of thymic production before the initiation of IL-2.

Table 1. Naive cell recovery under IL-2 therapy.

Patient	Average recovery rate of naive cells (cells/mm^3/day)	Confidence interval	No. of observations
Group A			
P1	0.06	0.05–0.07	10
P2	0.23	0.17–0.29	9
P3	0.25[a]	0.17–0.32	8
P4	0.05	0.03–0.07	8
P5	0.05	0.03–0.08	7
P6	0.27[b]	0.17–0.38	7
P7	0.53	0.40–0.73	6
Group B			
P8	0.07	0.02–0.12	9
P9	0.24	0.17–0.32	9
P10	0.32	0.18–0.53	4
P11	0.27[c]	0.19–0.37	6
P12	0.19	0.16–0.23	9
P13	0.40	0.31–0.50	9

[a]For patient 3, the model with non-zero net disappearance rate under highly active antiretroviral therapy (HAART) and thymic output constant between HAART and HAART plus IL-2 fitted best. Parameter estimates are: $\sigma = 0.22$ [confidence interval (CI) 0.17–0.27], and $\delta_{N1} = 0.066$ (CI 0.035–0.102).

[b]For patient 6, the model with a non-zero net disappearance rate fitted better than the linear model; for that model, $\sigma = 1.03$ (CI 0.68–1.51), and $\delta_N = 0.046$ (CI 0.025–0.076).

[c]For patient 11, the scenario with increased thymic output under IL-2, and the negligible net disappearance rate of cells under HAART but not under IL-2 fitted best. Parameter estimates are: $\sigma_1 = 0.08$ (CI 0.06–0.10), $\sigma_2 = 0.99$ (CI 0.63–1.64), $\delta_{N2} = 0.075$ (CI 0.042–0.133).

Effects of IL-2 on mature T cell activation and proliferation

In order to understand further the wide range of TREC recovery, we analysed the influence of peripheral activation and proliferation on peripheral mature CD4 T cells during IL-2 therapy. The proportion of CD4 cells displaying the CD25 α-chain (the high-affinity IL-2 receptor) significantly increased during IL-2 therapy in all cases, as previously reported [24] (data not shown). In contrast, we did not observe any changes in late surface markers induced by T-cell activation as HLA-DR during IL-2 therapy (data not shown). We also examined whether there is support for the hypothesis that naive cells increase as a result of enhanced naive cell survival. The median percentage of *Fas*-expressing CD45RO-negative cells increased from 23% (range 2–42) at baseline to 43% (range 2–69) after 52 weeks of IL-2 (Fig. 5). The slope of the regression line fitted to the number of *Fas*-expressing CD45+ RO-negative cells over time under IL-2 is positive, except in one patient (data not shown). Finally, we also evaluated the proportion of cells entering the cell cycle during IL-2 therapy, by analysing Ki67 expression in CD45RO+ and CD45RO-negative CD4 T cells in seven patients for whom cells were available. For all patients, Ki67 expression predominated on CD4+ CD45RO+ T cells compared with CD4+CD45RO-negative cells, with a median of 6.9% (range 3.8–13.6) and 1.3% (range 0.3–4.9) positive cells, respectively (Fig. 6a). No difference was observed between the average percentage of Ki67+CD45RO-negative and Ki67+ CD45RO+ CD4+ T cells under HAART alone and under HAART with IL-2 ($P > 0.99$ and $P = 0.37$, respectively) (Fig. 6a). Patients from group B

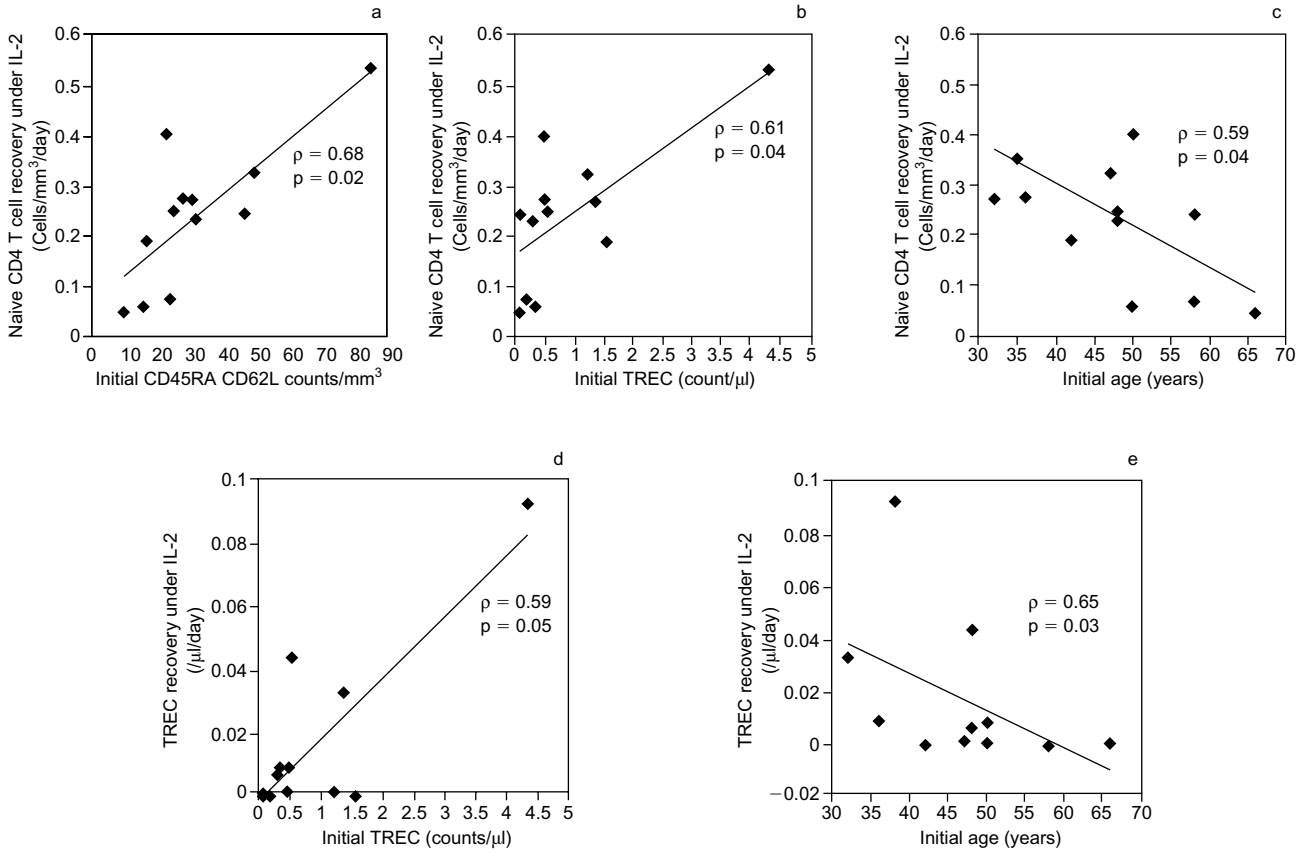

Fig. 4. Correlations of naive CD4 T cell and T-cell receptor rearrangement excision circle recovery with parameters of thymic production before the initiation of IL-2. Correlations between naive CD4 T cell recovery (cell/mm^3 a day) and (a) initial CD45RA CD62L counts; (b) initial TREC counts and (c) initial age. Correlation between TREC recovery (TREC/μl a day) and (d) initial TREC counts and (e) initial age. Non-parametric correlations were established through a Spearman rank correlation calculated with Statview 4.02 software. *P*-values below 0.05 were considered significant.

with a poor increase in TREC/μl over time had the highest percentages of Ki67+CD45RO-CD4+ cells (Fig. 6b). However, we did not observe any trend for an increase in the percentage of Ki67+ cells in either subset over time under IL-2 (data not shown).

In conclusion, TREC recovery was not associated with specfic changes in surface markers of T cell activation or death. As mentioned above, TREC recovery appears to be influenced by parameters of peripheral T-cell homeostasis at entry.

Discussion

To investigate whether IL-2 acts on thymopoiesis, we quantified TREC and naive CD4 T cells during the course of intermittent IL-2 therapy in HIV-1 patients with severe CD4 cell depletion. TREC measurements allowed us to evaluate whether IL-2 acts before or after the TCR rearrangement events. A mathematical model helped to discriminate between the role of thymic production and peripheral proliferation in naive T-cell reconstitution.

Our results and the mathematical model suggest that in these HIV-1-infected patients with severe CD4 T-cell depletion, the thymus plays an important role in naive T-cell recovery. Indeed, the fit of the model for the naive T-cell dynamics suggests that in most cases the naive cells are contributed by the thymus. However, as the patients were in steady state under HAART alone according to the entry criteria, thymus output cannot be estimated before the initiation of IL-2 therapy. Consequently, an enhanced survival of naive cells in the periphery cannot be ruled out with these models. Peripheral proliferation could still be substantial, but its effect, together with the conversion of naive to memory T cells, would be nullified by the death rate of cells.

To test the hypothesis of thymic involvement in naive T-cell recovery further, we analysed biological parameters of proliferation and death on CD4 naive cells. The more than twofold increase in TREC seen in half

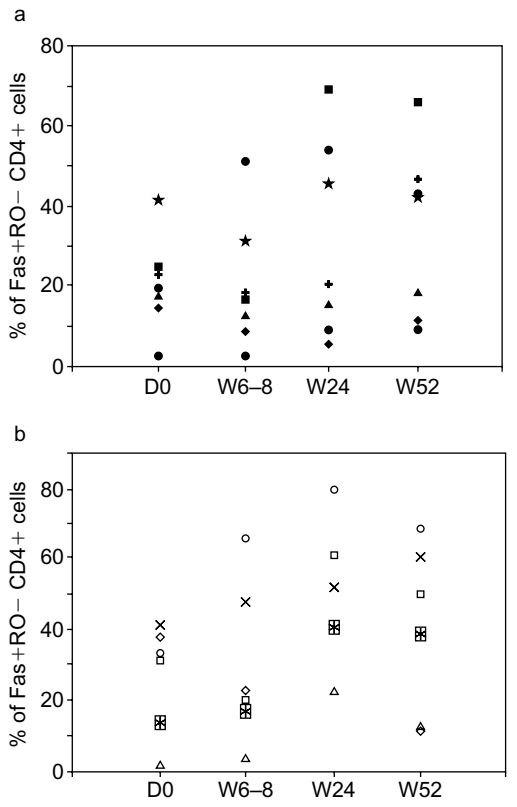

Fig. 5. Changes in *Fas*-positive RO-negative CD4 T-cell percentages under IL-2. Naive (CD45RO-) Fas+ CD4 T cells are determined on whole blood. The results are represented from D0 to W52 for patients of group A and patients of group B.

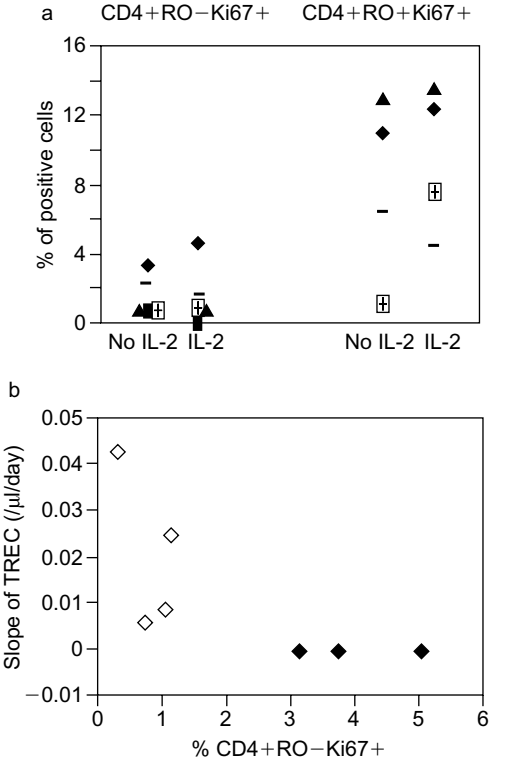

Fig. 6. Ki67+RO−/RO+ CD4 T-cell percentages according to treatment group. Naive (CD45RO-negative) and memory (CD45RO) Ki67 CD4 T cells are determined on whole blood. A comparison of the average percentage of Ki67+CD45RO-negative and Ki67+CD45RO+ CD4 T cells under highly active antiretroviral therapy (HAART) alone and under HAART with IL-2 (a). For six patients the T-cell receptor rearrangement excision circle increase is represented according to percentages of Ki67+CD45RO-CD4 cells (b).

the patients could result either from an enhanced thymic production, or from an overall decrease of naive cell death in the periphery as a result of IL-2 treatment. According to the thymic hypothesis, IL-2 would act on thymopoiesis by inducing the proliferation of thymocytes before TCR rearrangement. Such an effect of IL-2 on the intrathymic development of mature T cells has been strongly debated. *In vitro*, IL-2 has been shown to promote proliferation and differentiation of human pro-T cells into CD3+CD4+CD8+ mature thymocytes [7,8]. Although IL-7 is the main known immune modulator of thymic production, a smaller but direct effect of IL-2 on thymus production has recently been proposed in mice [10]. We observed at baseline a low seric level of IL-7 in patients compared with healthy individuals. These low values were even lower than those reported by Napolitano *et al.* [25], and may be linked to the incapacity of our patients to reconstitute their CD4 cell numbers under HAART. These values of IL-7 decrease even further in our patients under IL-2 therapy. Altogether, the low seric level of IL-7 and its decrease in our patients under IL-2 does not support the hypothesis of an indirect effect of IL-2 through increased production of IL-7. However, we cannot rule out a pharmacodynamic effect of increased IL-7 binding to IL-7R, which is highly expressed on naive T cells.

Alternatively, IL-2 could increase absolute TREC numbers by decreasing naive T-cell death in the periphery. However, assuming that *Fas* is a marker for susceptibility to cell death, this hypothesis is not supported by the changes observed in *Fas* expression, which rather increases on naive CD45RO-negative T cells. Therefore, pre-TCR rearrangement expansion of thymocytes seems the likely mechanism through which both naive cells and TREC increase under IL-2.

In half the patients, however, TREC do not increase in contrast to naive T cells, raising the hypothesis of naive T-cell proliferation. It is generally considered in normal individuals that naive cell proliferation is antigen dependent and results in rapid conversion to memory T cells. However, some homeostatic proliferation might be possible in profoundly lymphopenic mice [26]. A higher percentage of Ki67+CD45RO-negative cells in group B suggests that at least in some of these

profoundly lymphopenic individuals, homeostatic proliferation of naive CD45RO-negative T cells might indeed occur. However, this phenomenon is observed at entry and was not enhanced by IL-2. In addition, the linear increase of naive T cells suggests that if naive cell proliferation exists, it would take place in the thymus. Indeed, if this proliferation had occurred in the periphery, the naive increase would have been exponential. The hypothesis of proliferation after TCR rearrangement is supported by recent findings *in vivo* in a severe combined immunodeficiency human model. In that model, IL-2 may prevent HIV-induced depletion of thymocytes by the expansion of a relatively mature subset of CD4+CD8+ thymocytes [9]. Finally, the priming rate of naive cells remains an unknown factor. Indeed, one could argue that because IL-2 stimulates memory T-cell proliferation, there might be less need for the conversion of naive cells into memory cells [21]. However, we could not investigate the effect of IL-2 on the conversion rate of naive to memory cells. Furthermore, the absence of correlation of naive cell recovery with memory cell recovery under IL-2 does not support the hypothesis of a peripheral homeostatic mechanism.

We have assumed that the effect of IL-2 was continuous, i.e. not limited to the 5-day period of the IL-2 therapy itself. The reality probably lies somewhere in between: the effect of therapy extends well beyond the 5-day cycle. Indeed, in many patients the total CD4 cell count decreases during the course of an IL-2 cycle, but then increases to levels higher than before the cycle, suggesting that there is a long-term effect of IL-2 (data not shown). Sereti *et al.* [27] also observed an increase of activation and apoptosis markers during the 5-day IL-2 cycle, and a decrease to previous levels after IL-2 therapy. In conclusion, altered proliferation and increased death rates of CD4 cells during an IL-2 cycle may well play a role in naive cell reconstitution under IL-2, but they cannot explain the observed increase in TREC seen in at least half the patients.

The recovery rate of naive cells under IL-2, on average, is similar to that observed in other studies under HAART ($0.24 \pm 0.04/mm^3$ a day, mean $\pm$ SEM, versus $0.34 \pm 0.04/mm^3$ a day, respectively). Cohen Stuart *et al.* (in preparation) noted that recovery rates in these patients were similar to those in patients after anti-CD4 monoclonal antibody treatment. Similarly, we found that the recovery rates were not significantly different between the larger cohort on IL-2 therapy [17,18] and those in patients after bone marrow transplantation (0.21 ± 0.25 versus 0.16 ± 0.24) [28].

The variability in TREC and naive cell recovery rates during IL-2 therapy in this study can be explained by parameters of T-cell homeostasis and thymic production before the initiation of IL-2 therapy, such as the differences in numbers of TREC, naive cells and age at initiation of IL-2. IL-2 therapy thus appears to enhance the pre-existing mechanisms of T-cell homeostasis that were ineffective under HAART alone in these profoundly lymphopenic patients. Dependency on these three parameters adds support to the hypothesis of thymus involvement in the process of regeneration. We cannot rule out the hypothesis that several non-exclusive mechanisms might be involved in the naive T-cell reconstitution under discontinuous IL-2 therapy, such as altered proliferation or death rate, and future larger studies will be needed to analyse these points. Our results strongly suggest, however, that the thymus plays an important role in the long-term-recovery of naive T cells under IL-2 therapy.

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