Reconstitution of naive T cells during antiretroviral treatment of HIV infected adults is dependent on age.
Abstract

Introduction: The mechanism of regeneration of naive CD4+ and CD8+ T cells during highly active antiretroviral therapy (HAART) is unclear. If it were mainly dependent on thymic function, the regeneration rate should decrease with age, due to thymic involution.

Objective: To determine the influence of age on the regeneration rate of naive and memory T cells in 45 adults on HAART.

Methods: The number of naive and memory T cells was determined in whole blood. Naive cells were defined as CD45RA+CD27+. Cells negative for CD45RA and/or CD27 were considered memory type cells.

Results: The age of the patients ranged from 25 to 57 years. On average (±SEM), the regeneration rates of naive CD4+ and CD8+ T cells were 0.34±0.04 and 0.36±0.04 cells/mm³/day, respectively, which is not significantly different (p=0.5). The recovery rates of naive CD4+ and CD8+ T cells were negatively correlated to age (r= -0.41 and r= -0.47, respectively). The recovery rate of memory T cells showed no relation to age. The regeneration rate of naive CD4+ T cells during HAART is similar to previously reported regeneration rates in adults following cytotoxic chemotherapy or CD4 mAb therapy. This suggests a little, or at least reversible, impact of HIV on naive T-cell production.

Conclusion: The equal regeneration rate of naive CD4+ and CD8+ T-cells during HAART, and the inverse correlation between age and the recovery rate of naive T cells, both suggest that the thymus contributes considerably to the regeneration of naive T cells in adults on HAART.

Keywords: Antiretroviral therapy, CD4, CD8, Naive/memory, Immune Reconstitution
Introduction

Infection with HIV causes progressive depletion of both naive and memory CD4⁺ T cells. The CD8 count of HIV infected patients is usually increased until shortly before the onset of AIDS. This increase is due to expansion of the memory CD8⁺ T-cell population. Naive CD8⁺ T-cell counts decrease gradually from early after seroconversion (1,2).

In most patients, highly active antiretroviral therapy (HAART) leads to a substantial rise of the CD4 count, primarily due to an increase of memory CD4⁺ T cells (3-5). The effect of HAART on the CD8 count varies per study from a slight increase (4,6,7) to a decrease (3,8,9). Naive CD4⁺ and naive CD8⁺ T cells increase slowly during HAART (3,4,10).

The mechanism of regeneration of the naive T cells during HAART is unclear. It has been attributed to thymus dependent production (10-13), proliferation of naive T cells (11,12,14,15) and reversion from the memory to the naive phenotype (15). To understand the mechanism of naive T-cell regeneration is important because HIV induced deletions in the T-cell receptor repertoire (16,17) can exclusively be restored via thymus dependent production of naive T cells.

The generation and production of T cells is primarily located in the thymus. Because of thymic involution with increasing age (18,19), the capacity to regenerate T cells after depletion diminishes with age. It has been demonstrated that the recovery rate of peripheral CD4⁺ T cells after antineoplastic chemotherapy decreased with the age of the patients. In adults over 20 years of age, CD4⁺ T-cell recovery after depletion is slower than in children and appears to be predominantly due to thymus-independent peripheral proliferation (20-24).

Here, we tested whether in HIV infected adults the speed of regeneration of naive T cells during HAART decreases with age. Therefore, we determined the influence of age on the rate of reconstitution of naive and memory CD4⁺ and CD8⁺ T cells in 45 adults with successful suppression of plasma HIV RNA levels during HAART.
Material and methods

Study population
The recovery of naive and memory T cells was analyzed in all patients from the previously described CHEESE study cohort (25) with a sustained plasma HIV RNA response to less than 50 copies/ml (n=45). Briefly, this is a randomized study comparing antiviral efficacy of zidovudine (Retrovir, Glaxo-Wellcome, Research Triangle Park, N.C.) plus lamivudine (Epivir, Glaxo-Wellcome, Research Triangle Park, N.C.) plus saquinavir-soft-gelatin-capsules (SQV-SGC, Fortovase, Hoffmann-La Roche, Inc., Nutley, New Jersey) versus zidovudine plus lamivudine plus indinavir (Crixivan, Merck, West Point, Pa), in HIV-1 infected patients. Antiretroviral naive patients were eligible for study treatment if at the moment of screening plasma HIV RNA levels were at least 10,000 copies/ml and/or if CD4 counts were less than 500 cells/mm³ and/or if they had a history of HIV related symptoms (CDC stage B or C). During 48 weeks of treatment, the virologic and the CD4 count response was not different between the two treatment arms (26). Of the selected patients, 23 were from the indinavir arm and 22 from the SQV-SGC arm.

Blood sampling
Blood samples were obtained at week -2, at week 0 and every 4 weeks through week 24 and every 8 weeks from week 24 through week 48 of treatment.

Plasma Viral Load
Plasma HIV RNA levels were measured using an investigational version of the ultra sensitive quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor HIV-1 Monitor, Roche Diagnostic Systems). The lower limit of detection was 50 copies / ml.

Monoclonal antibodies
Peridinin chlorophyll protein (PerCP)-labeled CD4, PerCP-labeled CD8 and phycoerythrin (PE)-labeled CD45RA Mab’s were obtained from Becton Dickinson (San Jose, CA). FITC labeled CD27 Mab’s were obtained...
from CLB (Amsterdam, the Netherlands). Flow Cytometry. The fraction of naive and memory (CD45RA− and CD45RA+/CD27+) T cells was determined by three color FACS analysis using monoclonal antibodies against CD4 (or CD8), CD45RA, and CD27 on EDTA anticoagulated venous blood (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA).

Statistical Analysis
Parametric Pearson’s correlation coefficients were computed to measure bivariate correlations. All variables used to compute Pearson’s correlation coefficients were normally distributed, as tested by the Kolmogorov-Smirnov one-sample test. The paired-samples t test for two related samples was used to detect a difference between the recovery rates of naive CD4+ and CD8+ T cells. All reported p-values are two-sided. All statistical analyses were performed using SPSS for Windows (8.0.0).

Results
Because immune recovery during HAART is dependent on virus suppression to levels below the limit of detection (27), we investigated the reconstitution of T cells during 48 weeks of therapy in a group of patients who all achieved plasma HIV RNA levels below 50 copies/ml. The baseline characteristics of the patients were as shown in Table 1. The mean age of the patients at baseline was 37.2 years (range 25-57 years). The median plasma HIV RNA level at baseline was 72,000 copies/ml. The patients achieved plasma HIV RNA levels below 50 copies/ml within a median period of 16 weeks (range 4-44).

In the analysis of T-cell reconstitution, T cells co-expressing CD45RA and CD27 were considered truly naive cells (28,29). Memory T cells were defined as CD45RA+/CD27− and as CD45RA− (both CD45RA−/CD27+ and CD45RA−/CD27−). During the initial 48 weeks of therapy, the mean (±SEM) total, naive and memory CD4+ T-cell count in peripheral blood increased with 206±34, 112±17 and 53±23 cells/mm³, respectively (Figure 1). The total and memory CD8+ T-cell count decreased with
140±60 and 240±55 cells/mm³ in 48 weeks. The number of circulating naive CD8⁺ T cells increased with 126±16 cells/mm³ during 48 weeks of therapy.

To estimate the average recovery rate of naive and memory T cells in the blood during the initial 48 weeks of HAART, linear regression analysis was used. The mean (± S.E.M.) recovery rates of naive CD4⁺ and CD8⁺ T cells were 0.34±0.04 and 0.36±0.04 cells/mm³/day, respectively. These rates are similar to previously reported recovery rates of naive T cells during HAART in adults (3,4,13). The recovery rates of naive CD4⁺ and naive CD8⁺ T cells were not significantly different (paired samples t test, p=0.5) and correlated positively (r = 0.7, p<0.001; Figure 2). The recovery of memory CD4⁺ T cells was biphasic, with a rapid recovery rate in the first 4 weeks of therapy (1.46±0.4 cells/mm³/day) and a slow recovery rate between week 4 and week 48 (0.17±0.06 cells/mm³/day), consistent with findings of others (4). The mean recovery rate of memory CD4⁺ T cells over the whole study period (week 0-week 48) was 0.22±0.05.
Figure 1a. Reconstitution of absolute number of circulating (a) CD4$^+$ and (b) CD8$^+$ T cells during 48 weeks of HAART: total count (△), memory cells (■) and naïve cells (○).
The daily decrease of memory CD8+ T cells was $0.72 \pm 0.13$ cells/mm$^3$/day. No differences of recovery rates were observed between the two treatment groups (data not shown).

To eliminate the contribution of lymphocyte redistribution on the recovery of naive T cells in the blood, the recovery rates were also estimated for the period when plasma HIV RNA levels were below 50 copies/ml. Redistribution of lymphocytes from lymphoid tissue to the blood predominantly occurs during the first weeks of HAART, due to decreased inflammation in the lymphoid tissue (4). The recovery rates of naive CD4+ and CD8+ T cells in the period after the patients achieved plasma HIV RNA levels <50 copies/ml were $0.34 \pm 0.06$ and $0.35 \pm 0.05$ cells/mm$^3$/day, respectively. These recovery rates were not significantly different from the recovery rates based on the entire 48 weeks of treatment ($p=0.99$ and $p=0.7$, respectively), suggesting that redistribution had no significant influence on our estimates of the recovery rate of naive T cells.

Because thymic function diminishes with increasing age, we determined the influence of age on the speed of naive T-cell regeneration. The recovery rates of the naive CD4+ and CD8+ T cells correlated negatively with the age of the patients ($r = -0.41$, $p=0.005$ and $r = -0.47$, $p=0.001$,
Figure 3. Correlations between age and recovery rates of T-cell subsets. (a) Correlation between age and the recovery rate of naive CD4+ T cells ($r = -0.41$, $p=0.005$). The decrease of the recovery rate of naive T cells with increasing age fitted to an exponential function: $R(a) = p_1 e^{p_2 a}$, where $R(a)$ is the recovery rate and $a$ is the age. The parameters $p_1$ and $p_2$ (± S.D.), found by non-linear regression analysis (excluding 2 negative recovery rates) were as follows: $p_1=1.7$ (±1.8), $p_2= -0.050$ (±0.016), corresponding to a decay rate of 5.0% per year.

(b) Correlation between the age and the recovery rate of naive CD8+ T cells ($r = -0.47$, $p=0.001$). The parameters of the $R(a)$ function (excluding 4 negative recovery rates) were: $p_1=1.2$ (±1.7), $p_2= -0.036$ (±0.016), corresponding to a decay rate of 3.6% per year.
Figure 3. Correlations between age and recovery rates of T-cell subsets.
(c) Correlation between the age and the recovery rate of memory CD4$^+$ T cells ($r = -0.07$, $p=0.6$).
(d) Correlation between the age and the decrease rate of memory CD8$^+$ T cells ($r=0.1$, $p=0.2$).
Figure 4. Correlation between baseline naive T-cell counts and the recovery rate of naive T cells during HAART. Figure 4a. Naive CD$^+$ T cells. Figure 4b. Naive CD$^+$ T cells.
respectively; Figure 3). The decrease of the recovery rates with increasing age fitted best to an exponential function (Figure 3a, 3b). The recovery rates of naive CD4+ and CD8+ T cells decreased 5.0% per year (p=0.003) and 3.6% per year (p=0.02), respectively. The recovery rate of memory CD4+ T cells was not correlated to the age (r=-0.07, p=0.6), nor was the daily decrease of memory CD8+ T cells (r=0.1, p=0.2). Thus, our results indicate that the age of the patients is an important factor in naive T-cell regeneration.

To investigate whether the regeneration rate of naive T cells depends on the number of pre-existent naive T cells, we determined the relation between pretreatment counts of naive CD4+ and CD8+ T cells and the recovery rate of the respective T-cell subsets. No significant correlations were observed between naive T cell baseline counts and recovery rates (r = 0.2 p=0.2 and r = -0.1, p=0.4, respectively, Figure 4). This suggests that peripheral expansion plays a limited role in the regeneration of naive T cells.

Next, we determined the contribution of other factors on the speed of regeneration of naive T cells. First, the reduction of plasma HIV RNA levels during 48 weeks of HAART did not correlate to the recovery rate of the naive CD4+ and CD8+ T cells (r = 0.1, p=0.3 and r = 0.07, p=0.6, respectively). Secondly, no correlation was found between the rate of naive T-cell recovery and surrogate markers for the stage of HIV disease at baseline, total CD4 count and HIV RNA plasma levels (each p value > 0.3). Thus, baseline naive T-cell counts, viral load reduction during therapy and disease stage prior to therapy, do not contribute in a major way to the regeneration rate of naive T cells during HAART.

Discussion

In this study, we investigated the changes in the blood of the numbers of phenotypically naive and memory T cells during HAART. For the first time, we demonstrate that the recovery rate of naive CD4+ and CD8+ T cells during HAART is inversely correlated to the age of the patients (figure 3). The rate of increase in memory CD4+ T cells and the decrease
rate of memory CD8+ T cells showed no relation to the age of the patients. Regeneration of naive T cells during HAART may involve a thymus dependent and a thymus independent pathway. Although an influence of age on the thymic independent pathway cannot be excluded, the most plausible explanation for the inverse correlation between age and the regeneration rate of naive T cells is that the thymus dependent pathway plays an important role in naive T-cell regeneration, because thymic function diminishes with age (18,19). Moreover, the exponential decrease of the regeneration rates during aging (5.0% and 3.6% per year for naive CD4+ and CD8+ T cells, respectively, Figure 3) is in close agreement with the reported exponential decay of the volume of the thymic epithelial space at a rate of 4.1% per year in adults above 20 years of age (18). Apparently, the amount of thymic parenchyma plays a rate limiting role in the regeneration of naive T cells (13).

The inverse correlation between age and the recovery rate of naive T cells should be considered with one caveat, because the older patients may also have the longest duration of HIV infection. The duration of HIV infection was unknown in this study. However, laboratory markers of duration of infection, baseline CD4 count and plasma HIV RNA levels, did not correlate with either age nor with the recovery rates of naive T cells. The duration of infection is therefore not likely to be a confounding factor.

The finding that the recovery rates of naive CD4+ and CD8+ T cells during HAART are not significantly different (p=0.5) appears in contrast with previous observations that naive CD8+ T-cell regeneration is faster than naive CD4+ T-cell regeneration after antineoplastic chemotherapy (21,24). The higher regeneration rate of naive CD8+ T cells was attributed to extrathymic production of naive CD8+ T cells. However, these studies used CD45RA expression as the marker of naive CD8+ T cells, which seems inaccurate because the CD45RA+/CD8+ T-cell population contains besides naive cells also fully differentiated effector cells (28,29). The very similar regeneration rate of truly naive CD4+ and CD8+ T-cells (CD45RA+/CD27+) in our patients suggests that generation of both cell types is under control of a common mechanism, consistent with thymic production of naive T cells.

Interestingly, the rate of increase of naive CD4+ T cells in our patients is similar to the regeneration rates observed in HIV seronegative adults with
iatrogenic CD4⁺ T-cell depletion due to either antineoplastic chemotherapy or treatment for rheumatoid arthritis with CD4 monoclonal antibodies (20,22,23,24,30). In our patients, the mean rate of increase of naive CD4⁺ was 0.34 cells/mm³/day, corresponding to an 38 year old subject in Figure 3. After iatrogenic T-cell depletion, the regeneration rates of naive CD4⁺ T cells vary from 0.005 to 1.1 cells/mm³/day. For the total body, the mean regeneration rate of naive CD4⁺ T cells in our patients is 0.85 x 10⁸ cells/day, assuming that the total blood volume is 5 liters and that 2% of the lymphocytes are in the blood compartment. This number is in close agreement with estimates of a total body production rate of naive CD4⁺ T cells of ~10⁸ cells/day (31), which was based on the decay of lymphocytes with stable chromosome damage after radiation. Thus, in HIV infected patients, the capacity to regenerate naive T cells during HAART is comparable to the situation after iatrogenic T-cell depletion.

There is considerable evidence that the thymus dependent pathway to develop new naive T cells from bone marrow progenitors is downregulated during HIV infection. First, it was shown that CD34⁺ hematopoietic progenitor cells are susceptible to HIV infection (32), and have impaired survival and clonogenic capacity during HIV infection (33). Second, it was demonstrated that the thymic tissue is affected in individuals with AIDS (34). In studies with SCID-hu mice it was shown that HIV infection results in depletion of CD4⁺/CD8⁺ thymocytes and reduction of the number of CD4⁺ T-lymphocytes from the thymic implants (35-37). Finally, measuring T-cell receptor excised circles (TREC's) to identify recent thymic emigrants showed that the number of TREC positive T cells is decreased as compared to HIV negative individuals, which was taken as evidence for a reduced export of new T cells from the thymus in HIV infection (11,12). However, the decreased number of TREC positive T cells in HIV infected individuals may also be explained by the high division rate of T cells due to HIV infection (38,39). Our finding that the regeneration rate of naive T cells during HAART is comparable to that after iatrogenic T-cell depletion suggests that the HIV-induced dysfunctions of progenitor cells or thymus are either of minor importance or they are rapidly reversed after introduction of HAART.

The relatively low correlation coefficients between age and naive T-cell
recovery rates (Figure 3) may be explained in several ways. First, it may be due to a large variation of thymic output between individuals, in agreement with large differences in the numbers of TREC positive T cells found in healthy individuals (11,12). Secondly, several studies have suggested that thymus independent pathways of naive T-cell regeneration also play a role during HAART.

In one study, it was reported that the number of circulating naive (CD45RA+/CD62L+) CD4+ T cells increased during HAART in a thymectomized patient (14). However, the increase of naive CD4+ T cells was exclusively observed in the first 12 weeks of HAART and the number remained constant thereafter. The increase of naive CD4+ T cells during 95 weeks of HAART was approximately 25 cells/mm³ (0.038 cells/mm³/day).

In our study, and in studies by others with thymus-bearing patients, naive T-cell counts increased continuously throughout the complete course of HAART with a ~10 fold higher rate. The initial rise of naive CD4+ T cells in the thymectomized patient (14) could therefore be explained by a redistribution of naive T cells from (lymphoid) tissue to blood, which typically occurs in the first weeks of HAART (4).

In two other studies, TRECs were measured to identify recent thymic emigrants during HAART (11,12). It was shown that the increases in TREC-positive T cells during HAART were numerically insufficient to account for the increases in phenotypically naive (CD45RA+/CD62L+) T cells. This suggests that naive T cells may proliferate without converting to the memory phenotype. Increased expression of the proliferation marker Ki67 on naive T cells has been observed in untreated HIV-1 infected patients with less than 100 naive T cells/mm³ (40). However, Ki67 expression on naive T cells was shown to decrease rapidly during the first weeks of HAART (40), whereas in our study the increase of naive T cells is relatively constant, suggesting that proliferation plays a limited role in the regeneration of naive T cells. The absence of a positive correlation between baseline naive T-cell counts and the regeneration rates (Figure 4) also argues against peripheral expansion as a mechanism of naive T-cell regeneration.

Theoretically, reversion of memory cells may also contribute to the recovery of naive T cells during HAART (12,15). It has been suggested that CD45RO- (memory) T cells may revert to the CD45RA (naive)
isoform (41,42). However, using a combination of CD27 and CD45RA Mab’s we were able to distinguish between truly naive T cells (CD45RA+/CD27+) and memory/effector T cells of the CD45RA+/CD27- phenotype, that may have developed via the CD45R0+ stage (28,29). It is therefore unlikely that reversion of memory cells contributes to the recovery of truly naive (CD45RA+/CD27+) T cells in our study.

In conclusion, the similar regeneration rate of naive CD4+ and CD8+ T cells during HAART, and the inverse correlation between age and recovery rate of naive T cells, suggest that the thymus plays a considerable role in the regeneration of naive T cells in adults during HAART. In addition, our observation that the regeneration rate of naive CD4+ T cells is similar to the rates observed after iatrogenic T-cell depletion, suggests that HIV-induced dysfunctions of progenitor cells and the thymus are either of minor importance or they are rapidly reversed after initiation of HAART.

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