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## Immune Reconstitution after 2 Years of Successful Potent Antiretroviral Therapy in Previously Untreated Human Immunodeficiency Virus Type 1-Infected Adults

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Today's antiretroviral combination regimens can induce significant and sustained decreases in human immunodeficiency virus (HIV)-RNA levels, allowing the immune system to recover. To what extent immune reconstitution is possible and what factors determine the outcome have thus far not been resolved. We studied 19 subjects, treated for 2 years with protease inhibitor-containing triple therapy, who had a strong suppression of HIV-RNA levels. CD4<sup>+</sup> T-cell numbers increased from medians of 170 to 420 × 10<sup>6</sup> cells/L, but in a number of subjects T-cell numbers did not further increase after week 72, without having reached normal values. Long-term CD4<sup>+</sup> T-cell change was mainly caused by a slow but continuous increase in naive CD4<sup>+</sup> T cells (CD45RA<sup>+</sup>CD62L<sup>+</sup>) and was predicted by the baseline number of these cells. Our data indicate that long-term immunological recovery is gradual, even during strong suppression of viral replication, not always complete, and dependent on the preexisting level of naive CD4<sup>+</sup> T cells.

Infection with human immunodeficiency virus (HIV) causes progressive immune deterioration as well as chronic immune activation. Besides a gradual decrease in the number of CD4<sup>+</sup> T lymphocytes, T-cell function, measured as in vitro proliferative response on stimulation with mitogens or monoclonal antibodies (mAbs), decreases over the course of infection and becomes a strong independent prognostic marker for disease progression [1, 2]. In addition, the number of naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells declines with HIV infection, even though HIV preferentially infects activated CD4<sup>+</sup> memory T cells [3]. HIV-induced immune activation, measured by an increase in the expression of CD38 and human leukocyte antigen (HLA)-DR

on CD8<sup>+</sup> cells, can also predict development of AIDS [4, 5]. Furthermore, CD28 expression on CD8<sup>+</sup> T cells, associated with the capacity of these cells to proliferate on stimulation, decreases during disease progression [6, 7].

Antiretroviral combination therapy that includes a protease inhibitor (PI) leads to strong suppression of viral replication, reflected by a rapid and sustained decline of HIV type 1 (HIV-1)-RNA levels in the peripheral blood and lymphoid tissue [8-10]. In response, the CD4<sup>+</sup> cell numbers increase and T-cell function improves significantly [11-15].

We and others have shown that the initial increase in CD4<sup>+</sup> T-cell numbers in the blood after start of antiretroviral therapy consists mainly of cells with the memory phenotype, probably reflecting redistribution of these cells from lymphoid tissue into the circulation. After this initial increase, the number of memory CD4<sup>+</sup> T cells remains relatively stable. [11, 12, 16, 17]. Naive CD4<sup>+</sup> T-cell numbers increase at a slower but continuous rate, both in peripheral blood and lymphoid tissue [11-13, 16, 17].

The question of to what extent immune reconstitution is possible has not yet been answered. Immune recovery during potent antiretroviral therapy is expected to be a slow process, as can be inferred from immune recovery after bone marrow transplantation, chemotherapy, or CD4 antibody treatment in multiple sclerosis patients [18-20]. Furthermore, at this time it is not known which factors determine the extent of immune recovery, given a strong inhibition of HIV replication. In this

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This study has been approved by the medical ethics committees of each participating hospital. All participating patients gave written informed consent.

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study we report the results of 2 years of treatment with a PI-containing triple therapy in previously antiretroviral therapy-naïve subjects. Immune reconstitution was studied by measuring changes in CD4<sup>+</sup> and CD8<sup>+</sup> T cells, naïve and memory T-cell subsets, CD8 T-cell activation, and in vitro proliferative response to stimulation with mAb to CD3 and CD28. Factors predicting the extent of immune reconstitution and the observed individual variation were examined.

**Materials and Methods**

*Study participants.* Nineteen of 33 HIV-1-infected adults, originally included in an open-label PI-containing triple combination study, who remained on therapy for 2 years were studied [9]. At entry, all subjects were antiretroviral-naïve adults and had CD4<sup>+</sup> lymphocyte counts of at least 50 × 10<sup>6</sup> cells/L and a plasma HIV-RNA level of ≥30,000 copies per milliliter. Treatment consisted of the PI ritonavir (600 mg twice a day) and 2 nucleoside analogue reverse transcriptase inhibitors (NRTIs), zidovudine (300 mg twice a day) and lamivudine (150 mg twice a day). Participants were randomized to receive triple therapy from the start or to use ritonavir monotherapy for 3 weeks, followed by the addition of zidovudine and lamivudine. Three subjects switched to a different antiretroviral triple combination, consisting of 1 or 2 other PIs with 2 NRTIs, without interruption of treatment and remained included in the present study. One subject switched at week 20 and 2 at week 84.

Patients were followed up at weekly intervals from 2 weeks before until 4 weeks after start of therapy, with additional visits at days 3 and 10 and at weeks 6 and 8; every 4 weeks until week 52; and at 12-week intervals from weeks 60 through 108. At each visit blood was drawn for HIV-RNA and T-lymphocyte subset measurements.

*T-lymphocyte subsets.* Lymphocyte immunophenotyping for peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells was done by 2-color immunofluorescence flow cytometry (FACS). Naïve and memory T-lymphocyte subsets were measured by 3-color FACS for the surface proteins CD45RA, CD45RO, and CD62L (L-selectin), as described elsewhere [12]. T-cell subsets coexpressing CD45RA and CD62L were regarded as truly naïve T cells, whereas remaining T cells, including cells expressing CD45RO and cells expressing CD45RA without CD62L, were regarded as memory lymphocytes. Activation of CD8 cells was determined by the percentage of CD8<sup>+</sup>CD45RO<sup>+</sup> cells positive for CD38. Furthermore, the percentage of CD3<sup>+</sup>CD8<sup>+</sup> cells positive for CD28 was measured.

*T-cell function.* The in vitro proliferative response to CD3 and CD28 mAb was determined in whole blood lymphocyte culture, as previously described in detail [2]. In short, proliferative responses were measured after 4 days of culture by means of incorporation of [<sup>3</sup>H]-thymidine, added 24 h before harvest. Proliferative capacity was calculated to counts per minute per 10<sup>3</sup> CD3<sup>+</sup> T cells and expressed as percentage of the median response of 5 daily uninfected controls tested simultaneously.

*HIV-RNA levels.* Plasma HIV-1-RNA levels were measured with the Amplicor-HIV Monitor Test (Roche Molecular Systems, Branchburg, NJ), with a variable lower limit of quantification (LLQ), by following the manufacturer's instructions. In this study, the Amplicor assay had a mean LLQ of 204 (2.31 log<sub>10</sub>) copies/

mL (SD = 0.19 log<sub>10</sub>). For the 3 subjects who changed to a different combination without interruption of treatment, RNA levels were measured with the NucliSens assay (Organon Teknika BV, Boxtel, The Netherlands), with an LLQ of 400 copies/mL (2.60 log<sub>10</sub>), for their last 3 visits. Results obtained by Amplicor and by NucliSens were combined.

*Statistical analysis.* Immunological and virological baseline values were calculated as the mean value of 3 measurements prior to treatment (week 2, week 1, and week 0) for HIV-RNA, total CD4<sup>+</sup> and CD8<sup>+</sup> T-cell counts, and T-cell function, and as the mean of week 1 and week 0 for the naïve and memory T-cell subsets. Because previous analyses showed no differences between the 2 treatment arms in immunological or virological responses up to 36 weeks, results were combined and the participants analyzed as 1 group [9, 12].

Time-dependent analyses were done by using a repeated measurement (RM) model, Proc mixed, SAS 6.12 (SAS Institute, Cary, NC), including the respective baseline value and time since the start of treatment as covariates. SPSS 8.0 (SPSS, Chicago) was used for the remaining statistical analyses. T-cell subset changes over time were analyzed by linear slope estimation for each subject. Correlation coefficients were obtained by Spearman rank correlation. Differences between paired variables were analyzed with the Wilcoxon signed ranks test and between groups with the Mann-Whitney *U* test.

**Results**

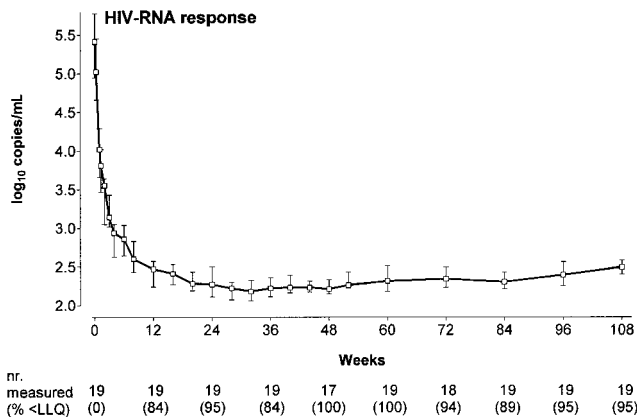
Baseline characteristics of the 19 subjects are depicted in table 1. A large fraction of the participants could be considered advanced in HIV disease, because 10 subjects had symptomatic HIV disease, and median CD4<sup>+</sup> T-cell counts were 170 × 10<sup>6</sup> cells/L before start of therapy.

*HIV-RNA response.* HIV-RNA levels strongly declined to levels below the LLQ in 83%–100% of the subjects at each visit from week 12 onward (figure 1; for significance of changes over time, *P* = .0001, repeated measurements). Ten subjects had quantifiable RNA levels on ≥1 occasions during this period,

**Table 1.** Baseline virological and immunological characteristics (*n* = 17 men, 2 women).

Characteristic	Median (interquartile range)
Age (years)	38 (32–41)
HIV-RNA levels (log <sub>10</sub> copies/mL)	5.41 (4.95–5.78)
T lymphocytes (10 <sup>6</sup> cells/L)	
CD4 <sup>+</sup> T cells	170 (127–230)
Naïve T cells	42 (19–56)
Memory T cells	109 (82–187)
CD8 <sup>+</sup> T cells	960 (627–1300)
Naïve T cells	103 (61–140)
Memory T cells	775 (497–1047)
Proliferative response per T cell (% of median of daily controls)	
CD3 + CD28 mAb	14 (5–22)

NOTE. HIV, human immunodeficiency virus; mAb, monoclonal antibody. Patients' CDC classifications were: class A, 9; B, 6; and C, 4. For HIV disease classification, CD4<sup>+</sup> cell counts <200 × 10<sup>6</sup>/L were not regarded as CDC class C.



**Figure 1.** Human immunodeficiency virus (HIV)-RNA response measured with Amplicor (Roche Molecular Systems), with a mean lower limit of quantification (LLQ) of 200 copies/mL, and, for 3 subjects who changed therapy, measured with NucliSens (Organon Teknika) during their last visits. Depicted are medians and interquartile ranges. No. of participants analyzed and percentage below LLQ are given.

but their RNA levels never exceeded 500 copies/mL and were unquantifiable again at the following visit.

**CD4<sup>+</sup> T-cell responses and naive and memory subsets.** The CD4<sup>+</sup> T-lymphocyte numbers showed a strong and sustained increase to a median of  $420 \times 10^6$  cells/L at week 108 ( $P_{RM} = .02$ ). CD4<sup>+</sup> T cells appeared to increase in a 3-phasic pattern of a rapid increase during the first 3 weeks, a gradual increase until week 72, and a stabilization in a large proportion of the participants thereafter. This was analyzed in more detail by measuring naive and memory T-cell subsets and by estimation of the individual linear slopes of cell counts over time (figure 2A and figure 3A, B, and C).

During the first 3 weeks, the median increase rate in CD4<sup>+</sup> T-cell numbers was  $4.52 \times 10^6$  cells/L per day, which was mostly determined by a rapid increase of memory cells at a median rate of  $4.61 \times 10^6$  cells/L per day, whereas the median naive cell increase rate was  $0.33 \times 10^6$  cells/L per day.

During the subsequent period, until week 72, the number of CD4<sup>+</sup> T cells continuously increased but at a significantly lower median rate of  $0.32 \times 10^6$  cells/L per day (compared with week 0–3,  $P = .02$ , Wilcoxon [W]). The median memory cell increase rate declined to  $0.19 \times 10^6$  cells/L per day ( $P_w < .001$ ), whereas the median naive cell increase rate was  $0.09 \times 10^6$  cells/L per day ( $P_w = .12$ ).

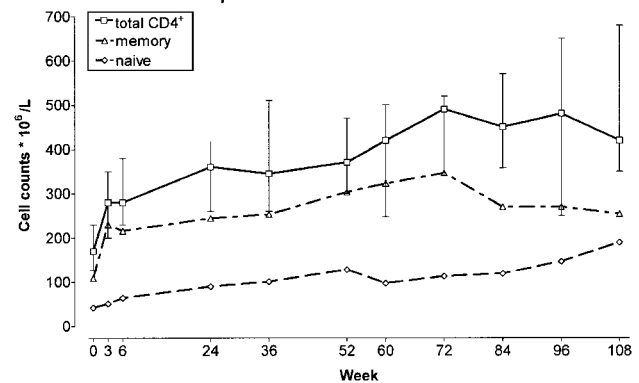
A third phase became apparent after week 72, with stabilization of the median CD4<sup>+</sup> T-cell numbers. The median increase rates of CD4<sup>+</sup> T cells between week 72 and 108 changed to  $0.02 \times 10^6$  cells/L per day (compared with weeks 3–72,  $P_w = .07$ ). In 7 of the 19 participants, the change rate was clearly negative (below  $-0.04$ ), and in 4 participants, it was close to zero (between  $-0.04$  and  $0.04$ ). The memory CD4<sup>+</sup> T-cell

change rate declined to  $-0.230 \times 10^6$  cells/L per day ( $P_w = .004$ ), whereas the median naive cell increase rate was  $0.27 \times 10^6$  cells/L per day. During the full follow-up of 108 weeks, the median percentage of CD4<sup>+</sup> T cells with the naive phenotype increased from 22% to 36%, with a considerable increase occurring during the third phase.

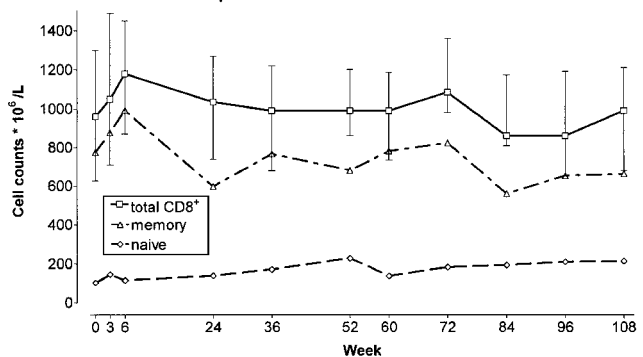
**CD8<sup>+</sup> T-cell responses.** CD8<sup>+</sup> T-lymphocytes showed an immediate increase during the first 6 weeks, with a subsequent decrease to baseline values, and remained stable for the duration of the study (figure 2B). The initial changes were mainly caused by an initial increase in CD8<sup>+</sup> memory T-cell numbers, which slowly declined thereafter. Naive CD8<sup>+</sup> cell numbers increased at a more steady rate of  $0.11 \times 10^6$  cells/L per day during the whole study period, which correlated with the CD4<sup>+</sup> naive increase rates between weeks 0 and 108 ( $R = 0.64$ ,  $P = .003$ , Spearman [S]). During the 108 weeks, the median percentage of CD8<sup>+</sup> T cells with the naive phenotype increased from 9% to 21%. The median CD4/CD8 ratio increased from 0.17 to 0.48 during the follow-up.

The percentage of activated CD8<sup>+</sup> memory cells,

**A. CD4<sup>+</sup> T-cell subset responses**



**B. CD8<sup>+</sup> T-cell subset responses**



n (total CD4&CD8)	19	19	18	18	18	17	18	17	19	18
n (subsets)	19	18	15	18	14	11	14	15	17	14

**Figure 2.** CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses, with naive and memory subsets. Naive cells are CD45RA<sup>+</sup>CD62L<sup>+</sup>. A. Median and interquartile range (IQR) CD4<sup>+</sup> T-cell response. B. Median and IQR CD8<sup>+</sup> T-cell response.

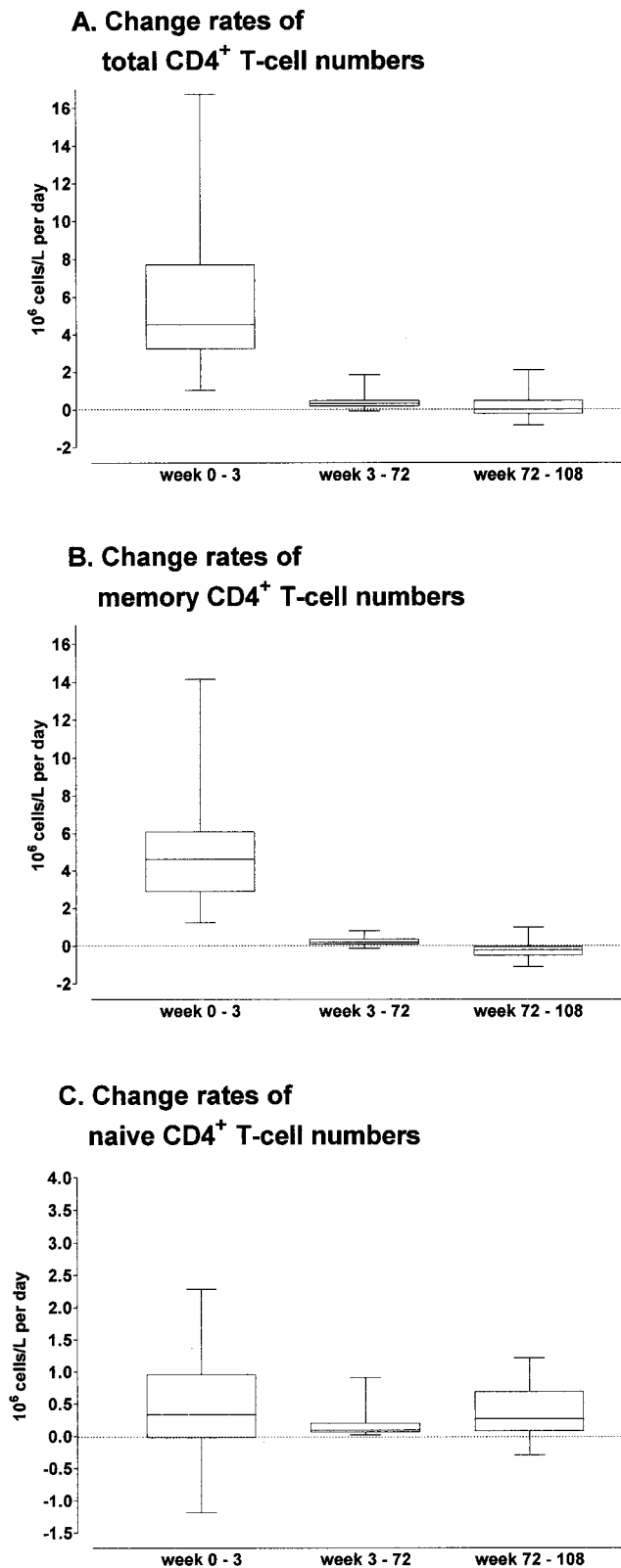


Figure 3. Change rates of (A) total, (B) memory, and (C) naive CD4<sup>+</sup> T-cell numbers during 3 respective periods.

CD8<sup>+</sup>CD45RO<sup>+</sup>CD38<sup>+</sup>, declined from a median of 32% at week 0 to a stable level between 8% and 12% after week 16. The percentage of CD8<sup>+</sup> T cells expressing CD28, CD8<sup>+</sup>CD3<sup>+</sup>CD28<sup>+</sup>, increased rapidly from a median of 32% before therapy to 41% at week 4 and slowly to 52% at week 96 (figure 4).

**T-cell function.** T-cell function, measured as in vitro proliferative response to stimulation with mAb to CD3 and CD28, calculated per 10<sup>3</sup> T cells and expressed as percentage of uninfected daily controls, increased rapidly during the first 3 weeks from a median of 14% to a median of 42% and gradually increased to 56% of the normal controls at week 108 (figure 5).

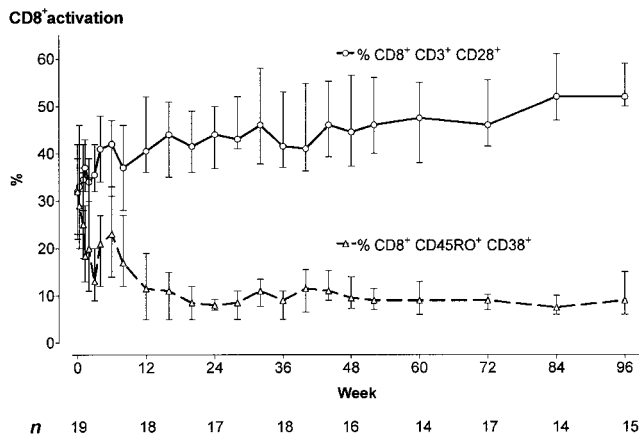
**Prediction of long-term responses.** Because the long-term gain in total CD4<sup>+</sup> T-cell numbers after week 3 was associated with the increase in naive T-cell numbers, the influence of baseline CD4<sup>+</sup> naive T-cell numbers on the CD4<sup>+</sup> T-cell response was further analyzed. CD4<sup>+</sup> T-cell increase rates between weeks 3 and 72 correlated significantly with the baseline CD4<sup>+</sup> naive T-cell counts ( $R_s = 0.62, P = .005$ ), whereas it did not correlate well with baseline total CD4<sup>+</sup> T-cell counts, baseline HIV-RNA levels, or age, which ranged from 26 to 47 years ( $R_s = 0.43, P = .07; R_s = -0.32, P = .18; \text{ and } R_s = -0.05, P = .84$ , respectively). The naive CD4<sup>+</sup> T-cell increase rates between weeks 3 and 72 also correlated with the baseline CD4<sup>+</sup> naive T-cell counts ( $R_s = 0.51, P = .03$ ) but not with the other baseline parameters (data not shown).

For further analysis, the baseline naive CD4<sup>+</sup> T-cell numbers were divided in tertiles, and the 2 outer groups were compared (figure 6A–D). Median baseline CD4<sup>+</sup> naive T-cell counts were 8 and 70 × 10<sup>6</sup> cells/L and total CD4<sup>+</sup> T-cell counts were 109 and 278 × 10<sup>6</sup> cells/L, respectively. Total CD4<sup>+</sup> T-cell increase rates between weeks 3 and 72 were significantly lower in the group with the low baseline naive CD4<sup>+</sup> T-cell numbers (medians, 0.13 vs. 0.41 × 10<sup>6</sup> cells/L per day,  $P = .02$ , Mann-Whitney [MN]). Median naive CD4<sup>+</sup> T-cell increase rates were 0.044 and 0.10 × 10<sup>6</sup> cells/L per day ( $P_{MN} = .004$ ), and memory CD4<sup>+</sup> T-cell increase rates were 0.09 and 0.27 × 10<sup>6</sup> cells/L per day, respectively ( $P_{MN} = .09$ ; figure 6A–D).

The CD4<sup>+</sup> T-cell change rates after week 72 did not correlate significantly with the baseline naive or total CD4<sup>+</sup> T-cell numbers or with the total or naive CD4<sup>+</sup> T-cell levels reached at week 72 (data not shown). Between the low and high baseline naive CD4<sup>+</sup> T-cell groups, median CD4<sup>+</sup> T-cell change rates were 0.006 and 0.23 × 10<sup>6</sup> cells/L per day ( $R_{MN} = .49$ ), respectively. Absolute CD4<sup>+</sup> T-cell levels reached at weeks 72 and 108 correlated with both baseline total and naive CD4<sup>+</sup> T-cell counts (data not shown).

**Discussion**

The extent of immune recovery during potent antiretroviral therapy has been shown to depend on the achievement of ad-



**Figure 4.** Percentage of activated CD8<sup>+</sup> memory cells (CD8<sup>+</sup> CD45RO<sup>+</sup> CD38<sup>+</sup>) and CD8<sup>+</sup> T cells capable of proliferation (CD8<sup>+</sup> CD3<sup>+</sup> CD28<sup>+</sup>).

equate suppression of viral replication [14]. To study the maximal immunological recovery that can be obtained and to search for other factors possibly determining the recovery, we studied subjects in whom plasma viral RNA levels were strongly suppressed for 2 years.

In this study, in which a considerable fraction of the participants had advanced HIV disease, we showed that immune recovery can continue during 2 years of potent antiretroviral therapy and that long-term CD4<sup>+</sup> T-cell increase is mainly caused by a slow but continuous increase in naive CD4<sup>+</sup> T-cell numbers. However, in a considerable number of participants, CD4<sup>+</sup> T-cell numbers stabilized or even slightly decreased after ~1½ years of therapy, sometimes without having reached normal levels.

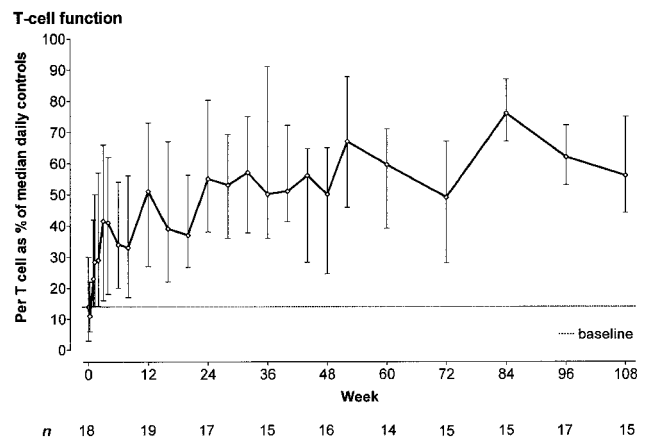
Directly after start of therapy, HIV-RNA levels strongly decreased and CD4<sup>+</sup> T-cell numbers strongly increased, the latter mostly as a result of the release of memory CD4<sup>+</sup> T cells from the lymphoid tissue [12]. Simultaneously, the percentage of activated memory CD8<sup>+</sup> T cells rapidly decreased, while T-cell proliferative capacity, corrected for the number of repopulating cells, strongly increased. Cytokine levels in lymphoid tissue have been shown to decrease shortly after use of antiretroviral therapy [21]. This suggests that the immediate strong immunological improvement might be caused by the decrease in inflammatory cytokines in the lymphoid tissue, after the strong suppression of viral replication in the lymphoid tissue [8].

Specific immunity to a number of antigens has been shown to improve with potent therapy [11, 14, 22, 23]. Lymphoproliferative responses to a number of recall antigens, reflecting CD4 helper cell function, improved after 40 weeks of treatment in several of our subjects, although cytotoxic T-cell frequency specific for HIV antigens did not improve substantially [23]. The increase in CD4<sup>+</sup> T-cell numbers and the improvement of T-cell function, measured as in vitro proliferative response to

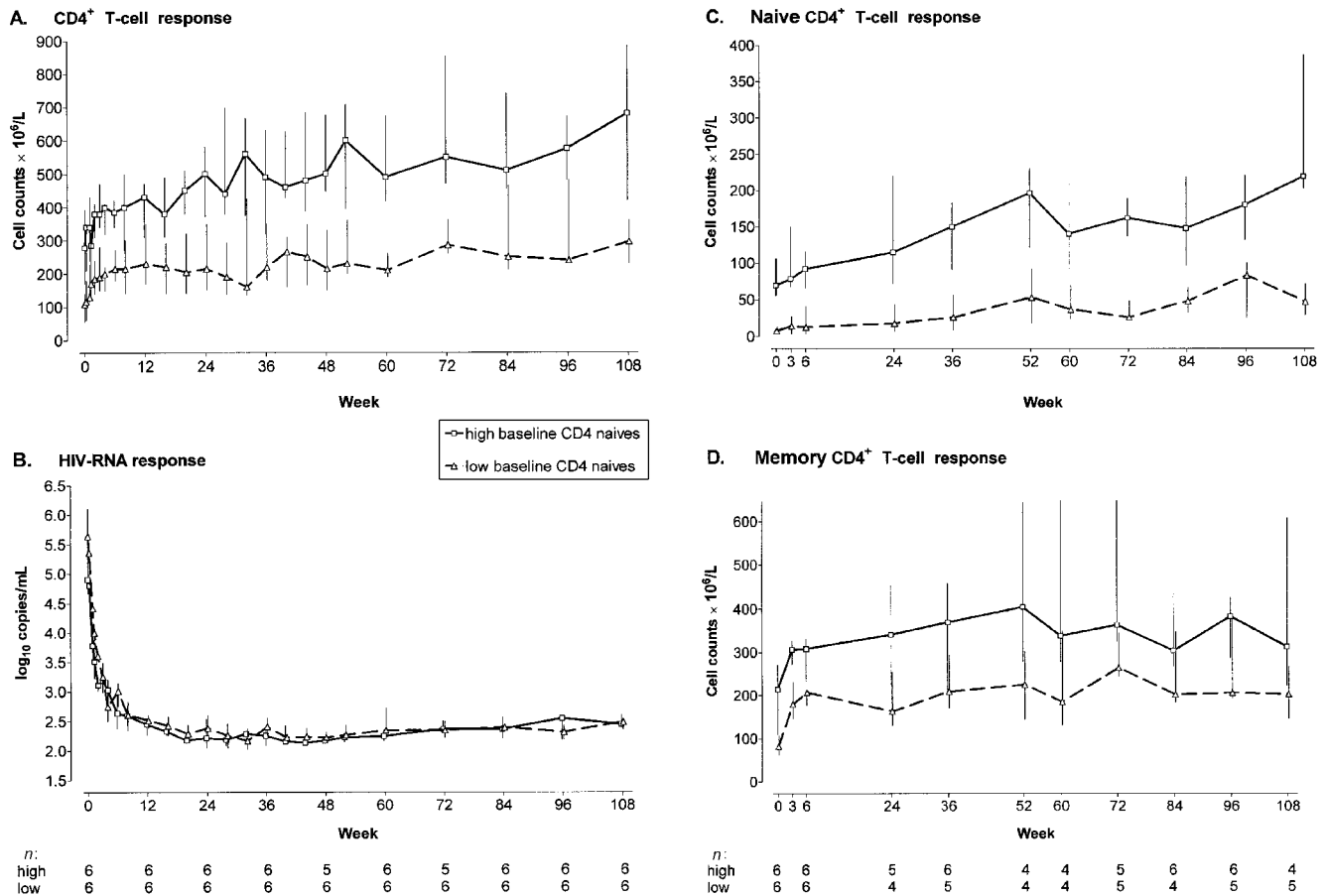
CD3 and CD28 mAb, thus reflect improved immunity to specific opportunistic pathogens. The median T-cell function reached at week 108, 56%, is close to the lower 95% confidence limit of 58% for uninfected controls [2].

Despite the limited number of participants, we showed that the number of naive CD4<sup>+</sup> T cells at the start of therapy is an important predictor of the long-term immunological outcome. Subjects with low pretreatment numbers of naive CD4<sup>+</sup> T cells had a poor recovery of their CD4<sup>+</sup> T cells after 2 years of treatment. In these subjects, after the initial increase in memory CD4<sup>+</sup> T cells in the first 3 weeks, the second repopulation period was deficient, compared with patients starting with higher numbers of naive CD4<sup>+</sup> T cells, in whom naive CD4<sup>+</sup> T cells mainly determined the total CD4<sup>+</sup> T-cell increase. The low number of participants in our study did not allow for extensive analysis of a larger number of possible factors in a multivariate analysis, to distinguish confounding factors.

In a subset of our study participants, the capacity of peripheral circulating precursor cells to generate mature T cells in in vitro thymic organ cultures was found to improve strongly during therapy. Furthermore, the increase in this capacity correlated with the increase in naive T-cell numbers [24]. Abundant thymic tissue has been detected in a considerable proportion of HIV-1-infected adults, and the amount of tissue correlated with the number of circulating naive T cells [25]. This suggests that the capacity to generate new T cells from precursor cells on suppression of viral replication depends on the amount of thymic tissue present, which then determines the recovery of CD4<sup>+</sup> T-cell numbers. This is further supported by a recent observation that thymic function improves during antiretroviral therapy [26]. Furthermore, in 4 of the subjects we analyzed, naive CD4<sup>+</sup> T cells appearing in circulation showed a slow improvement in T-cell receptor Vβ repertoire during 1 year of



**Figure 5.** In vitro T-cell function: proliferative response to stimulation with monoclonal antibody to CD3 and CD28, calculated to counts per minute per 10<sup>3</sup> CD3<sup>+</sup> T cells and expressed as percentage of median response of 5 daily uninfected controls tested simultaneously.



**Figure 6.** Human immunodeficiency virus (HIV)-RNA and immunological responses in 2 groups with high and low baseline CD4-naive cells, respectively. Medians and interquartile range. *A*, Total CD4<sup>+</sup> T-cell responses of 2 groups. *B*, HIV-RNA responses. *C*, Naive CD4<sup>+</sup> T-cell responses. *D*, Memory CD4<sup>+</sup> T-cell responses.

therapy, although a normal distribution was not yet reached [27].

The estimated T-cell change rates reflect changes in absolute counts in the peripheral blood and are the result of new production, redistribution, cell death, and maturation of naive into memory cells and thereby do not represent production rates.

In other long-term studies on immune reconstitution and possible determining factors, the influence of total but not of naive CD4<sup>+</sup> T-cell numbers at baseline on long-term CD4 reconstitution has been investigated [28, 29]. Renaud et al. found a significant relationship between long-term CD4<sup>+</sup> T-cell count changes and the extent of HIV-RNA reduction and the CD4<sup>+</sup> T-cell decline rate before initiation of therapy, but not with the baseline total CD4<sup>+</sup> T-cell counts. Staszewski et al. also found a relationship between increase in CD4<sup>+</sup> T-cell numbers and the suppression of HIV-RNA levels. A correlation of baseline naive CD4<sup>+</sup> T-cell counts with short-term (4-month) increases of these cells has been reported [30].

After 2 years of treatment, only a few participants had reached the lower limits of normal values for several of the

measured immune parameters, and there was considerable individual variation in the rate of CD4<sup>+</sup> T-cell repopulation. In a number of subjects, the increase in CD4<sup>+</sup> T cells appeared to slow down or stop completely after week 72, sometimes before reaching normal CD4<sup>+</sup> T-cell counts. No factors predicting this phenomenon could be identified, and this should be further investigated in larger groups of patients after even longer follow-up. Because the number of naive cells continued to increase after week 72, exhaustion of production of new cells, possibly from thymic origin, seems unlikely. The age distribution of our participants, aged from 26 to 47 years, might have been too narrow to detect influences of age.

Although the state of immune activation strongly decreased with the therapy, after 2 years of treatment the percentage of activated memory CD8<sup>+</sup> T cells did not return to the 2% found in uninfected people [31]. This might suggest residual continuing viral replication and antigen expression [32].

In line with previous findings of immune recovery in other strongly immunosuppressed patients, we conclude that long-term immunological recovery is a very gradual process, even

during strong suppression of viral replication, and might not be complete in a number of HIV-infected persons. The long-term increase in CD4<sup>+</sup> T-cell numbers is dependent on the preexisting level of naive CD4<sup>+</sup> T cells. Therefore, antiretroviral therapy should be initiated before the number of naive T-cells drops to low levels.

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