Central memory CD4 T cells are associated with incomplete restoration of the CD4 T cell pool after treatment-induced long-term undetectable HIV viraemia

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Objectives: It is unclear to what extent T cell reconstitution may be possible in HIV-1-infected individuals on continuous successful highly active antiretroviral therapy (HAART). Herein, we analysed distinct phenotypic markers of immune recovery in patients with undetectable viraemia for 8 years, taking as reference untreated patients and healthy controls.

Methods: Seventy-two subjects were examined: 28 HIV-1+ patients on successful long-term HAART, 24 HIV-1+ untreated viraemic patients and 20 age-matched healthy controls. Analysis of naive and memory CD4 and CD8 T cells was combined with measurements of activation status (expression of CD38) and with thymic function (expression of CD31). Statistical significance was determined by non-parametric tests.

Results: After long-term HAART, the majority of parameters were normalized compared with age-matched control values, including T cell activation and thymic function. However, absolute counts of naive and central memory CD4 T cells remained below normal levels. The only parameters significantly associated with CD4 counts at the end of follow-up were the pre-HAART CD4 count (β ± SD = 0.54 ± 0.16, P = 0.003) and the level of CD4 central memory cells at the end of follow-up (β ± SD = 1.18 ± 0.23, P < 0.0001). Only patients starting HAART with CD4 counts >350 cells/mm³ reached a complete normalization of CD4 counts.

Conclusions: Even after long-term successful HAART, complete CD4 restoration may be attainable only in patients starting therapy with moderately high CD4 counts, prompting early initiation of antiretroviral therapy. Incomplete CD4 restoration may be associated with a defective restoration of central memory CD4 T cells, a cell subset with a pivotal role in T cell homeostasis.

Keywords: long-term HAART, immune reconstitution, thymopoiesis

Introduction

Since the introduction of highly active antiretroviral therapy (HAART), many studies have addressed its effect on the reconstitution of the CD4 T cell pool. Although CD4 recovery is observed in most patients starting HAART, the degree is highly variable and it is unclear to what extent complete T cell reconstitution may be possible in patients on long-term successful HAART.1 There is a general consensus that T cell reconstitution is a three-phase process, differentiated by the speed of reconstitution and by the mechanisms responsible for it. The first fast phase occurs during the first 6 months and is mostly due to memory T cell redistribution. The second slower phase extends to the second year; finally, the slowest phase takes several additional years.2,3 The level of CD4 restoration is highly variable, depends on several factors4 and complete restoration of CD4 levels (taking as reference seronegative controls) after long-term suppression of viral replication with HAART is controversial, with some studies supporting this possibility,5 but not others.6–8

Immune restoration after HAART is not only a matter of the CD4 level, but also of many other aspects that are altered in HIV infection, such as T cell subset distribution and T cell activation among others.9–11 The suppression of viral replication with HAART restores many of these alterations, although not always to normal levels, at least in short- and mid-term HAART.7,12,13 Few studies have explored the effect of long-term HAART on these parameters and it is not known if they can be completely normalized in this situation.9 Normalization of these alterations may be important for at least two reasons: first, as surrogate markers of restored T cell functionality; and, second, some of them (such as T cell activation) are important pathogenic factors of HIV disease and thus their normalization is necessary in order to halt CD4 T cell depletion. In fact,
several studies have shown the interdependence between the level of restoration of such alterations and the level of CD4 count restoration.\textsuperscript{4,14–17}

Another important factor in the degree of CD4 restoration under HAART is the level of de novo T cell production in the thymus.\textsuperscript{18} Several studies have shown that the level of CD4 restoration is linked to the proportion of naive cells among CD4 T cells.\textsuperscript{4,14–19} Moreover, a link between naive CD4 T cell recovery and the abundance of thymic tissue has also been reported\textsuperscript{20} and a reduced thymic output has been proposed as a major mechanism of CD4 restoration failure in patients on long-term HAART.\textsuperscript{8} Thus, determining the level of de novo production of T cells is an important factor to consider when analysing CD4 restoration in the context of long-term successful HAART. CD31 is a marker of truly naive cells that have recently migrated from the thymus (recent thymic emigrants (RTEs))\textsuperscript{21} and has been employed as a marker of thymus functionality in the context of antiretroviral therapy.\textsuperscript{5,8,22,23}

To test the hypothesis of a complete recovery of the CD4 T cell pool and other immunological parameters after long-term viral suppression with HAART, herein we analysed distinct phenotypic markers of immune recovery (T cell activation, differentiation status and fraction of CD31-expressing CD4 and CD8 T cells) in patients on HAART with undetectable plasma HIV viraemia for 8 years, taking as reference untreated patients and healthy controls.

Methods

Study population
This was a cross-sectional study conducted in 52 patients with chronic HIV-1 infection on regular follow-up at Hospital Carlos III, a reference HIV clinic in Madrid, Spain. Subjects were split into two groups: (i) 28 patients on long-term HAART and with sustained virological suppression (<50 HIV-RNA copies/mL) and (ii) 24 untreated viraemic patients. Twenty HIV-seronegative healthy volunteers were included as controls to establish the normal values for the different parameters evaluated. To participate in the study, written informed consent was obtained from all individuals and the study protocol was evaluated and approved by the Hospital Ethics Committee.

Viral load measurement
Plasma HIV-RNA was measured using Versant HIV-1 RNA v3.0 (Siemens, Barcelona, Spain), which has a lower limit of detection of 50 HIV-RNA copies/mL.

Cell samples
All analyses were done in cryopreserved peripheral blood mononuclear cells (PBMCs). Only one cell sample was analysed per patient. In the group of patients on long-term HAART, sampling was done at the end of follow-up. EDTA-anticoagulated blood was obtained by venepuncture; PBMCs were immediately isolated by density gradient centrifugation using Ficoll-Hypaque (Sigma Chemical Co., St Louis, MO, USA) and frozen in fetal calf serum plus 10% DMSO. The viability of thawed PBMCs was always >85%.

Immunophenotypic analysis by flow cytometry
Analysis of the differentiation stage of CD4+ and CD8+ T cells was combined with measurements of activation status (expression of CD38). The post-thymic history of T cells was determined considering the fraction of CD31-expressing CD4+ and CD8+ T cells. All parameters were evaluated using five-colour flow cytometry. The following antibodies were used for flow-cytometric analysis: anti-CD4-PE/Cy7 (SFC112T4D11; Beckman Coulter, Fullerton, CA, USA), anti-CD8-PE/Cy7 (SFC121Thy2D3; Beckman Coulter), anti-CD45RA-PEC (2H4; Beckman Coulter), anti-CD27-PE (M-T271; BD Biosciences, San Diego, CA, USA), anti-CD38-PECy5 (LS198-4; Beckman Coulter) and anti-CD31-FTC (WM59; BD Biosciences). Each sample was stained with two different antibody panels: CD31-FTC/CD27-PE/CD45RA-EC/CD38-PECy5/CD4-PECy7 and CD31-FTC/CD27-PE/CD45RA-EC/CD38-PECy5/CD8-PECy7. PBMCs (1 million) were washed with 2 mL of PBS and stained for surface markers by incubation with the appropriate antibody panel for 30 min at 4°C. Cells were then washed with 2 mL of PBS and resuspended in 250 μL of PBS. Five-colour acquisition was performed on a Cytoflex FC 500 flow cytometer (Beckman Coulter). For each sample, a minimum of 50000 CD4+ and 50000 CD8+ events were acquired. Data analysis was performed using CXP software (Beckman Coulter). Gating was done on CD8+bright and CD4+ cells. Based on the expression of CD45RA and CD27 markers, four different subsets of CD4+ and CD8+ cells were defined: naive (CD45RA+CD27-), central memory (CD45RA–CD27+), effectector (CD45RA–CD27–) and effector (CD45RA+CD27–) cells. The proportion of each of these subsets expressing CD38 and/or CD31 was further analysed. Figure 2 shows a representative example of flow cytometry data with the gating strategy used.

Statistical analyses
The characteristics of the study population and the different immune parameters analysed were recorded as median (IQR) and comparisons were made using the non-parametric tests Mann–Whitney U-test, Wilcoxon test or Kruskal–Wallis test as appropriate. Correlations between quantitative parameters were explored using Spearman’s ρ test. All statistical analyses were performed using SPSS software version 13 (SPSS Inc., Chicago, IL, USA). All P values were two-tailed and were considered significant when <0.05.

Results

Study population
Table 1 shows some characteristics of the three different groups of subjects included in the study. At the time of sampling, treated patients were on successful HAART for a median of 96 (92–100) months and exhibited a median CD4 count of 691 (545–12617) cells/mm$^3$. Thirty-six percent (10 out of 28) of them started HAART with CD4 counts <200 cells/mm$^3$ and 68% (19 out of 28) with CD4 counts <350 cells/mm$^3$. The majority (26 out of 28) of treated patients were receiving a non-nucleoside reverse transcriptase inhibitor (‘NNRTI’)–based HAART regimen and two patients were receiving a protease inhibitor-based HAART regimen. Fifty-eight percent (14 out of 24) of untreated patients had been previously exposed to antiretroviral therapy, but were off therapy for ≥1 year prior to the sampling. In untreated HIV-1 patients, the median plasma HIV-RNA was 4.6 (4.4–5.0) log copies/mL. CD8 cells were significantly increased in both groups of patients compared with controls. Figure 2 shows the CD4 count
evolution in treated patients during the 8 years of successful HAART. The increase in CD4 counts with respect to the pre-HAART level (timepoint 0 in Figure 2) was higher during the first 2 years on therapy, reaching a plateau after 5 years with no significant increase afterwards. All patients experienced a CD4 count increase after 8 years of HAART, with a median increase of 432 (346–580) cells/mm³. However, even after 8 years of continuous successful HAART, patients still had significantly lower CD4 values compared with uninfected controls [691 (545–948) versus 934 (812–1169) cells/mm³, respectively, $P = 0.001$ (Mann–Whitney $U$-test; two-group comparison)].

**Differentiation stage of CD4 and CD8 T cells**

Figure 3 shows the percentages and absolute counts of the four different subsets of CD4 and CD8 cells based on the expression of

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**Figure 1.** Representative example of flow cytometry data with the gating strategy used. Data for CD4+ (a) and for CD8+ (b) cells are shown. Gating was done on CD4+ or CD8+ lymphocytes and then the expression of CD38 and CD31 analysed on total CD4+ or CD8+ cells and on the four cell subsets defined by the expression of CD27 and CD45RA markers (naive, central memory, effector memory and effector subsets). Numbers inside the plots indicate percentages of cells in each quadrant. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*. 

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**Figure 2.** Time course of CD4+ and CD8+ lymphocyte counts in treated patients during the 8 years of successful HAART.
CD45RA and CD27 markers in the three groups of subjects analysed. After 8 years of successful HAART, patients exhibited percentages, for the different CD4 subsets, similar to those found in uninfected controls. Surprisingly, the percentages observed in untreated patients were also similar to those in healthy controls, despite the low CD4 counts and high viral loads in this group of patients at the moment of study. However, absolute counts for all the different CD4 subsets were significantly decreased in untreated patients compared with healthy controls. In treated patients, absolute counts of effector memory and effector CD4 cells were similar to those found in controls, whereas effector memory cells were increased with respect to healthy controls.

**Activation levels of CD4 and CD8 T cells**

Expression of the CD38 marker was used to evaluate the level of activation in the different subsets of CD4 and CD8 T cells. Figure 4 shows the percentages and absolute counts of cells expressing CD38. Untreated patients showed very high percentages of CD38 expression on central memory, effector memory and effector CD4 T cells compared with healthy controls. All these alterations were normalized in treated patients after 8 years on successful HAART. However, given the lower levels of CD4 counts in untreated and in treated patients compared with healthy controls, absolute counts of activated naive and central memory CD4 T cells were decreased in untreated patients and remained so in patients on HAART.

Percentages of activation in the different subsets of CD8 T cells were even more increased than in CD4 cells in untreated patients compared with healthy controls, affecting also naive CD8 T cells. As for CD4 T cells, percentages of activation in CD8 subsets were similar to control group values after 8 years of successful HAART. Absolute counts of activated subsets of CD8 T cells were increased in untreated patients, whereas in treated patients only activated central memory and effector memory CD8 subsets were slightly increased.

**Expression of CD31 marker**

CD31 expression was analysed to evaluate the thymic history of CD4 and CD8 T cells. Figure 5 shows the percentages and absolute counts of CD31 expression in the different subsets of CD4 and CD8 T cells. In all studied groups, CD31 expression on CD4 T cells was almost restricted to naive cells, with the expression in the rest of the subsets being very low. Untreated patients showed decreased percentages of CD31 expression on naive CD4 T cells, whereas values in treated patients were similar to control group values. Absolute counts of naive CD31+ cells were very low in untreated patients and similar to those of healthy controls in treated patients.
The behaviour of CD31 expression was different in CD8 T cells. There was not a clear predominance of expression in the naive subset compared with the rest of the subsets. As for CD4 T cells, both percentages and absolute counts of naive CD8 cells expressing CD31 were significantly decreased in untreated patients, but similar to those of healthy controls in treated patients.

Using another approach, the percentages of RTEs, defined as CD4 or CD8 T cells co-expressing CD45RA, CD27 and CD31 markers, were also analysed. Levels of CD4 RTEs were significantly decreased in untreated patients and similar to controls in long-term HAART patients. However, for CD8 T cells, percentages of RTEs were also decreased after long-term HAART (data not shown).

**Factors associated with level of CD4 count restoration**

To ascertain what parameters could potentially be associated with the level of CD4 restoration after HAART, we sought the potential associations between the different immunological parameters measured on CD4 and CD8 T cells and the level of CD4 gains ($\Delta$CD4), as well as the CD4 count level after long-term HAART. $\Delta$CD4 was significantly associated with absolute counts of CD4 effector memory cells (Spearman’s $r=0.44$, $P=0.02$) and CD4 central memory cells (Spearman’s $r=0.51$, $P=0.003$). Interestingly, $\Delta$CD4 was not associated with baseline CD4 counts. Regarding CD4 counts after HAART, they were significantly associated with absolute counts of CD4 naive (Spearman’s $r=0.45$, $P=0.02$), CD4 effector memory (Spearman’s $r=0.58$, $P=0.001$) and CD4 central memory (Spearman’s $r=0.74$, $P<0.0001$) cells, as well as with baseline (pre-HAART) CD4 counts (Spearman’s $r=0.62$, $P<0.0001$). In agreement with this last association, only patients starting HAART with CD4 counts $>350$ cells/mm$^3$ reached a complete normalization of CD4 counts after treatment with levels similar to those found in healthy controls [952 (642–1069) versus 934 (812–1169) cells/mm$^3$ in patients and controls, respectively, $P=0.5$]. Using a linear regression model, both absolute counts of CD4 central memory and baseline CD4 counts were significantly and independently associated with CD4 counts after HAART, with the CD4 central memory count being the parameter with the highest contribution to the model (Table 2).

Using another approach, patients were stratified according to the $\Delta$CD4 (using the median $\Delta$CD4 in the whole group as the cut-off value) into two groups, those showing $\Delta$CD4 above...
the median value (ΔCD4H group) and those showing ΔCD4 below
the median value (ΔCD4L group), and the different parameters
were compared between these two groups and between each
group with healthy controls. There were no significant differences
between both groups, either in the percentages or in the absolute
counts of the different CD4 and CD8 T cell subsets analysed,
except for the absolute counts of central memory CD4 cells that
were significantly higher in ΔCD4H compared with ΔCD4L patients
(407 (339 – 459) versus 288 (239 – 382) cells/mm³, respectively,
P = 0.04). On the other hand, only ΔCD4H patients had levels of
CD4 counts and levels of naive CD4 counts similar to healthy con-
trols, whereas central memory CD4 counts were significantly
decreased in both ΔCD4H and ΔCD4L groups. Data not shown.

Discussion
In spite of the extensive literature on CD4 restoration after HAART,
much controversy still exists regarding the ability of patients on
HAART to fully replenish the pool of CD4+ T cells. Our data support
the idea that even after long-term successful HAART, CD4 counts
are not completely restored using as reference age-matched
seronegative controls. Several previous studies addressing CD4
restoration have failed to test this hypothesis for different
reasons, such as selection bias,19,24,25 lack of age-matched sero-
negative controls,25–27 persistence of detectable HIV load25,26
and short-term HAART.24,26,28 Moreover, studies performed
under conditions that permit assessment of CD4 reconstitution
after long-term successful HAART are very scarce. Even studies per-
formed under such conditions do not yield the same conclusions.
Our results agree with the findings of some of these studies,6–8
but not with those of others.5 However, the three studies conclud-
ing that CD4 restoration is not complete had some important dif-
fferences in design compared with our study. First, they had a
much shorter follow-up period;7,8 second, and even more import-
ant, the patients included had very low pre-HAART CD4 counts.6,8
In fact, in one of these studies analysing a large population of
patients, the level of CD4 counts after 3 years of HAART was
clearly correlated with baseline CD4 counts.7 Interestingly,
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Thus, the apparent disagreement between our study and the study by Vrisekoop et al. may be explained by different percentages of patients with low CD4 counts at baseline. In our study, 36% (10 out of 28) of patients started HAART with CD4 counts <200 cells/mm³ and 68% (19 out of 28) with CD4 counts <350 cells/mm³. In fact, CD4 levels at the end of follow-up in our study were normalized only in those patients starting HAART with CD4 counts >350 cells/mm³ (9 out of 28 patients). Thus, our data also support that a complete CD4 restoration is likely to be achieved only in patients initiating antiretroviral treatment with a moderate level of CD4 depletion, as has been suggested by other authors. 

We analysed different important parameters of HIV pathogenesis to test if their alterations could be completely normalized after a long-term period of undetectable viraemia. Our results suggest that some important alterations still remain after a long-term period of undetectable HIV replication. First, absolute counts of naive (CD45RA⁺CD27⁺) and central memory (CD45RA⁻CD27⁺) CD4⁺ T cells were still significantly decreased after long-term

![Figure 5](https://academic.oup.com/jac/article-abstract/68/11/2616/833762)
HAART and these populations have an important role in maintaining the T cell repertoire (naive cells) and as precursors of effector cells (central memory cells). However, it must be highlighted that both the fraction and absolute counts of CD31-expressing naive CD4 cells were normalized after long-term HAART. Since CD31 expression is linked to RTEs, these results suggest that thymic function can be restored in the context of long-term viral suppression, as has been proposed by some authors. Alternative, since HIV replication induces T cell proliferation and this may have a diluting effect on the CD31 T cell content, as has already been shown for T cell receptor excision circle (TREC) content, the increase in the fraction of naive CD4 cells expressing CD31 after long-term viral suppression could be the consequence of T cell proliferation being halted by HAART-induced viral suppression. However, because absolute counts of CD31-expressing T cells are not affected by this diluting effect, the finding that absolute counts of CD4 naive cells expressing CD31 were normalized in HAART patients supports the thymus function restoration hypothesis.

On the other hand, T cell activation is one of the main mechanisms driving CD4 depletion and is largely dependent on HIV replication. Thus, normalization of this parameter after treatment-induced viral suppression is highly desirable in order to halt CD4 destruction. In our study, a complete normalization of the proportion of activated CD4 and CD8 T cells was observed after long-term HAART independently of complete CD4 recovery. Since previous studies have shown that persistent activation is associated with impaired CD4 restoration in treated patients, our results support the hypothesis that mechanisms other than T cell activation are involved in the incomplete CD4 restoration even after long-term HAART. The existence of residual viraemia after successful HAART could be involved in the incomplete normalization of the CD4 T cell pool. However, this is a very controversial issue and it was not evaluated in our study. The level of residual viraemia has been previously associated with persistent T cell activation and poor immune reconstitution (gain of <200 CD4 cells/mm³). However, in our study, patients on successful long-term HAART achieved a median gain of 432 CD4 cells/mm³, which is far from the concept of poor immunological response (gain of <200 CD4+ T cells/mm³). Moreover, in our study, T cell activation was completely normalized in all patients on HAART. Notably, a very recent study with a similar design to ours did not find a correlation between the level of persistent viraemia, T cell activation and the level of CD4 restoration.

An interesting finding in our study is that although absolute counts of CD4 naive cells expressing CD31 (RTEs) were normalized after long-term HAART, absolute counts of total CD4 naive (CD45RA+CD27+) cells were decreased, meaning that the fraction of CD4 naive cells not expressing CD31 was not completely restored, probably as a consequence of diminished peripheral expansion of this cell subset. Another important subset of CD4 T cells not completely restored after long-term HAART were central memory (CD45RA−CD27+) cells, a subset considered a self-renewing source of expendable effector memory and effector cells and with a pivotal role in progression to AIDS. Different observations in our study support the role of this population in the restoration of the CD4 T cell pool. First, the absolute count of central memory CD4 cells is the only parameter significantly associated with both the level of CD4 gains and the level of CD4 counts after HAART. Second, in a multivariate analysis, central memory CD4 cells show the strongest association with the CD4 count after HAART. Third, absolute counts of this population are significantly higher in the subset of patients showing complete restoration of the CD4 T cell pool. However, it has to be mentioned that even in those patients showing complete restoration of the CD4 T cell pool, central memory cells are still significantly decreased, suggesting either a persistent defect in their renewal capacity or, alternatively, an increased transition to other populations such as effector memory cells.

The main strengths of our study are the long observation period in a population of patients maintaining complete suppression of plasma HIV viraemia over the entire period of follow-up and the detailed immunological analysis in terms of the different aspects involved in HIV pathogenesis. The main limitations are the cross-sectional design, the relatively small sample size and the lack of an exact matching between patients and controls in terms of age and sex. However, it is important to note that the difference in age, although statistically significant, was very small and thus this probably does not represent an important bias. Moreover, age was not statistically significant when comparing only long-term HAART patients and healthy controls (data not shown). Regarding sex, 50% of control subjects were female whereas this was the case in only 18% of long-term HAART patients. However, CD4 counts were very similar between females and males in the control group (data not shown), excluding the possibility of a bias in the CD4 comparison due to this difference in sex distribution between controls and patients.

In summary, our results demonstrate a defect in complete CD4 restoration even after a prolonged period of complete viral suppression in patients on HAART. Nonetheless, several of the immune parameters analysed were normalized after long-term viral suppression, some of them having a pivotal role in HIV disease pathogenesis, such as T cell activation and thymic function. This suggests that the incomplete restoration of the CD4 T cell pool is not the consequence of persistent immune activation or decreased thymic function. Our findings point towards a defect in specific T cell populations, such as central memory T cells, as potentially involved in this defective CD4 restoration. Also, our data support that complete CD4 restoration may be attainable only in those patients starting therapy with a moderate level of immune suppression, prompting the initiation of therapy in all patients before they reach a critical CD4 level.

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Transparency declarations
None to declare.
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