Changes in T-cell subpopulations during 4 years of suppression of HIV-1 replication in patients with advanced disease

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SUMMARY

We compared the number/percentages of naive and memory $CD4^+$ T-cells, $CD38^+CD8^+$ T-cells and $CD28^+CD4^+$ and $CD28^+CD8^+$ T-cells in patients with advanced (baseline CD4+ count < 100) with those with less advanced (baseline CD4+ cell count > 100) HIV-disease during 4 years of suppressive HAART. This prospective, longitudinal study included 30 treatment-naive patients and 32 controls. Flow cytometry was done at baseline and after 1, 3, 6, 12, 24 and 48 months (mo). Advanced HIV-infected patients (n=13; median baseline $CD4^+$: 23/µl) gained more $CD4^+$ T-cells than less advanced patients (n=11; median baseline $CD4^+$ 189/µl) at 1 mo (median: 60 vs 36/µl), 3 mo (86 vs 14), 6 mo (111 vs 23), 12 mo (174 vs 47), 24 mo (162 vs 72) and 48 mo (257 vs 123) (p=0.15; p<0.001; p=0.026; p=0.021, p=0.1; p=0.06 respectively). Advanced patients gained relatively more naive $CD4^+$ T-cells at 48 mo compared to less advanced patients (27.3 vs 11.4%, p=0.05). The relative gain in memory $CD4^+$ T-cells was greater in advanced versus less advanced patients at 1 mo (median: 6.4 vs 1.4%), 3 mo (4.3 vs 2.0), 6 mo (6.7 vs 1.6), 12 mo (6.9 vs 2.4), 24 mo (7.5 vs 3.1) and 48 mo (11.3 vs 6.8) (p=0.002; p=0.013; p<0.001; p=0.004; p=0.001; p=0.015, respectively). At 48 mo, $CD38^+CD8^+$ T-cells and naive $CD4^+$ T-cells reached normal values (9.2%, p=0.869 vs controls and 47.5%; p=0.699, respectively) in less advanced patients as well as $CD38^+CD8^+$ T-cells in advanced patients (4.7%, p=0.309 vs controls). The kinetics of naive and memory $CD4^+$ T-cell reconstitution is different in less advanced compared to advanced HIV-patients.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection induces a progressive loss in the number of $CD4^+$ T-cells, which is parallel to the development of severe immunodeficiency, increased susceptibility to opportunistic infection and subsequently death. The ability of highly active antiretroviral therapy (HAART) to reconstitute the immune system of HIV-infected patients is usually estimated by monitoring the increase in the number of $CD4^+$ T-cells during viral suppression [1].

It has been hypothesised that in HIV-infected patients with undetectable viremia the number of $CD4^+$ T-cells will increase for a certain period of time before reaching a plateau. This concept has been supported by several studies which demonstrated significantly slower increase in the number of $CD4^+$ T-cell after the first or second year of treatment in virological responders [2-4]. Contrary to these findings, Hunt et al have recently provided evidence that $CD4^+$ T-cell counts continue to rise after four years in people on continuously successful HAART, regardless of the baseline $CD4^+$ T-cell value [5].

The complete assessment of the quality of immune reconstitution during HAART does not only depend on the increase in the number of $CD4^+$ T-cells but also includes monitoring the correction in virus-mediated changes in T-cell phenotype and function [6-13].

The aim of this study was to compare the effect of four years of undetectable plasma viremia on the percentage of naive $CD45RA^+CD62L^+$ and memory $CD45RO^+$ $CD4^+$ T-cells, activated $CD38^+CD8^+$ T-cells and the expression of the costimulatory molecule CD28 on $CD4^+$ and $CD8^+$ T-cells in the peripheral blood of HIV-infected adults. We compared the kinetics of selected immune parameters between HIV-

infected persons who began HAART with less than 100 $CD4^+$ T-cells (advanced) and those who began therapy with higher $CD4^+$ T-cell counts (moderately advanced). We limited our analysis to patients who become undetectable after 6 months of HAART and maintained <50 copies/ml during the follow-up period.

METHODS

Study design and patients:

This prospective, four-year longitudinal study was conducted at the University Hospital for Infectious Diseases "Dr. Fran Mihaljević", Zagreb, Croatia between the December of 1998. and November of 2002. We enrolled 30 treatment-naive HIV-1 infected adults. HIV-patients were treated with a combination of zidovudine or stavudine, lamivudine and indinavir at standard doses. Included in this analysis were 24 patients who become undetectable (less than 50 copies of HIV-1 RNA per ml of plasma) at 6 months and stayed so thereafter.

Immunological (flow cytometry) and virological analysis (quantitative RT-PCR for HIV-1 RNA) were done before and after 1, 3, 6, 12, 24 and 48 months of treatment. Plasma samples for HIV-1 RNA quantification and peripheral blood samples for flow cytometry were collected as a part of routine diagnostic follow-up.

The study also included 32 apparently healthy controls. Informed consent was obtained from all patients and healthy controls. The study excluded children (< 17 years of age) and pregnant women. The study was approved by the Ethics committee of the Hospital.

Viral load

The quantification of HIV-1 RNA in the plasma of HIV-1-infected persons was performed by using Cobas Amplicor^{TM} HIV-1 monitor Test, version 1.5,

UltraSensitive specimen preparation (Roche Diagnostic Systems, Inc., Branchburg, New Jersey, USA) with lower limit of detection of 50 copies of HIV-1 RNA/ml.

Flow cytometry

To assess the quantity of T-cell immune reconstitution, we compared several lymphocyte subpopulations in HIV-1-infected patients before and during treatment to healthy controls. Percentage of $CD28^+CD4^+$ and $CD28^+CD8^+$ T-cells, naive $CD45RA^+CD62L^+$ and memory $CD45RO^+$ $CD4^+$ T-cells and activated $CD38^+CD8^+$ T-cells were determined by three-colour flow cytometry by using panel of monoclonal antibodies specific for CD3, CD4, CD8, CD14, CD16, CD19, CD45, CD56, HLA-DR, CD38, CD28, CD45RA, CD45RO and CD62L (DAKO, Denmark) conjugated with FITC, PE or RPE-Cy5. Absolute counts of $CD4^+$ T-lymphocytes were determined directly on the cytometer by using Flow-Count Fluorospheres and four-colour flow cytometry panel CYTO-STAT tetra CHROME (CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 and CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5, Beckman Coulter, Inc., FL, USA) as recommended [14].

Peripheral blood samples were prepared for flow cytometry by using a whole blood non-wash method on Multi-Q-Prep System with ImmunoPrep Reagent System (Beckman Coulter, Inc., FL, USA). The samples were analysed on Epics XL-MCL flow cytometer (Beckman Coulter, Inc., FL, USA).

Statistical analysis

The changes in immunological parameters from baseline throughout the follow-up period were analysed by Wilcoxon signed rank test ($p<0.05$ was considered significant). The comparison between two independent groups was done by Mann-Whitney test. All reported p-values are two-sided and exact. A generalized additive model was fit and trajectories of T-cell counts were described by drawing a smooth curve using robust local regression [15]. Statistical analysis was performed with SAS software version 8.2 [16] and StatXact release 5.0.3. (Cytel Software Corporation, USA). The loess option of PROC GAM was used to plot T-cells over time [16].

RESULTS:

Patients

Out of 30 patients enrolled, 24 met the inclusion criteria related to the undetectable viral load within 6 months of starting their first HAART regimen and completed all follow-ups. Pre-treatment viremia in 24 patients included in this analysis was 5.3 $\log_{10}$ copies of HIV-1 RNA/ml (range 3.4-6.7 $\log_{10}$ copies of HIV-1 RNA/ml). After 3 months of HAART, plasma viremia was undetectable in 14 patients. Ten out of 24 patients had detectable plasma viremia after 3 months of treatment (median 3.6 $\log_{10}$ copies of HIV-1 RNA/ml, range 3.0-4.8 $\log_{10}$ copies of HIV-1 RNA/ml) but subsequently achieved undetectable plasma viremia after 6 months of HAART.

The patients were divided into two groups, based on their initial $CD4^+$ T-cell counts (< or > 100 cells/μl). HIV-infected patients with < 100 $CD4^+$ T-cells per μl before treatment (n=13) were considered advanced (Table 1). At baseline, median percentages of naive and memory $CD4^+$ T-cells in advanced patients were 0.9% and 2.1%, respectively. Patients with initial $CD4^+$ T-cell counts > 100 cells per μl (n=11) were classified as moderately advanced. Median baseline percentages of naive and memory $CD4^+$ T-cells in moderately advanced patients were 32.6% and 10.1%, respectively.

Absolute counts of $CD4^+$ T-cells

Absolute counts of $CD4^+$ T-cells in advanced HIV-infected patients increased from a median of 23 cells/μl (IQR 4-55) before treatment to a median of 298 cells/μl (IQR 188-322) after 4 years of HAART (Table 2). Following an initial rapid increase

in $CD4^+$ T-cell counts in the first three months of treatment, there continued to be a less marked but sustained increase in median $CD4^+$ T-cell count during the remainder of the first year. There were statistically significant increases in the $CD4^+$ T-cell counts at the end of the first and fourth year of treatment.

In less advanced patients, $CD4^+$ T-cell counts increased from a median of 189 cells/µl (IQR 164-225) before treatment to 341 cells/µl (IQR 260-495) after 4 years of treatment (Table 2). The changes in CD4+ T-cell counts in moderately advanced patients were more gradual compared with advanced patients.

Advanced HIV-infected patients gained more $CD4^+$ T-cells than less advanced patients at 1 month (median: 60 vs 36 cells/µl), 3 months (86 vs 14 cells/µl), 6 months (111 vs 23 cells/µl), 12 months (174 vs 47 cells/µl), 24 months (162 vs 72 cells/µl) and 48 months (257 vs 123 cells/µl) ($p=0.15$; $p<0.001$; $p=0.026$; $p=0.021$, $p=0.1$; $p=0.06$, respectively, Table 2, Figure 1).

Naive $CD4^+$ T-cells

Percentages of naive $CD4^+$ T-cells increased in advanced patients increased from baseline (median 0.9%, IQR 0-15%) to a median of 40.2% (IQR 37.0-45.6%) after 48 months of treatment (Table 3). There was an early and statistically significant increase in naive $CD4^+$ T-cells within 3 months of HAART followed by the period of little change until the end of the first year. Significant changes in naive $CD4^+$ T-cells were also observed at the end of the second year of treatment but with little change thereafter.

In less advanced patients, naive $CD4^+$ T-cells increased from a median 32.6% (IQR 21.2-48.7%) at baseline to 50.6% (IQR 38.9-56.9%) after 48 months of HAART (Table 3). Unlike advanced HIV-patients, there was no early (first 3 months) increase

in naive $CD4^+$ T-cells in moderately advanced patients. Significant increase in the percentage of naive $CD4^+$ T-cells in moderately advanced patients was observed between the third and sixth month of treatment but we failed to observe any additional increase until the end of the first year. Percentage of naive $CD4^+$ T-cells in moderately advanced patients increased significantly at the end of the second and third year of treatment. At 48 months, percentages of naive $CD4^+$ T-cells in less advanced patients (median 50.6%) reconstituted to the levels of healthy controls (median 47.5%, p=0.699).

Advanced patients gained relatively more naive $CD4^+$ T-cells at 48 months compared to less advanced patients (27.3 vs. 11.4%, p=0.05, Figure 2).

Memory $CD45RO^+$ T-cells

Suppressive HAART enabled an increase in memory $CD4^+$ T-cells in advanced HIV-infected patients from baseline median 2.1% (IQR 5.0-6.9%) to a median of 15.6% (IQR 12.0-20.5%) after 48 months (Table 3). Following an initial rapid and significant increase within the first month of treatment, percentages of memory $CD4^+$ T-cells slightly decreased during the next two months with little change until the second significant increase observed at the end of the fourth year of HAART.

In less advanced patients, percentages of memory $CD4^+$ T-cells increased from a baseline median 10.1% (IQR 6.6-13.0%) to a median 17.8% (IQR 16.5-20.1%) at the end of follow-up (Table 3). Similarly to advanced patients, an early and statistically significant increase in memory $CD4^+$ T-cells occurred during the first month of HAART. There was a period of sustained but not significant increase in memory $CD4^+$ T-cells during the remainder of the first year as well as during the

second year of HAART followed by the significant increase at the end of the follow-up.

The relative gain in memory $CD4^+$ T-cells was greater in advanced versus less advanced patients at 1 month (median: 6.4 vs 1.4%), 3 months (4.3 vs 2.0%), 6 months (6.7 vs 1.6%), 12 months (6.9 vs 2.4%), 24 months (7.5 vs 3.1%) and 48 months (11.3 vs 6.8%) ($p=0.002$; $p=0.013$; $p<0.001$; $p=0.004$; $p=0.001$; $p=0.015$, respectively, Figure 3).

$CD8^+$ T-cells

Four years of HAART had little influence on the percentages of $CD8^+$ T-cells in both advanced and moderately advanced patients. Percentages of $CD8^+$ T-cells in advanced HIV-infected patients slightly decreased from a median of 57.1% (IQR 43.5-63.0%) before treatment to a median of 51.0% (IQR 44.9-56.7%) after 4 years of treatment (Table 2.). In less advanced HIV-infected patients, percentages of $CD8^+$ T-cells also decreased from a pre-treatment median of 58.0 (IQR 44.5-63.7) to 48.1% (IQR 43.1-50.5%) after 48 months of HAART (Table 2.).

Other lymphocyte subpopulations

There was no difference in the kinetics of $CD38^+CD8^+$ T-cells, $CD28^+CD4^+$ and $CD28^+CD8^+$ T-cells between advanced and less advanced patients during follow-up.

HAART-induced suppression of viral replication enabled a decrease in the percentage of activated $CD38^+CD8^+$ T-cells in advanced HIV-infected patients from a pre-treatment median of 50.0% (IQR 45.3-51.3%) to 4.7% (IQR 3.1-11.7%) after 48 months (Table 2). Percentages of $CD38^+CD8^+$ T-cells in less advanced patients

decreased from baseline median of 47.7% (IQR 40.2-51.0%) to 9.2% (IQR 6.5-12.6%) (Table 2).

The most dramatic decrease in the percentage of $CD38^+CD8^+$ T-cells in both patient groups occurred within the first month of HAART. In moderately advanced patients, statistically significant decrease in the percentages of these cells was observed during the first six months of treatment. Initial rapid decrease in the percentages of $CD38^+CD8^+$ T-cells was parallel to the treatment-induced reduction of plasma viremia to undetectable levels in 10 out of 24 patients after 3 months and all patients after 6 months of HAART (inclusion criteria). Percentages of $CD38^+CD8^+$ T-cells showed little change between the sixth month of HAART and the remainder of the first year. Interestingly, percentages of $CD38^+CD8^+$ T-cells decreased even further at the end of the second and fourth year of suppressive HAART. At month 48, $CD38^+CD8^+$ T-cells reached normal values (median 9.2% for controls) in both advanced (4.7%, p=0.309 vs. controls) and less advanced patients (9.2, p=0.869 vs. controls).

Percentages of $CD4^+$ T-cells expressing costimulatory molecule CD28 increased from baseline median of 60.2% (IQR 56.0-75.0%) before treatment to a median of 90.1 (IQR 80.9-93.6%) after 48 months of HAART (Table 3). Antiretroviral treatment appeared to have little influence on the percentages of $CD4^+$ T-cells expressing a costimulatory molecule CD28 during the first year in advanced patients. The majority of increase in the percentages of $CD28^+CD4^+$ T-cells in advanced patients occurred during the second and fourth year of treatment.

In less advanced patients, percentages of $CD28^+CD4^+$ T-cells increased from pre-treatment median of 59.1% (IQR 45.1-85.9%) to a median of 89.5% (IQR 80.5-91.8%) after 48 months of HAART (Table 3). Similarly to advanced patients, a period

of little change during the first six months of treatment was followed by statistically significant increases in the percentage of these cells at the end of the first, second and fourth year of HAART.

Percentages of $CD8^+$ T-cells expressing costimulatory molecule CD28 in advanced patients increased from baseline median of 39.6% (IQR 19.6-44.8%) before treatment to a median of 73.0 (IQR 70.6-75.6%) after 48 months of HAART (Table 3). Statistically significant increase in the percentages of $CD28^+CD8^+$ T-cells was observed within the first month of treatment as well as at the end of the second and fourth year.

In less advanced patients, percentages of $CD28^+CD8^+$ T-cells increased from baseline median of 34.3 (IQR 25.1-50.1%) to a median of 69.5% (IQR 64.5-75.9%, Table 3). Similarly to advanced patients, percentages of these cells significantly increases within the first month of treatment. After a 2 month period of little change, percentages of $CD28^+CD8^+$ T-cells continued a statistically significant increase at all time intervals until the end of follow-up.

DISCUSSION

Our results have shown that the kinetics of HAART-induced increase in truly naive and memory subpopulations of $CD4^+$ T-cells is different in moderately advanced compared to advanced HIV-infected patients. Advanced HIV-infected patients had a more pronounced gain in the percentage of memory $CD4^+$ T-cells during the first and at the end of the second and fourth year of HAART compared to less advanced patients. In addition, the relative gain in naive $CD4^+$ T-cells was also significantly higher in advanced compared to moderately advanced HIV-patients after four years of HAART. After four years of suppressive HAART enabled the complete reconstitution of naive $CD4^+$ T-cells in less advanced but not in advanced patients. Additionally, long-term control of viral replication enabled the decrease of activated $CD38^+CD8^+$ T-cells to the levels of healthy controls.

The reason for the observed difference in the gain of memory $CD4^+$ T-cells in advanced vs. less advanced HIV-infected patients during suppressive HAART is not clear. A model of T-cell reconstitution as a composite of memory cell redistribution from lymphoid tissues to the periphery and a regeneration of naive T-cells from thymic origin provides a possible explanation for the observed difference [17]. The kinetics of memory $CD4^+$ T-cells observed in this study appeared to be triphasic: an early and rapid increase followed by the more gradual increased and a subsequent significant increase at the end of follow-up. The initial increase in the percentage of these cells probably corresponds to the redistribution phase of memory T-cell from lymphoid tissues to the peripheral blood.

It is believed that the amount of memory T-cells “entrapped” in the peripheral lymphoid tissues is much higher in advanced HIV-disease compared with asymptomatic patients or those with early HIV-disease. Therefore, the number of

memory T-cells migrating to the periphery upon HAART initiation is expected to be much higher in advanced patients and this could explain the observed difference in memory $CD4^+$ T-cell gains between our two patient groups.

According to a different model of $CD4^+$ T-cell depletion during HIV infection being a result of disturbed homeostasis, it is possible that the homeostatic pressure to compensate for the loss of this cellular subpopulation was more pronounced in patients with lower pre-treatment $CD4^+$ T-cells count [18].

Naive $CD4^+$ T-cells are often regarded as a measure of true immune reconstitution because they enable a diversification of T-cell receptor repertoire in the infected host. Our study has shown that a complete restoration of naive $CD4^+$ T-cells after four years of successful virological control only in less advanced patients. Our study has also shown an early increase in naive $CD4^+$ T-cells of advanced patients, similarly to that observed by Mezzaroma et al [19]. The importance of naive $CD4^+$ T-cell in long-term immunological recovery during HAART was also confirmed by Notermans et al. showing that long term increase in total $CD4^+$ T-cells was predicted on the baseline number of naive $CD4^+$ T-cells [20].

Literature data on the gain in the number of $CD4^+$ T-cells during suppressive HAART in longitudinal studies is quite variable [3,4,5,21,22]. Lederman et al monitored $CD4^+$ T-cell increase in 643 HIV-infected patients during 144 weeks and demonstrated [22]. According to their results, $CD4^+$ T-cells increased from pre-treatment median of 266 $CD4^+$ T-cells/µl to a median of 358 cells/µl at the end of follow-up, showing similar kinetics of $CD4^+$ T-cell reconstitution to that observed in our study.

The ability of the immune system to continuously reconstitute $CD4^+$ T-cells for longer periods of time is still a controversial issue. The results of several

longitudinal studies suggested that the ability of the immune system to continuously reconstitute total $CD4^+$ T-cells for more than one or two years was limited [2,3,4]. A recent study by Hunt et al showed that virological responders to HAART continue to increase their $CD4^+$ T-cell count even after four years of treatment, regardless of the initial $CD4^+$ T-cells count [5]. However, this view was contradicted by Viard et al (2004) who failed to demonstrate any significant gain in CD4 T-cells by prolonging HAART for more than three years and questioned the value of life-long HAART [21].

The results of our study have show a continuous increase in the number of $CD4^+$ T-cells between the second and fourth year of HAART in both advanced and moderately advanced HIV-infected patients. Unfortunately, we were not able to analyse the selected parameters after three years of HAART. Therefore, we do not know it this was a difference between the second and third or third and fourth years of HAART. Therefore, we can not claim that the immune reconstitution continues for four years without reaching a plateau.

The ability of HAART to correct virus-induced downregulation of a costimulatory molecule CD28 on $CD4^+$ and $CD8^+$ T-cells has been described previously [3, 23]. Arno et al have shown a normalisation in the expression of CD28 on $CD8^+$ T-cells in nine HIV-1-infected asymptomatic persons treated with monotherapy or double antiretroviral therapy who maintained undetectable plasma viremia for two years [23]. Valdez et al observed the complete reconstitution of CD28 on $CD8^+$ T-cells but not on $CD4^+$ T-cell in patients who began treatment in moderately advanced HIV-disease after three years of HAART [3]. However, our study has shown that four years of HAART were not sufficient to completely reconstitute the expression of CD28 on $CD4^+$ and $CD8^+$ T-cells in both less advanced and advanced HIV-infected patients.

Sousa et al have proposed a concept concerning the role of immune hyperactivation as an inducer of $CD4^+$ T-cells depletion in HIV-disease [24]. Therefore, a reduction in the expression of activation markers such as CD38 (on $CD8^+$ T-cells) is considered to be an essential part of immune reconstitution. Valdez et al have shown that three years of HAART did not completely abrogate abnormal $CD8^+$ T-cell activation in moderately advanced HIV-disease. However, our study has shown a complete normalisation of CD38 overexpression on $CD8^+$ T-cell after four years of HAART in both moderately advanced and advanced HIV-infected persons.

Both groups of HIV-infected persons included in our study showed the highest reduction in the percentage of $CD38^+CD8^+$ T-cells within the first month of treatment which is probably related to the marked decrease in the plasma viremia observed in that period of time. HAART-mediated decrease in the expression of CD38 on $CD8^+$ T-cells parallel to the reduction in the plasma viremia has been previously described [25]. Additionally, both groups of HIV-infected persons included in the study (regardless of their initial $CD4^+$ T-cell counts) demonstrated a significant decrease in the percentage of $CD38^+CD8^+$ T-cells after two and four years of HAART.

In summary, this study indicates that the kinetics of the reconstitution of $CD4^+$ T-cells, naive and memory $CD4^+$ T-cells induced by HAART might be different in patients with more advanced HIV-infection compared to those with less advanced disease. The pattern was similar, however the magnitude of the response compared to the baseline value was different at various time points. There was no difference in the pattern or magnitude of $CD38^+CD8^+$ T-cells as well as in $CD28^+CD4^+$ and $CD28^+CD8^+$ T-cells between patients with less than 100 $CD4^+$ T-cells compared to those with more than $CD4^+$ T-cells/µl.

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Table 1. Baseline demographic and immunological parameters of the study population

Parameter	Healthy controls	advanced HIV-patients (baseline $CD4^+$ T-cell count < 100 cells/µl)	moderately advanced HIV-patients (baseline $CD4^+$ T-cell count > 100 cells/µl)
Number	24	13	11
Age in years	37.5 (31-44)	36 (34-42)	41 (28-44)
Sex ratio (Male/Female)	20/4	10/3	10/1
$CD4^+$ T-cells/µl	936.5 (785.5-1123)	23 (4-55)	189 (164-225)
$CD4^+$ T-cells (%)	47.3 (42.8-49.7)	2.6 (0.7-6.1)	15,6 (11.8-19.7)
$CD45RA^+CD62L^+$ $CD4^+$ T-cells (%)	47.5 (41.6-52.3)	0.9 (0-15)	32.6 (21.2-48.7)
$CD45RO^+CD4^+$ T-cells (%)	23.3 (21.3-25.9)	2.1 (5.0-6.9)	10.1 (6.6-13.0)
$CD8^+$ T-cells (%)	21.6 (16.7-25.8)	57.1 (43.5-63.0)	58.0 (44.5-63.7)
$CD38^+CD8^+$ T-cells (%)	9.2 (7.0-11.0)	50.0 (45.3-51.5)	47.7 (40.2-51.0)
$CD28^+CD4^+$ T-cells	95.6	60.2	59,1

(% of total $CD4^+$ T-cells)	(93.5-97.1)	(56.0-75.0)	(45.1-85.9)
$CD28^+CD8^+$ T-cells (% of total $CD8^+$ T-cells)	77.0 (75.0-85.6)	39.6 (19.6-44.8)	34.4 (25.1-50.1)

-values expressed as median, values in parentheses are interquartile range (IQR)

Table 2. Number of $CD4^+$ T-cells and percentages of, $CD8^+$ and $CD38^+$ $CD8^+$ T-cells in uninfected healthy controls and, advanced and moderately advanced HIV infected patients before and after 1, 3, 6, 12, 24 and 48 months of highly active antiretroviral therapy.

	Advanced patients (N=13)	Moderately advanced patients (N=11)	P^a	P^b
Treatment time points (months)	Median (IQR)	Median (IQR)		
$CD4^+$ T-cells (cells/µl)				
0	23 (4-55)	189 (164-225)		
1	72*** (59-205)	213 (200-256)	0.15	
3	123* (82-201)	216 (171-241)	<0.001	
6	125 (107-205)	221 (200-299)	0.026	
12	215** (104-235)	242 (211-336)	0.021	
24	237 (115-262)	301 (326-387)	0.1	
48	298** (188-322)	314 (260-495)	0.06	
Controls	936.5 (785.5-1123)			<0.0001
$CD8^+$ T-cells %				
0	57.1 (43.5-63.0)	58.0 (44.5-63.7)		
1	48.7 (42.3-60.6)	56.5 (43.6-59.7)	NS	

3	50.4 (45.7-60.1)	54.6 (42.9-60.4)	NS	
6	54.4 (44.7-59.4)	53.2 (45.5-61.3)	NS	
12	55.2 (44.7-58.2)	51.3 (46.9-60.1)	NS	
24	51.2 (45.1-55.2)	50.9* (48.3-55.6)	NS	
48	51.0 (44.9-56.7)	48.1** (43.1-50.5)	NS	
Controls	21.6 (16.7-25.8)			<0.0001
$CD38^+CD8^+$ T-cells %				
0	50.0 (45.3-51.5)	47.7 (40.2-51.0)		
1	28.0* (21.4-36.9)	32.2* (29.1-36.1)	NS	
3	29.8 (17.0-32.0)	21.9* (20.0-28.3)	NS	
6	21.0 (18.5-25.3)	19.5* (17.6-23.2)	NS	
12	17.0 (14.5-22.2)	17.3 (13.2-19.0)	NS	
24	10.1*** (8.6-16.9)	11.0** (8.9-14.6)	NS	
48	4.7* (3.1-11.7)	9.2* (6.5-12.6)	NS	
Controls	9.2 (7.0-11.0)			0.44 (0.42-0.45)

Statistically significant increase from the previous measurement are marked *($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$); Wicoxon signed ranked test.

[a]Between group comparisons of the difference of values at particular time points and the baseline value.

[b]Comparison between values after 48 months of treatment in moderately and advanced HIV infected patients and healthy controls using the Kruskal-Wallis test with Monte Carlo estimations of exact p-values and their 99% CI.

IQR, interquartile range; NS, not significant.

Table 3. Percentages of $CD28^+$ $CD4^+$,$CD28^+$ $CD8^+$ T, $CD45RO^+CD4^+$ and $CD45RA^+CD62L^+$ T cells in uninfected healthy controls and, advanced and moderately advanced HIV infected patients before and after 1, 3, 6, 12, 24 and 48 months of highly active antiretroviral therapy.

	Advanced patients (N=13)	Moderately advanced patients (N=11)	P^a	P^b
Treatment time points (months)	Median (IQR)	Median (IQR)		
$CD28^+CD4^+$ T-cells				
0	60.2 (56.0-75.0)	59.1 (45.1-85.9)		
1	74.0 (58.9-86.7)	70.1 (60.2-85.6)	NS	
3	76.2 (60.1-85.0)	73.4 (64.5-87.6)	NS	
6	77.7 (60.1-88.8)	79.7 (60.3-82.3)	NS	
12	76.6 (64.5-81.3)	85.2** (74.5-89.7)	NS	
24	83.2*** (70.9-90.1)	88.1* (77.8-89.6)	NS	
48	90.1* (80.9-93.6)	86.9** (80.5-91.8)	NS	
Controls	95.6 (93.5-97.1)			<0.0001
$CD28^+CD8^+$ T-cells				
0	39.6 (19.6-44.8)	34.4 (25.1-50.1)		
1	40.6* (35.0-52.1)	42.4** (30.1-52.3)	NS	
3	44.3 (37.7-47.8)	43.6 (30.1-52.3)	NS	

6	45.4 (39.5-51.0)	49.8** (39.1-60.1)	NS	
12	45.8 (41.4-88.1)	57.6* (45.6-64.5)	NS	
24	59.6** (55.6-64.5)	59.6* (55.6-65.6)	NS	
48	73.0*** (70.6-75.6)	69.5* (64.5-75.9)	NS	
Controls	77.0 (75.0-86.0)			<0.0001
CD45RO$^+$CD4$^+$ T-cells				
0	2.1 (5.0-6.9)	10.1 (6.6-13.0)		
1	9.5*** (5.2-11.5)	10.6** (8.1-15.2)	0.002	
3	8.3 (5.2-13.2)	12.3 (7.9-15.3)	0.013	
6	9.1 (6.9-12.5)	12.5 (7.6-14.0)	<0.001	
12	10.2 (3.3-12.9)	13.2 (9.5-13.6)	0.004	
24	10.4 (9.6-11.2)	13.6 (11.0-14.5)	0.001	
48	15.6** (12.0-20.5)	17.8** (16.5-20.1)	0.015	
Controls	23.3 (21.3-26.0)			<0.0001
CD45RA$^+$CD62L$^+$ CD4$^+$ T-cells				
0	0.9 (0-15)	32.6 (21.2-48.7)		
1	20.1* (18.5-20.6)	35.2 (20.1-45.6)	NS	
3	22.3** (18.9-	40.1 (22.6-50.2)	NS	

	24.6)			
6	21,5 (19.2-24.6)	38.0* (20.6-54.6)	NS	
12	25.9** (20.4-30.8)	39.8 (22.3-54.2)	NS	
24	33.2* (27.7-36.8)	40.5* (25.6-55.1)	NS	
48	40.2 (37.0-45.6)	50.6* (38.9-56.9)	0.05	
Controls	47.5 (52.3-41.6)			0.056 (0.05-0.06)

Statistically significant increase from the previous measurement are marked *($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$); Wicoxon signed ranked test.

[a]Between group comparisons of the difference of values at particular time points and the baseline value.

[b]Comparison between values after 48 months of treatment in moderately and advanced HIV infected patients and healthy controls using the Kruskal-Wallis test with Monte Carlo estimations of exact p-values and their 99% CI.

IQR, interquartile range; NS, not significant.

Figure 1. Loess estimates differences of $CD4^+$ T-cell counts

Figure 2. Loess estimates differences of percentage of memory $CD4^+$ T-cells

Figure 3. Loess estimates differences of percentage of naive $CD4^+$ T-cells