Thymic volume is associated independently with the magnitude of short- and long-term repopulation of CD4+ T cells in HIV-infected adults after highly active antiretroviral therapy (HAART)[1]

E. RUIZ-MATEOS†, A. RUBIO†‡, A. VALLEJO†, R. DE LA ROSA†, A. SANCHEZ-QUIJANO†, E. LISSEN† & M. LEAL†
†*Viral Hepatitis and AIDS Study Group, Virgen del Rocio University Hospital, Seville, and* ‡*Department of Medical Biochemistry, University of Seville, Spain.*

SUMMARY

Age is one of the main factors involved in the rapidity and the magnitude of CD4+ T cell repopulation in human immunodeficiency virus (HIV)-infected patients on highly active antiretroviral treatment (HAART). Improved thymic function has been suggested as the main factor associated with CD4+ T cell restoration after HAART. This work was undertaken to determine, among host factors, the predictor variable at baseline involved in the magnitude of short- and long-term recovery of CD4+ T cells after HAART. HIV-RNA levels and CD4+ T cell numbers were determined in 54 HIV-infected adults at baseline and at weeks 4, 12, 48 and 96 after HAART. T cell subpopulations were determined by flow cytometry, thymic volume by computed tomography, T cell receptor excision circle (TREC)-bearing cells by quantitative polymerase chian reaction (PCR) and interleukin (IL)-7 levels by enzyme linked immunosorbent assay at baseline. The phenotype of patients' isolates was determined by infecting GHOST cells expressing CCR5 and CXCR4. The possible interference of phenotype with thymic function was also analysed. Baseline thymic volume was associated independently with the magnitude of short- and long-term recovery of CD4+ T cells after HAART, despite the patients' viral phenotype. The measurement of thymic volume before therapy may predict the magnitude of T cell increase. This result could have important clinical implications not only in HIV-infected patients, but also in other scenarios of T cell depletion such as bone marrow transplantation and chemotherapy.

Keywords   CD4+   HIV   HAART   thymic function   TREC

INTRODUCTION

Several virological and host factors have been associated with CD4+ T cell recovery after highly active antiretroviral therapy (HAART). Among the virological factors, the magnitude of virological suppression has been correlated with increases in CD4+ T cell numbers [1,2]. Among host factors, age is one of the main players involved in the rapidity and the magnitude of CD4+ T cell repopulation in human immunodeficiency virus (HIV)-infected patients on HAART [3]. Moreover, naive T cell recovery has been associated with young individuals [4]. All such studies tried to explain that the magnitude of CD4+ T cell recovery is age related, and they suggested that improved thymic function could be associated with CD4+ T cell restoration after HAART. In fact, the role of the thymus after HAART is becoming accepted as one of the sources of CD4+ T cell recovery [5]. Hence, concomitant thymic volume increments, naive T cells and/or T cell receptor excision circle (TRECs)-bearing cells (the three most used thymic function-related markers) have been observed in children and adults under HAART [6,7]. Moreover, a poor CD4+ T cell recovery after HAART has been associated with impaired thymic T cell production [8]. We have reported recently that a larger thymic volume when starting HAART is associated with earlier CD4+ T cell repopulation [9]. In fact, among the most used thymic function-related markers, the baseline thymic volume has been reported to be the best predictor for the rapidity of CD4+ T cell recovery after HAART [10]. However, the association between these parameters and the magnitude of CD4+ T cell recovery after HAART has not yet been analysed.

Correspondence: Manuel Leal MD, PhD, Viral Hepatitis and AIDS Study Group, Department of Internal Medicine, Virgen del Rocío University Hospital, Sevilla, PC: 41013, Spain.
E-mail: mleal@cica.es

On the other hand, the syncytium-inducing (SI) phenotype has been associated traditionally with a lower CD4$^+$ T cell level and a rapid progression to AIDS [11], although nowadays this association is not clear. Hence, T-tropic strains might affect thymic function by inhibiting new T cell production. In relation to this, *in vitro* data have shown a high expression of the CXCR4 co-receptor in immature thymocytes [12] suggesting that the T-tropic phenotype might be associated with poorer CD4$^+$ T cell recovery after HAART because T-tropic strains use this co-receptor preferentially. For this reason, it would be of special interest to study viral phenotype before treatment, predicting that the predominance of a T-tropic phenotype would be associated inversely with thymic function-related markers and with a poorer recovery of CD4$^+$ T cell after HAART.

This work was undertaken to determine at baseline which variables among the host factors could predict the magnitude of short- and long-term CD4$^+$ T cell recovery after HAART. We analysed T cell subsets, thymic function-related markers and other variables related to thymopoiesis, i.e. age and interleukin (IL)-7 levels. We also analysed the potential association between viral phenotype and the magnitude of CD4$^+$ T cell gains and thymic function-related markers.

MATERIALS AND METHODS

Patients

In August 1998, the Viral Hepatitis and AIDS Study Group of Virgen del Rocio University Hospital began to study the role of the thymus in T cell repopulation in HIV-infected adults after HAART. The characteristics of this cohort have been described previously in other works [7,9,10,13]. Briefly, a total of 65 consecutive antiretroviral-naive HIV-infected patients starting HAART were included in an observational dynamic cohort. Up to March 2003, 54 patients were seen in at least two visits during follow-up (including baseline) and reported therapy compliance equal to or higher than 95%. All these patients agreed to participate in the study, providing written informed consent, and the Ethical Committee approved the study. HAART was defined as a combination of at least two nucleoside reverse transcriptase inhibitors plus a protease and/or a non-nucleoside reverse transcriptase inhibitor. Patients were evaluated at baseline and at weeks 4, 12, 48 and 96 after treatment. Each time-point was analysed for CD4$^+$, CD8$^+$ and CD3$^+$ T cell subsets and HIV-1 RNA levels in fresh samples. Isolated peripheral blood mononuclear cells (PBMCs) were frozen in liquid nitrogen and serum samples were frozen at –20°C until further determinations. Thoracic computed tomography (CT) to measure thymic volume, T cell phenotype and TREC-bearing cell determinations were studied at baseline.

Thoracic computed tomography

Mediastinic CT was performed with a modified previously described method [14]. Briefly, coded samples were always measured by the same radiologist using a 3000 GE Sytec Scanner with 5 mm thick contiguous sections at 5 mm intervals. Thymic tissue was delimited carefully in all the slices between the first and the last in order to exclude mediastinic fat infiltration (a high density for soft tissue and a low density for surrounding fat). CT Sytec software (version 4·0, General Electrics Medical Systems, Milwaukee WI, USA) was used to analyse all the thymic areas along the slices to calculate thymic volume.

Flow cytometry

At each time-point, absolute numbers of CD4$^+$, CD8$^+$ and CD3$^+$ T cells were determined in fresh samples by conventional flow cytometry. Percentages of naive CD4$^+$ T cells (CD4$^+$CD45RA$^+$CD45RO$^-$), memory CD4$^+$ T cells (CD4$^+$CD45RA$^-$CD45RO$^+$), naive CD8$^+$ T cells (CD8$^+$CD45RA$^+$CD11a^{low}) and memory CD8$^+$ T cells (CD8$^+$CD45RA$^-$CD11a^{high}) were determined using a frozen aliquot as described previously [15]. Naive and memory CD4$^+$ and CD8$^+$ T cell absolute numbers were calculated according to the CD4$^+$ and CD8$^+$ T cell counts obtained from fresh blood samples.

TREC-bearing cells: quantification in PBMCs

A modified polymerase chain reaction (PCR)-based method was used to quantify the δRec-ΨJα TREC number [16]. This method has been adapted to a real-time PCR using a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) to quantify the characteristic signal-joint sequences of the generated TREC and the β-globin gene [7]. TREC levels were measured in PBMC samples and given as TREC number per 10^6 PBMCs; in addition, the absolute count of TRECs/μl was calculated by multiplying the absolute T cell count by the proportion of TRECs in the CD3$^+$ T cell subpopulation of PBMCs.

IL-7 quantification

Determination of IL-7 levels in serum samples was performed using a high sensitivity colourimetric enzyme-linked immunosorbent assay (ELISA) (QuantikineHS IL-7 immunoassay kit, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations. This assay has a lower detection threshold of 0·1 pg/ml.

HIV-1 RNA quantification

Plasma HIV-1 RNA was measured by a quantitative PCR (HIV Monitor™ Test kit version 1·5, Roche Molecular Systems, Hoffman-La Roche, Basel, Switzerland) according to the manufacturer's instructions. This assay has a detection limit of 50 HIV-1 RNA copies/ml.

Hepatitis C virus (HCV) co-infection

Serum RNA HCV was measured by PCR (COBAS Amplicor, Roche Diagnostics, Barcelona, Spain).

Primary HIV-1 isolation

Patients' PBMCs were co-cultured with phytohaemagglutinin (PHA, Boerhinger Ingelheim)-stimulated HIV–HCV-negative donor's PBMCs in equal proportion. Cells were cultured in RPMI-1640 media (Biochrom AG) supplemented with 20% fetal calf serum (FCS) (FCS, Biochrom AG), 2 μg/ml of IL-2 (R&D Systems), 20 μM glutamine, streptomycin and penicillin (Sigma Aldrich). Cultures were maintained for 3 weeks and supernatants were harvested three times a week and stored at –80°C. Cell-free supernatants with HIV-p24 antigen (ELISA, Zeptometrix, Belgium) above 500 pg/ml were used for further infection assays.

Determination of HIV-1 co-receptor usage

Co-receptor usage was determined using GHOST cell lines, a human osteosarcoma (HOS) cell line transfected with the human CD4 gene and one of the co-receptors, CCR5 or CXCR4. Cultures were maintained in Dulbecco's modified Eagle medium

(DMEM; Biochrom AG) containing 10% FCS (Biochrom AG), streptomycin and penicillin (Sigma Aldrich). GHOST cells were plated in 48-well plates at 10^4 cells per well. Approximately 200 pg of HIV-p24 antigen of virus stocks from 30 patients were used to infect GHOST cells. Cultures were maintained for 7 days and supernatants were harvested every other day and tested for HIV-p24 antigen. We used the T-tropic NL4·3 strain and the M-tropic BaL strain as positive controls. HIV-p24 antigen levels above 20 pg/ml were considered positive. For statistical purposes, we used the ratio between the HIV-p24 antigen concentration obtained using GHOST-CD4-CXCR4 cells and the concentration obtained using GHOST-CD4-CCR5 cells at day 7 in order to transform a dichotomous variable into a continuous variable. A similar approach has been reported previously elsewhere [17,18].

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software package (SPSS 11·0, Chicago, IL, USA). All continuous variables were expressed as median [interquartile (IQR) range] while categorical variables were expressed as number of cases (percentage). Pearson's correlation coefficient analysis (univariate analysis) was used to assess bivariate correlations between all the variables. All variables analysed that had a statistically significant association ($P < 0.1$) with changes in T cell counts at any point during follow-up *versus* baseline were included in a multivariate analysis using a multiple linear regression model with a stepwise procedure. Multivariate analysis was considered significant when $P < 0.05$.

RESULTS

Patients' baseline characteristics

The patients' baseline characteristics are summarized in Table 1. The study population was composed preferentially of young males with moderate immunosuppression (median 255·5 CD4+ T cells/μl) and with a high viral load. Almost half the patients were injecting drug users and HCV-co-infected.

Thymic volume is associated independently with the magnitude of CD4+ T cell recovery both short- and long-term after HAART

The Pearson test was used to correlate the changes in CD4+ T cells during follow-up with baseline parameters. Several variables correlated with the changes in CD4+ T cells, although only thymic volume correlated at each time-point throughout follow-up (Table 2). Variables showing an univariate association ($P < 0.1$) with changes in CD4+ T cells at weeks 4, 12, 48 and 96 were included in the multivariate analysis as potentially independently associated variables (age and HCV-co-infection were also included). Multiple linear regression using a stepwise procedure was performed considering changes in CD4+ T cell counts as dependent variables at weeks 4, 12, 48 and 96 *versus* baseline. Only the baseline thymic volume was associated independently with changes in CD4+ T cells at weeks 4, 12, 48 and 96 *versus* baseline (Table 3).

Table 1. Patients' characteristics at baseline ($n = 54$)

Risk group	
IDU[a]	25 (46·3)
Homosexual[a]	15 (27·8)
Heterosexual[a]	14 (25·9)
Sex	
Male[a]	42 (77·8)
HCV-co-infection[a]	26 (48·1)
Age (years)[b]	37·0 [29·8–43·3]
Total CD4+ count (cells/μl)[b]	255·5 [44·0–439·5]
Total CD8+ count (cells/μl)[b]	756·0 [456·8–977·0]
Naive CD4+ count (cells/μl)[b,c]	60·2 [7·90–140·5]
Naive CD8+ count (cells/μl)[b,c]	45·1 [23·8–116·2]
Memory CD4+ count (cells/μl)[b,c]	122·8 [41·8–181·0]
Memory CD8+ count (cells/μl)[b,c]	248·5 [156·0–491·0]
IL-7 (pg/ml)[b,c]	26·7 [19·5–41·8]
Viral load (log_{10}copies/ml)[b]	4·9 [4·4–5·2]
Thymic volume (cm³)[b,d]	3·4 [1·8–6·2]
TREC-bearing cells (cells/μl) [b,c]	0·74 [0·2–2·5]

[a]Number of subjects (percentage); [b]median (interquartile range); [c]available in 42 patients; [d]available in 49 patients. IDU, injecting drug user; HCV, hepatitis C virus; TREC, T cell excision circle.

Table 2. Univariate analysis between changes in total CD4+ T cells and different baseline parameters

Variable	Week 4 ($n = 49$)		Week 12 ($n = 50$)		Week 48 ($n = 33$)		Week 96 ($n = 27$)	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Age	−0·032	0·826	−0·239	0·095	0·072	0·692	−0·850	0·674
Total CD4+ counts	0·180	0·216	0·094	0·517	−0·048	0·791	−0·109	0·589
Total CD8+ counts	−0·041	0·778	−0·260	0·068	−0·348	0·047	−0·342	0·081
Naive CD4+ T cells	0·267	0·105	0·307	0·051	0·046	0·802	0·045	0·831
Naive CD8+ T cells	0·226	0·173	0·358	0·022	−0·049	0·789	0·142	0·499
Memory CD4+ T cells	0·070	0·676	−0·013	0·934	−0·086	0·640	−0·125	0·552
Memory CD8+ T cells	−0·078	0·640	−0·430	0·005	−0·245	0·176	−0·239	0·250
IL-7 levels	−0·215	0·194	−0·060	0·710	−0·146	0·425	−0·107	0·609
Viral load	−0·083	0·585	−0·247	0·094	0·019	0·921	−0·301	0·144
Thymic volume	0·326	0·031	0·479	0·001	0·422	0·016	0·432	0·031
TREC+ bearing-cells	0·152	0·361	0·084	0·602	−0·174	0·342	0·175	0·402
X4/R5 ratio	−0·084	0·712	−0·105	0·632	0·081	0·774	0·489	0·107

Significant values are underlined ($P < 0.1$). At weeks 4,12, 24, 48 and 96 a Pearson test was used. TREC, T cell excision circle.

Table 3. Multivariate analysis between changes in total CD4$^+$ T cells and different baseline parameters

	Week 4 ($n = 49$)		Week 12 ($n = 50$)		Week 48 ($n = 33$)		Week 96 ($n = 27$)	
Variable	P	R^2	P	R^2	P	R^2	P	R^2
Age	0·445	–	0·095	–	0·226	–	0·848	–
Total CD8$^+$ count	–	–	0·078	–	0·079	–	0·256	–
Naive CD4$^+$ T cells	–	–	0·996	–	–	–	–	–
Naive CD8$^+$ T cells	–	–	0·519	–	–	–	–	–
Memory CD8$^+$ T cells	–	–	0·025	–	–	–	–	–
Thymic volume	0·008	0·159	<0·0001	0·332	0·016	0·150	0·031	0·151
Viral load	–	–	0·128	–	–	–	–	–
HCV-co-infection	0·624	–	0·359	–	0·351	–	0·863	–

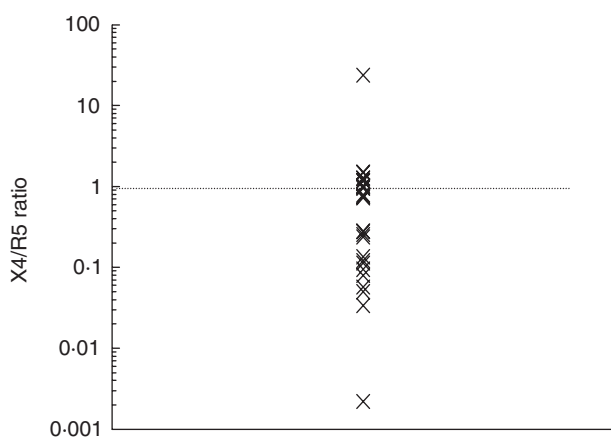Significant values are underlined. HCV, hepatitis C virus.

Fig. 1. X4/R5 co-receptor ratio distribution of patients' isolates. The median X4/R5 ratio was 0·73 [0·12–1·05]. Patients' isolates used both co-receptors and preferentially R5; only two virus isolates used almost exclusively one co-receptor.

Fig. 2. Correlation between X4/R5 ratio and total CD4$^+$ T-cells at baseline. Spearman's test was used.

Association of viral phenotype with baseline parameters and the magnitude of CD4$^+$ T cell recovery

Co-receptor usage was determined in 30 isolates at baseline. The median X4/R5 ratio was 0·73 [0·12–1·05], indicating that most isolates can use both co-receptors with a tendency to use CCR5 preferentially. Only two isolates used one of the co-receptors almost exclusively (Fig. 1). Viral phenotype was associated inversely with baseline CD4$^+$ T cells, i.e. total, naive and memory CD4$^+$ T cells ($P = 0·041$, $P = 0·013$, $P = 0·004$, respectively; Fig. 2). Nevertheless, the X4/R5 ratio was not associated with either the baseline thymic function-related markers or the rest of the parameters at baseline. Viral phenotype was not associated with the magnitude of CD4$^+$ T cell recovery at any week during follow-up (Table 2).

DISCUSSION

The results presented herein show for the first time that baseline thymic volume is associated independently with the magnitude of short- and long-term recovery of total CD4$^+$ T cells in HIV-infected adults after HAART. Unexpectedly, the viral phenotype
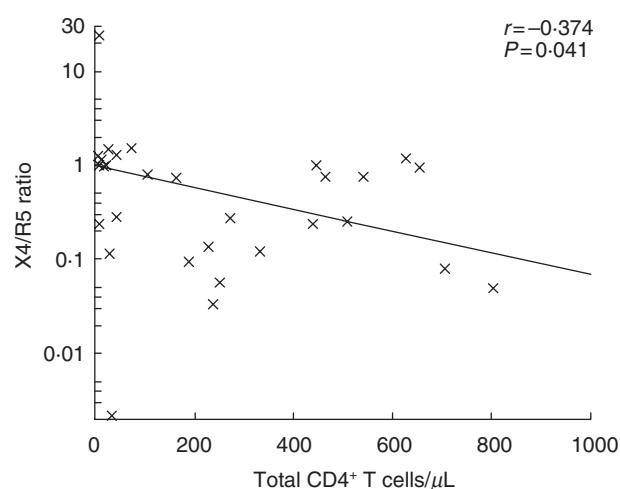
at baseline was not associated with either thymic function-related markers or the magnitude of CD4$^+$ T cell count increases.

In many T cell depletion scenarios, such as intensive chemotherapy and haematopoietic stem cell transplantation, age has been found to be associated with CD4$^+$ T cell recovery [19]. Among HIV-infected patients, younger subjects have also been associated with improved CD4$^+$ cell recovery, in terms of rapidity and intensity [3]. Moreover, reconstitution of naive T cells in adults has been described to be dependent on age [4]. All these studies suggest that the greater the thymic function patients have at baseline, the better the immunological response they will obtain after HAART. In fact, restoration of CD4$^+$ T cell numbers after HAART may be attributed partially to thymic function. Hence, increases in naive T cells, TREC-bearing cells, as well as increased thymic volume, after HAART support this affirmation [7]. Moreover, a small amount of thymic tissue after therapy has been associated with a poor CD4$^+$ T cell recovery after suppression of HIV replication under HAART [8].

We have reported recently that baseline thymic volume is the main factor and the best thymic function-related marker to predict the rapidity of CD4$^+$ T cell recovery after HAART [9,10]. Data presented herein support previous reported data among

children [20]. Moreover, this study provides evidence, for the first time, that baseline thymic volume is associated independently with the magnitude of short-term (4 and 12 weeks) and long-term (1 and 2 years) recovery of CD4+ T cell numbers in adults after HAART. At week 12, memory baseline CD8+ T cell counts were associated independently with CD4+ T cell gains. However, only thymic volume was a predictor of the studied event. These data are very interesting, as several reports have established that the initial increase in CD4+ T cell numbers after HAART is due exclusively to redistribution from peripheral lymphoid organs [21–23]. In this work, we report a clear association between thymic volume and the short-term increase in CD4+ T cell numbers, suggesting active thymic output in the first weeks after treatment. According to these results, we may speculate that thymic function is not a late event after HAART but is an early mechanism, as is redistribution from peripheral lymphoid organs. This affirmation agrees with our previous work that described an early increment in thymic volume, naive T cell numbers and TREC-bearing cells after HAART [7,13,24], and also supports data reported by others [25].

Several *in vitro* and animal model studies have shown that different HIV-1 strains can productively infect and destroy thymocytes [12,26–29]. The wide distribution of CXCR4 on human thymocytes [12,18,30–32] suggests that a predominance of X4-tropic isolates could induce a greater impact on thymopoiesis, impairing CD4+ T cell recovery after HAART. Nevertheless, in this work viral phenotype was not found to be associated with the magnitude of CD4+ T cell repopulation after HAART. Because thymic volume is associated independently with CD4+ cell recovery, it would have been expected that the predominance of an X4-phenotype would have been associated inversely with thymic volume and, hence, with lower CD4+ T cell levels. Surprisingly, co-receptor usage, expressed as X4/R5 ratio, was not correlated with any thymic function-related marker at baseline. However, it was associated inversely with total CD4+ T cell levels supporting the biological value of the X4/R5 ratio for expressing viral phenotype according to previous data using SI strains [11]. The absence of correlation between viral phenotype, the magnitude of CD4+ T cell repopulation during follow-up and thymic function-related markers at baseline could be explained by the predominance of either a dual-tropic virus or a mixture of X4 and R5 viruses (Fig. 1). Perhaps the absence of correlation is due to either the narrow spectrum of viral strains in these patients or the low number of isolates available. On the other hand, recent reports have shown that both X4 and R5 isolates can infect thymocytes and cause the same cytopathic effect, supporting our results [33]. In fact, co-receptor use is not the only determinant of HIV pathogenesis in the thymus; different cytokine levels could also influence thymus impairment [33].

Baseline IL-7 levels have been related to increments in thymic volume after HAART [13]. Nevertheless, we found no correlation between baseline IL-7 levels and CD4+ T cell increments during follow-up. This could be explained because some patients whose thymic volume did not increase during follow-up may have had a bigger thymic volume at baseline and so undergone a substantial CD4+ cell increment after treatment. We also did not find a correlation between HCV co-infection and CD4+ T cell recovery, supporting previous studies [34,35], although a great controversy is growing regarding this topic [36,37].

Nowadays, the thymus has been proposed to be an important source of T cell recovery after severe immunosuppression events, as occurs in HIV infection. Data presented herein show for the first time in HIV-infected adults on HAART that the baseline thymic volume is associated independently with the magnitude of CD4+ cell recovery, irrespective of viral phenotype. These results also show that this thymic function is maintained both short- and long-term after antiretroviral therapy providing further evidence for the important role of the thymus after HAART.

This work has provided important clinical implications since thymic volume may predict the magnitude of CD4+ T cell recovery not only in HIV infection but also in other T cell depletion scenarios, i.e. bone marrow transplantation and chemotherapy. The role of the thymus might explain why young subjects respond better to chemotherapy, as younger patients should have a bigger thymus. Moreover, immunodeficiency after bone marrow transplantation might be explained in part by the absence of T cell maturation in a more involuted thymus. According to this hypothesis, it would be relevant to know the thymic volume before beginning these therapeutic interventions. New strategies to improve and preserve thymic function are under research and will have important implications for this treatment and other T cell depletion situations.

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