Clin Exp Immunol 2004; **137**:570–577 doi:10.1111/j.1365-2249.2004.02583.x

HIV-infected children with moderate/severe immune-suppression: changes in the immune system after highly active antiretroviral therapy

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(Accepted for publication 28 June 2004)

SUMMARY

The objective of this study was to monitor the changes in the immune system of HIV-infected children with moderate or severe immunodeficiency after highly active antiretroviral therapy (HAART), comprising a follow-up study in 14 HIV-infected children on HAART at two time points separated approximately by 11·8 ± 0·4 (9·9; 15·4) months. HIV-infected children had significantly lower TREC levels than the control group, but 1 year after HAART the levels increased significantly ($P < 0·05$). In contrast, viral load (VL) did not change significantly. A positive correlation between T cell receptor excision circle (TREC) levels and both $CD4^+$ T cell absolute counts ($r = 0·558$; $P = 0·05$) and percentages ($r = 0·625$; $P = 0·030$) was found. During follow-up on HAART, the percentages and absolute counts of naive $CD4^+$ and $CD8^+$ T cell subsets were increased significantly ($P < 0·05$). $CD4^+$ $CD45RA^{hi+}$ $CD62L^+$, $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD38^+$ percentages, and the $CD8^+$ $CD45RA^{hi+}$ $CD62L^+$ counts reached similar values to the control group. Also, $CD8^+$ $CD45RO^+$ $CD38^+$ and $CD8^+$ $CD45RO^+$ percentages, and $CD8^+$ $CD45RO^+$ $CD38^+$ absolute counts ($P < 0·05$) decreased with respect to the baseline. Lymphoproliferative responses to pokeweed mitogen (PWM) before HAART were lower in HIV-infected children than the control group, but they recovered to normal levels after a year on HAART. Tumour necrosis factor (TNF)-α and interferon (IFN)-γ production by PHA-activated peripheral blood mononuclear cells (PBMC) was lower before HAART ($P < 0·001$), but reached similar levels to the control group 1 year after HAART. In HIV-infected children IgG, IgG_1 and IgG_3 plasma levels decreased significantly after HAART. The immune system reconstitution induced by HAART in HIV-infected children seems to be the consequence of decreased immune system activation and naive T cell reconstitution, mainly of thymic origin.

Keywords cytokine HAART HIV-infected children immunoglobulin T cell subsets TREC

INTRODUCTION

HIV infection leads to a severe depletion of $CD4^+$ T cells, phenotypic alterations of T cell subsets and a decline in thymic function which, in turn, produces a progressive impairment of functional immunity [1–3]. These alterations can be partially corrected after inhibition of HIV replication by highly active antiretroviral therapy (HAART) in HIV-infected children [4]. Thus, in HIV-infected children after severe depletion of T cells, HAART resulted in a consistent increase in $CD4^+$ T cell counts [5–7] and T cell receptor excision circle (TREC) levels in young HIV-infected patients [8–10], even in advanced stages of HIV disease [11], or in children with virological failure (virological non-responders) [8,12]. These findings indicate that recovery of thymic function is a pivotal event in immune reconstitution [12]. In children, the recovery of naive $CD4^+$ T cells occurs more rapidly if treatment is started at a younger age, but after 1 year of viral replication control, patients of all ages have achieved the same level of restoration. Markers of chronic activation in $CD8^+$ T cells persist after 1 year on HAART [13].

Moreover, HIV infection induces qualitative T cell abnormalities as a result of a decrease in lymphoproliferative response (LPR) to mitogens such as phytohaemagglutinin (PHA), pokeweed mitogen (PWM) or anti-CD3 plus anti-CD28 [2,14], as well as a desregulated production of cytokines [15], all of them more pronounced in advanced HIV disease. Thus, tumour necrosis factor-alpha (TNF-α) levels are elevated in serum and associated with elevated and immunoglobulin G (IgG) and IgA concentrations in HIV-infected children [16]. Several papers have described immune reconstitution in HIV-infected children, but each of them have focused on a limited set of characteristics of the

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immune system [12,13,17,18]. In this paper we underwent a more thorough analysis of the immune system, including changes in peripheral blood T cell subpopulations, thymic function, cellular response to mitogens, cytokine production in cellular culture and immunoglobulin plasma levels as a response to HAART during a follow-up study in HIV-infected children with moderate or severe immunodeficiency.

PATIENTS AND METHODS

Study population

Infants born to HIV-infected mothers were studied at the Immune-paediatric Unit of the General University Hospital 'Gregorio Marañón' in Madrid, and the 'Virgen del Rocío' Hospital in Sevilla, Spain. All infants were diagnosed as HIV-1-infected on the basis of positive results in both DNA polymerase chain reaction (PCR) and virus culture assays, as described previously [19]. The 14 children selected had $CD4^+$ T cells <25% at the entry of study. Drugs were prescribed by the attending paediatrician according to the Centre for Disease Control and Prevention (CDC) guidelines [20] upon obtaining written informed consent from parents or legal guardians. The study was conducted according to the Declaration of Helsinki and approved by the Ethical Committee of our hospital. Clinical classification was based on the 1994 revised guidelines of the CDC [21]. We also studied 26 age-matched uninfected children as a control group.

Quantification of TREC

Thymic function was studied by quantifying a specific marker of recently produced T cells by the thymus (TREC). The TREC values were determined in peripheral blood mononuclear cells (PBMC) by real-time quantitative PCR in a LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany), as described previously [1]. Samples were analysed in duplicate or triplicate, which never varied more than 10% from one to another, and the result was averaged. A β-globin control PCR was performed to verify that all the samples had the same DNA content. All samples from each child were measured in the same assay to avoid interassay variations.

Quantification of HIV-1 RNA

Blood samples were collected in EDTA tubes, separated within 4 h and plasma stored at −70°C. Viral load was measured in 200 μl plasma using an ultrasensitive quantitative reverse transcriptase PCR (RT-PCR) assay (Amplicor Monitor, Roche Diagnostic Systems, Brandenburg, NJ, USA).

Determination of antibody levels

Total IgG, IgA and IgM were determined by nephelometric method in a NR II (Dade Behrin, Munich, Germany).

Quantification of $CD4^+$ and $CD8^+$ T lymphocyte subsets

T lymphocyte subsets in peripheral blood were quantified by direct immunofluorescence. The monoclonal antibodies used for the analysis of T cell subsets were conjugated with fluorescein-isothiocyanate (FITC) (anti-IgG1, anti-HLA-DR, anti-CD45RA, anti-CD38), phycoerythrin (PE) (anti-IgG1, anti-CD45RO, anti-CD62L, anti-HLA-DR), and peridinin chloropyll protein (PerCP) (anti-CD4 and anti-CD8). The monoclonal antibodies were obtained from Becton-Dickinson (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA), except anti-CD38 (Immunotech, Marseille, France). T cell subsets were analysed by three-colour multi-parametric flow cytometry, in whole, lysed and washed blood [22]. Naive T cells were defined as $CD62L^+$ and $CD45RA^+$ bright T cells ($CD45RA^{hi+}$/$CD62L^+$) and $CD4^+$ $CD38^+$ 0. Memory cells were defined as $CD45RO^+$ T cells. Memory-activated cells were defined as $CD4^+$ $CD45RO^+$ $HLA\text{-}DR^+$ or $CD8^+$ $CD45RO^+$ $CD38^+$ 0. Effector $CD8^+$ T cells were defined as $CD8^+$ $CD57^+$ and $CD8^+$ $CD28^-$ $CD57^+$ 0.

Acquisition was performed in a FACScan (Becton-Dickinson, Immunocytometry Systems, San José, CA, USA) cytometer using Lysis II software (Becton-Dickinson) within 2 h of cell staining. The optimal parameters for acquisition (detector sensitivity, detector amplification and compensation) were determined periodically using calibrate (Becton-Dickinson) and the AUTOCOMP (Becton-Dickinson) program. Five thousand (5000) events were compiled using a collection gate for $CD4^+$ or $CD8^+$ T cells. The gate was defined using low SSC and high CD4 or CD8 expression [23,24]. Data were analysed using the Lysis II analysis program (Becton-Dickinson). Appropriate isotypic controls (IgG1-FITC; IgG1-PE) were used to evaluate the non-specific staining, which was deducted from the remaining results.

Proliferative response and cytokine production PBMC

Total PBMC cultured were seeded in 96-well flat-bottomed microtitre plates (2×10^5/200 μl per well) in RPMI-1640 medium supplemented with fetal calf serum (FCS) 10%. PBMC were stimulated with mitogenic stimulus: 1 μg/ml of PHA (Murex Biotech Limited, Dartford, UK) or 1 μg/ml of anti-CD3 monoclonal antibody (SPV3Tb) plus 1 μg/ml of anti-CD28 monoclonal antibody (L-293, Becton-Dickinson Immunocytometry Systems, San José, CA, USA) or 4 μg/ml of PWM (Sigma Chemical Co., St Louis, MI, USA). The assay was carried out in quadruplicate cultures. The cultures were incubated at 37°C and maintained in a humidified atmosphere containing 5% CO_2. At day 3 of mitogenic stimulus cultures and day 6 of antigenic stimulus cultures, 50% of the culture supernatants were harvested and stored at −70°C and the wells were replenished with an equivalent volume of fresh medium with 1 μCi [^{3}H]-thymidine (Amershan, Buckinghamshire, UK). Cell proliferation was estimated by incorporation of [^{3}H]-thymidine into DNA during the last 6 h of culture. The cells were harvested in glass fibre filters in an automatic cell harvester (Skatron, Norway) and radioactive incorporation was measured in a liquid scintillation spectrometer (1450 Microbeta Trilux, Wallac, Turku, Finland). The results were expressed as lymphocyte stimulation index (see statistical analysis).

Cytokine production was quantified in PBMC culture supernatants stimulated with PHA. We have used commercially available enzyme-linked immunosorbent assays (ELISAs), according to the manufacturer's instructions: interleukin (IL)-5 (Bio Source International, Camarillo, CA, USA), TNF-α and interferon (IFN)-γ (Bender Medical Systems Diagnostics, Vienna, Austria). Concentrations were assayed in duplicate.

Statistical analysis

In all analyses, viral load (VL) and TREC values were transformed to $\log_{10}$-scale in order to normalize their distribution. Cytokine production from PBMC stimulated with mitogens were corrected subtracting the cytokines values of unstimulated PBMC. PBMC proliferation is expressed as stimulation indexes (s.i.):

s.i. = proliferation of PBMC with mitogen or antigen/spontaneous proliferation of PBMC.

Differences in characteristics among groups of children were analysed using the non-parametric test (Mann–Whitney *U*-test). Statistical differences between basal and 1 year after on HAART were studied with Wilcoxon's non-parametric tests.

RESULTS

Characteristics of the HIV-infected children

We have carried out a follow-up study in 14 HIV-infected children on HAART with two points separated by approximately 1 year [mean 11·8 ± 0·4 (9·9; 15·4) months]. At study entry, all HIV-infected children had $CD4^+$ T cells <25%. All the HIV-infected children were treated previously with antirretroviral therapy with one or two nucleoside analogues of retro-transcriptase.

The immunological, virological and clinical characteristics of different groups at the entry to the study (baseline) are described in Table 1. Mean $CD4^+$ and $CD8^+$ T cells percentages values in peripheral blood and plasma VL of HIV-infected children before and after HAART are described in Table 2. After 1 year on HAART children had an increase of $CD4^+$ T cell absolute counts and percentages, although $CD4^+$ T cells and $CD8^+$ T cells never reached Control-group values.

Thymic function

To assess the effect of HAART in the thymus of HIV-infected children, we compared TREC levels at entry of study and after 1 year on HAART and compared with age-matched controls (Table 3). In this analysis, HIV-infected children showed a significant increase of TREC levels between the beginning and end of the study ($P < 0·05$). However, they did not reach values of TREC similar to those of the control groups.

Table 1. Individual characteristics of HIV-1-infected children before and after HAART

					Before HAART			After HAART		
No.	Age (years)	Sex	CDC	Antiretroviral treatment	CD4+ T cells (%)	CD4+ T cells/mm^3	Log_{10} VL (copies/ml)	CD4+ T cells (%)	CD4+ T cells/mm^3	Log_{10} VL (copies/ml)
1	8·35	Male	C	AZT + Indinavir + Abacavir	16·17	670	2·6	31·4	1382	3·1
2	5·63	Male	B	ddI + D4T + Nelfinavir	18·9	501	2·6	22·9	641	2·8
3	4·98	Male	A	ddI + D4T + Nelfinavir	21·33	579	3·74	28·8	1048	3
4	10·32	Female	C	ddI + D4T + Indinavir	20·8	599	3·57	26·4	661	2·8
5	7·24	Female	C	3TC + D4T + Nelfinavir	8·2	173	5·8	27·3	537	2·7
6	16·43	Female	B	3TC + D4T + Nelfinavir	12·8	233	3·53	10·3	317	2·5
7	12·41	Female	B	3TC + D4T + Nelfinavir	16	332	2·6	26·6	682	2·8
8	16·33	Female	A	3TC + D4T + Efavirenz	18·27	164	3·41	19	221	2·3
9	11·05	Female	A	3TC + D4T + Saquinavir + Indinavir	19	558	3·92	22·6	667	2·8
10	7·85	Male	C	3TC + D4T + Nelfinavir + Efavirenz	2·1	35	5·12	29·7	799	2·9
11	4·05	Male	C	3TC + D4T + Nevirapina + Nelfinavir	17·47	472	4·11	18·1	610	2·8
12	2·94	Female	C	D4T + Abacavir + Nelfinavir	16·4	475	4·72	30·8	993	3
13	5·29	Male	A	D4T + Nelfinavir + Saquinavir + Abacavir	23	827	2·6	26·5	1161	3·1
14	14	Female	C	D4T + Nelfinavir + Efavirenz	22·1	302	3·7	26·9	367	2·5

VL: viral load. CDCP: Centers for Disease Control and Prevention.

Table 2. Global characteristics of HIV-infected children after 1 year on HAART

Characteristics	Control	Basal	After 1 year on HAART	P^a	P^b	P^c
No. children	26	14	14			
Age (years)	10·2 ± 0·8 (3·6; 17·6)	9·1 ± 1·2 (2·9; 16·4)	10·1 ± 1·2 (3·9; 17·9)	0·481	0·958	<0·001
Lymphocyte subsets						
% $CD4^+$	41·9 ± 1·5 (29·5; 55·4)	16·6 ± 1·5 (2·1; 23)	24·8 ± 1·5 (10·3; 31·4)	<0·001	<0·001	0·003
% $CD8^+$	22·1 ± 0·9 (13·7; 28·1)	54·3 ± 3·3 (32·0; 73·1)	48·0 ± 2·7 (32·1; 68·3)	<0·001	<0·001	0·018
$CD4^+$/mm^3	1415 ± 164 (588; 3123)	423 ± 60 (35; 827)	720 ± 88 (221; 1382)	<0·001	0·001	0·001
$CD8^+$/mm^3	702 ± 74 (300; 1641)	1331 ± 152 (375; 2464)	1344 ± 143 (507; 2016)	0·001	0·001	0·873
Virological characteristics						
Log_{10} VL (copies/ml)	–	3·72 ± 0·26 (2·60; 5·80)	3·41 ± 0·26 (2·60; 4·80)	–	–	0·382
VL = 400 copies/ml	–	4	7	–	–	0·125

VL = viral load; AIDS = acquired immunodeficiency syndrome. Values are expressed as mean ± s.e.m. (min, max). *P*: Difference between groups (level of significance). [a]Difference between basal and healthy control. [b]Difference between HIV-children after 1 year on HAART and healthy-control. [c]Difference between basal and after 1 year on HAART.

Table 3. TREC values, and percentages and absolute counts of naive $CD4^+$ and $CD8^+$ T cells in HIV-infected children on HAART

	Control	Basal	After 1 year on HAART	P^a	P^b	P^c
TREC	19025 ± 3208	6101 ± 1644	9535 ± 1374	0·002	0·014	0·035
T cells (%)						
$CD4^+$ $CD45RA^{hi+}$ $CD62L^+$	59·1 ± 2·9	42·9 ± 4·6	60·2 ± 2·7	0·007	0·782	0·007
$CD4^+$ $CD45RA^+$	60·5 ± 2·6	44·4 ± 4·5	60·6 ± 2·7	0·005	0·983	0·007
$CD4^+$ $CD38^+$	75·4 ± 2·4	72·7 ± 2·4	80·2 ± 2·1	0·440	0·135	0·054
$CD8^+$ $CD45RA^{hi+}$ $CD62L^+$	70·2 ± 2·2	24·2 ± 4·4	35·6 ± 4·6	<0·001	<0·001	0·003
$CD8^+$ $CD45RA^+$	84·5 ± 1·5	48·4 ± 4·9	58·2 ± 4·2	<0·001	<0·001	0·033
T cells (cells/mm^3)						
$CD4^+$ $CD45RA^{hi+}$ $CD62L^+$	900 ± 133	201 ± 37	447 ± 64	<0·001	0·005	<0·001
$CD4^+$ $CD45RA^+$	914·5 ± 132·9	208·4 ± 38·5	450·9 ± 64·2	<0·001	0·011	<0·001
$CD4^+$ $CD38^+$	1118 ± 154	315 ± 48	581 ± 73	<0·001	0·004	0·001
$CD8^+$ $CD45RA^{hi+}$ $CD62L^+$	510 ± 60	264 ± 31	442 ± 55	0·001	0·411	0·002
$CD8^+$ $CD45RA^+$	603·1 ± 67·5	619·1 ± 93·5	776·8 ± 97·8	0·888	0·140	0·014

Control: age-matched HIV-negative healthy children. Values are expressed as mean ± s.e.m. *P*: Difference between groups (level of significance). [a]Difference between basal and healthy-control. [b]Difference between HIV-children after 1 year on HAART and healthy-control. [c]Difference between basal and after 1 year on HAART.

Table 4. Memory and effector $CD4^+$ and $CD8^+$ T cells in HIV-infected children on HAART

	Control	Basal	After 1 year on HAART	P^a	P^b	P^c
T cells (%)						
$CD4^+$ $CD45RO^+$ HLA-DR^+	2·7 ± 0·3	10·1 ± 2·8	5·7 ± 0·8	0·020	0·002	0·181
$CD4^+$ $CD45RO^+$	39·8 ± 3·2	51·1 ± 6·1	43·2 ± 4·9	0·117	0·579	0·158
$CD8^+$ $CD45RO^+$ $CD38^+$	12·1 ± 1·5	46·3 ± 4·7	35·7 ± 4·6	<0·001	<0·001	0·022
$CD8^+$ $CD45RO^+$	30·2 ± 1·9	68·7 ± 4·6	61·6 ± 4·7	<0·001	<0·001	0·008
$CD8^+$ $CD57^+$	10·5 ± 1·5	38·3 ± 4·3	34·1 ± 3·2	<0·001	<0·001	0·541
$CD8^+$ $CD28^-CD57^+$	8·82 ± 1·4	29·1 ± 4·2	28·4 ± 3·2	<0·001	<0·001	0·910
T cells (cells/mm^3)						
$CD4^+$ $CD45RO^+$ HLA-DR^+	33 ± 3	30 ± 5	39 ± 7	0·650	0·376	0·196
$CD4^+$ $CD45RO^+$	539 ± 81	182 ± 20	285 ± 34	<0·001	0·008	0·001
$CD8^+$ $CD45RO^+$ $CD38^+$	85 ± 13	652 ± 105	468 ± 74	<0·001	<0·001	0·025
$CD8^+$ $CD45RO^+$	200 ± 22	952 ± 139	837 ± 112	<0·001	<0·001	0·181
$CD8^+$ $CD57^+$	70 ± 14	549 ± 99	484 ± 78	<0·001	<0·001	0·804
$CD8^+$ $CD28^-CD57^+$	59 ± 13	429 ± 88	414 ± 75	<0·001	<0·001	1

Control: age-matched HIV-negative healthy children. Values are expressed as mean ± s.e.m. *P*: Difference between groups (level of significance). [a]Difference between basal and healthy-control. [b]Difference between HIV-children after 1 year on HAART. [c]Difference between basal and after 1 year on HAART.

During the follow-up study a positive correlation between the increase of TREC levels and an increase of $CD4^+$ T cell absolute counts ($r = 0{\cdot}558$; $P = 0{\cdot}05$), and percentages ($r = 0{\cdot}625$; $P = 0{\cdot}03$) at the end of the study were found. However, we did not find a correlation among changes in TREC values and $CD3^+$, $CD8^+$ T cells or VL at the end of the study.

$CD4^+$ and $CD8^+$ T cell subpopulations

HIV-infected children at the study entry had percentages and absolute counts of naive ($CD45RA^{hi+}$ $CD62L^+$) $CD4^+$ and $CD8^+$ T cells lower than the control group, except for $CD4^+$ $CD38^+$ percentages and $CD8^+$ $CD45RA^+$ counts (Table 3). After 1 year on HAART, the percentages and absolute counts of naive $CD4^+$ and $CD8^+$ T cells subsets were increased significantly ($P < 0{\cdot}05$). More interestingly, $CD4^+$ $CD45RA^+$, $CD4^+$ CD45RAhi+ $CD62L^+$ and $CD4^+$ $CD38^+$ percentages, and $CD8^+$ $CD45RA^{hi+}$ $CD62L^+$ counts reached after HAART similar values than the control group (Table 3).

At the study entry, activated memory T cells ($CD4^+$ $CD45RO^+$ HLA-DR^+ and CD8+ $CD45RO^+$ CD38+) were higher than the control group, but after 1 year on HAART we observed a significant decrease in the values of theses T cell subsets ($P < 0{\cdot}05$) with similar values to the control group. Memory $CD4^+$ T cells ($CD4^+$ $CD45RO^+$) increased and memory $CD8^+$ T cells ($CD8^+$ $CD45RO^+$) decreased at the end of study on HAART ($P < 0{\cdot}05$) (Table 4). We also analysed effector $CD8^+$ T cells ($CD8^+$ $CD57^+$, $CD8^+$ $CD28^-$ $CD57^+$) and we found that HIV-infected children had higher values of $CD8^+$ $CD57^+$ and $CD8^+$ $CD28^-$ $CD57^+$ than control group. However, these subsets did not decrease during follow-up (Table 4).

Responder and non-responder HIV-infected children to HAART

We have analysed the changes in TREC and T cells subsets in responder and non-responder HIV-children to HAART (children with VL = 400 copies/ml and VL > 400 copies/ml). We observed statistically significant differences only in $CD4^+$ T cells subsets (Table 5). Responder HIV-children to HAART (VL = 400 copies/ml) increased naive $CD4^+$ T cells and decreased memory T cells. However, we found statistically significant differences only in $CD4^+$ and $CD4^+$ HLA-DR^- $D38^+$ T cells percentages and $CD4^+$ $CD45RA^+$ $CD62L^+$ and $CD4^+$ HLA-DR^- $CD38^+$ T cells/mm^3 (Table 5). We did not observe statistically significant changes in TREC.

Functional activity of T cells

PBMC from HIV-infected children showed similar LPR to PHA and anti-$CD3^+$ anti-CD28 (measured as stimulation indexes) than the control group during the entire study. In contrast, lower LPR levels to PWM were recorded at study entry than after 1 year on HAART recovery to similar values to the control group (Table 6).

Cytokine production by PBMC stimulated with PHA was differentially affected. At the study entry, IL-5, TNF-α and IFN-γ production were lower than the control group. However, after 1 year on HAART, TNF-α and IFN-γ production showed a significant increase ($P < 0{\cdot}01$), reaching similar values to the control group (Table 6). In contrast, IL-5 production was not significantly affected by HAART.

Analysis of humoral immunity

The IgM, IgA, total IgG, IgG_1, IgG_2 and IgG_3 values were higher in HIV-infected children than the control group during follow-up. IgE and IgG_4 values were not statistically significantly different

Table 5. Summary of changes in TREC values, and percentages and absolute counts of $CD4^+$ T cell subsets in HIV-infected children after 1 year on HAART according to virological response

Characteristics	VL = 400 copies/ml	VL > 400 copies/ml	*P*
No. children	7	7	
Age (years)	1 (0·9; 1·0)	0·9 (0·8; 1·3)	0·710
TREC	4636 (–5022; 15719)	852 (376; 11610)	0·530
$CD4^+$ T cell subsets (%)			
$CD4^+$	10·6 (3·5; 27·6)	3·7 (–2·5; 14·5)	0·026
$CD4^+$ $CD45RA^+$ $CD62L^+$	20·9 (3·3; 63·1)	6·5 (–10; 17·3)	0·073
$CD4^+$ $CD45RO^+$	–6·3 (73·6; 1·9)	–0·3 (–9·9; 8·70)	0·097
$CD4^+$ HLA-$DR^-$$CD38^+$	3·1 (–5·0; 52·8)	–1·5 (–20·6; 6·1)	0·097
$CD4^+$ $CD38^+$	10·5 (– 1·8; 38·5)	2·8 (–10·2; 13·1)	0·105
$CD4^+$ T cell subsets (cells/mm^3)			
$CD4^+$	364 (62; 764)	109 (57; 518)	0·073
$CD4^+$ $CD45RA^+$ $CD62L^+$	392 (56; 600)	98 (12; 367)	0·026
$CD4^+$ $CD45RO^+$	144 (14; 265)	47 (35; 136)	0·053
$CD4^+$ HLA-$DR^-$$CD38^+$	325 (41; 694)	62 (–3·2; 378)	0·038
$CD4^+$ $CD38^+$	384 (38; 709)	88 (29; 431)	0·053

VL: viral load. TREC: TCR rearrangement excision circles. Values are expressed as median (min, max). *P*: level of significance.

Table 6. Proliferation and cytokine production by PBMC of HIV-1-infected children on HAART

	Controls	Basal	After 1 year on HAART	P^a	P^b	P^c
Mitogenic stimuli (s.i.)						
PHA	140 ± 14	104 ± 22	121 ± 25	0·180	0·505	0·746
PWM	33 ± 4	18 ± 6	26 ± 6	0·037	0·378	0·325
anti-$CD3^+$ anti-CD28	125 ± 10	99 ± 11	103 ± 16	0·101	0·272	0·715
Cytokine production(pg/ml)[a]						
IL-5	204 ± 61	53 ± 22	46 ± 27	0·029	0·025	0·767
TNF-α	1729 ± 324	586 ± 240	1497 ± 310	0·008	0·609	0·008
IFN-γ	3323 ± 593	1587 ± 592	4429 ± 1628	0·046	0·537	0·008

[a]After PHA stimulation. Control: age-matched HIV-negative healthy children. Values are expressed as mean ± s.e.m. s.i.: stimulation index. PHA: phytohaemagglutinin; PWM: pokeweed mitogen. *P*: Difference between groups (level of significance). [a]Difference between basal and healthy-control. [b]Difference between HIV-children after 1 year on HAART. [c]Difference between basal and after 1 year on HAART.

Table 7. Total immunoglobulin and IgG subclasses plasma levels on HIV-infected children after 1 year on HAART

	Controls	Basal	After 1 year on HAART	P^a	P^b	P^c
IgG[d]	237·9 ± 20·9	1172·8 ± 170·3	722·6 ± 68·5	<0·001	<0·001	0·041
IgA[d]	29·1 ± 4·9	174·9 ± 34·7	131·3 ± 31·9	0·001	0·009	0·086
IgM[d]	29·5 ± 3·7	113·4 ± 15·8	99·4 ± 18·8	<0·001	0·003	0·344
IgE	53·3 ± 19·3	285·0 ± 199·1	93·6 ± 58·7	0·280	0·537	0·257
IgG_1[e]	1688·6 ± 243·4	9899·2 ± 1338·6	6109·2 ± 693·6	<0·001	<0·001	0·020
IgG_2[e]	436·7 ± 53·1	1012·2 ± 142·3	869·5 ± 109·1	0·002	0·003	0·073
IgG_3[e]	141·7 ± 9·4	1082·5 ± 293·4	720·7 ± 163·3	0·008	0·005	0·050
IgG_4[e]	89·8 ± 22·5	104·4 ± 33·0	69·3 ± 22·6	0·719	0·526	0·143

Values expressed as median (min–max). Mann–Whitney *U*-test was used to compare between groups studied. [d]mg/dl, [e]:mg/l and [f]UI/ml. *P*: Difference between groups (level of significance). [a]Difference between basal and healthy-control. [b]Difference between HIV-children after 1 year on HAART. [c]Difference between basal and after 1 year on HAART.

between HIV-infected children and the control group. However, we observed a statistically significant decrease in total IgG, IgG_1 and IgG_3 levels ($P < 0·05$) in all HIV-infected children after 1 year on HAART. Moreover, there was a decrease of IgA and IgG_2 ($P < 0·1$) (Table 7).

DISCUSSION

HAART has dramatically modified the course of HIV infection [25,26] causing a drastic fall of VL in HIV-infected patients, enabling the recovery, at least partially, of the immune system [27,28]. Here, we have analysed the variations in the immune system of HIV-infected children that take place after HAART. T cell reconstitution may take place by either enhanced thymopoiesis, probably more operative in younger subjects, or by peripheral expansion of the already existing T cell pool, which more probably takes place in older subjects [29]. Thus, T cell reconstitution through thymopoiesis is associated with an increase in $CD45RA^+$ T cells, while peripheral expansion T cell-regeneration is associated with $CD45RO^+$ T cells [29–32]. The decrease in naive T cells has been shown to correlate with low $CD4^+$ T cell levels [33], whereas $CD4^+$ T cell recovery with HAART correlates with a naive T cells increase [6,32]. In our study, we found a $CD4^+$ T cells increase after HAART, which correlated positively with the increase in TREC values. HIV-infected children at entry study had lower percentages of naive $CD4^+$ and $CD8^+$ T cells than the control group, which could be ascribed to (a) preferential destruction of naive T cells infected by HIV as thymic precursors [34,35]; (b) to a defect in generation of new T cells lack as a consequence of thymic atrophy secondary to HIV infection [36]; and (c) to a continuous switch of naive to memory T cells due to chronic HIV infection [36]. However, after 1 year on HAART those 14 children had similar naive $CD8^+$ T cells to the control group, although the naive $CD4^+$ T cells were lower than the control group. Naive $CD4^+$ T cells could be infected by HIV and recently produced naive ($CD4^+$ $CD45RA^{hi+}$ $CD62L^+$) T cells switch continually to memory $CD4^+$ $CD45RO^+$ T cells, probably favoured by the chronic viral stimulation of T cells, because the children did not achieve undetectable VL levels. Regarding $CD8^+$ T cells, HAART induces a naive T cell increase associated with a consistent decrease in memory activated T cells ($CD8^+$ $CD45RO^+$ $CD38^+$). These results may be explained by the fact that naive and memory $CD8^+$ T cell pools are regulated independently, and each pool has its own niche. Thus, naive $CD8^+$ T cells survive by not dividing, while memory $CD8^+$ T cells survive by proliferating upon antigen recognition [37]. When thymus function is high and naive T cells emigrants are produced in large amounts, turnover is high in the peripheral naive T cell pool in order to make room for new thymic emigrants. As thymic output gradually dwindles with age, the naive T cells half-life becomes longer, thus ensuring the organism of a supply of naive T cells even when the thymic production has stopped [13]. We also observed a decrease of memory and memory-activated $CD8^+$ T cells during the follow-up study, although they did not reach similar values to the control group. This could indicate that an incomplete control of viral replication favours the transformation of naive T cells into memory $CD8^+$ T cells [38].

However, there is still controversy as to whether infection-associated changes in TREC values are due to alterations in thymic output of naive T cells or whether it is better explained by changes in the proportion of naive *versus* activated/proliferative T cells in the periphery [39,40]. Evidently, the natural pathway from naive $CD4^+$ T cells to memory does not occur and the number of naive increases after HAART. These naive T cells could accumulate, leading to an increase in the number of TREC. However, although the $CD8^+$ $CD45RO^+$ $CD38^+$ T cells/mm^3 decreased during follow-up, $CD8^+$ $CD45RO^+$, $CD8^+$ $CD57^+$ and $CD8^+$ $CD28^-$ $CD57^+$ T cells/mm^3 remain constant.

Whereas TREC and naive $CD4^+$ T cell levels were found to increase in all the study groups as after HAART, no significant differences in TREC levels were found in the separate group of HAART responders and non-responders. However, there were differences in naive $CD4^+$ T cells. This apparent discrepancy can be due to the different effects of viral replication in those two markers. TREC are circular DNA molecules that are not replicated during mitosis and are thus diluted with each round of cell division [41]. The lack of differences between responders and non-responders could indicate that the thymic function has not recovered completely in both groups. However, the

continuous switch of naive to memory T cells due to chronic HIV infection is stopped by HAART [36]. Moreover, memory T cells could revert to naive T cells in HIV responders with viral replication controlled [36], thus increasing the percentage of naive T cells.

On the other hand, HIV infection impairs the LPR to mitogens, antigens and alloantigens before $CD4^+$ T cell depletion takes place [2,42]. After HAART, those LPR return to normal values, further supporting that HIV replication itself is the immune dysfunction main cause [17,28,43]. In agreement with this, we have found an increase in the LPR of PBMC to PWM. Furthermore the depressed TNF-α and IFN-γ production to PHA reached similar values to the control group in children who respond to HAART, supporting a functional immune system reconstitution.

The helper T type 1 (Th1) function is presumed to be of key importance in host defence against HIV-1 and it is responsible for the production of different antibody isotypes [44]. In our study we have also found a decrease in IgG, IgG_1 and IgG_3 after 1 year on HAART, although it did not reach normal values. Previous *in vitro* studies have proposed various mechanisms to explain the hypergammaglobulinaemia and B cells activation in HIV infection, such as stimulation by HIV, HIV viral proteins, IL-6 or membrane-bound TNF-α on $CD4^+$ T cells [45–47]. Higher immunoglobulin values were found in HIV patients and they are used as prognostic markers of AIDS and survival [48,49]. The hypergammaglobulinaemia in HIV patients is usually polyclonal and comprises several Ig isotypes [50]. IgA levels were higher between patients with less complete virological suppression relative to patients with persistently undetectable plasma HIV-RNA [51]. A higher IgE production in HIV infection has been described [52–54], and has been related to low IFN-γ production, immunosuppression (<25% $CD4^+$ T cells), high VL and severe clinical symptoms [55,56]. In agreement with those studies, our children had low IFN-γ values and a tendency (not statistically significant) to have higher IgE than the control group at baseline. However, IFN-γ increased as IgE decreased after HAART.

In summary, the reconstitution of the immune system induced by HAART in HIV-infected children seems to be the consequence of a decrease in activation of naive T cell reconstitution, mainly of thymic origin. In spite of persistent viraemia, $CD4^+$ T cell increase is sustained by a continuous thymic output that compensates peripheral $CD4^+$ T cell depletion which might be slowed down by emerging viruses with reduced fitness. This immune system restoration supports the normalization of immune parameters (cytokine production, lymphoproliferative response and immunoglobulin levels in plasma).

ACKNOWLEDGEMENTS

We wish to thank to Dolores García Alonso and Consuelo Muñoz for their excellent technical assistance. This study received financial support from the Fundación para la Investigación y la Prevención del SIDA en España, FIPSE (grant no. 36365/02, 12456/03), Comunidad de Madrid (grant no. 08·5/0034/2001), the Red Temática Cooperativa de investigación en SIDA (grant no. RIS G03/173) from FIS, the Red Temática Cooperativa de Investigación en Genética Clínica y Molecular (grant no. RIG C03/07) from FIS, Bristol-Myers, SA Squibb and Plan Nacional de Salud (grant no. SAF 2003–09209). Salvador Resino is supported by a grant from FIS (grant no. CM0300038).

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