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Stimulated proliferative responses in vertically HIV-infected children on HAART correlate with clinical and immunological markers

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SUMMARY

The objective of the study was to investigate the relationship between various $CD4^+$ T cell subsets and the ability of peripheral blood mononuclear cells (PBMC) to proliferate to several stimuli in vertically human immunodeficiency virus type 1 (HIV-1)-infected children. We studied 29 HIV-1-infected children on highly active antiretroviral therapy (HAART) (median duration: 12·3 months). T cell subsets were determined by flow cytometry. Plasma viral load (VL) was quantified using a standardized molecular method. Proliferative responses were evaluated by [^{3}H]-thymidine incorporation. Decreased proliferative responses of PBMC to pokeweed mitogen (PWM) were found for HIV-1-infected children in Centers for Disease Control (CDC) clinical categories B and C when compared to the control group ($P < 0·05$). Similarly, children with ≤15% $CD4^+$ T cells showed a decrease in proliferative responses to PWM ($P < 0·01$), anti-CD3 + anti-CD28 ($P < 0·01$) and phytohaemagglutinin (PHA) ($P < 0·05$) with respect to the control group and to children with $CD4^+$ T cells ≥25%. Proliferative responses to PWM, anti-$CD3^+$, anti-CD28 and PHA had a statistically significant positive correlation with $CD3^+/mm^3$, $CD4^+/mm^3$, % CD4 T cells, CD4/CD8 ratio and the percentage of naive T cell subsets ($CD4^+CD45RO^-$ HLA-DR^-, $CD4^+$ $CD45RA^+$ $CD62L^+$, $CD4^+$ $CD45RA^+$), $CD4^+$ $CD62L^+$ and $CD4^+$ T cells co-expressing $CD38^+$ ($CD4^+$ HLA-DR^-CD38^+, $CD4^+$ $CD38^+$). Moreover, we found a negative correlation between PBMC proliferative responses and % CD8 T cells, memory, memory-activated and activated $CD4^+$ T cell subsets. Lower proliferative responses to PWM ($P < 0·01$) and PHA ($P < 0·01$) were associated with higher VL. Our data show that higher proliferative responses to PWM, anti-CD3 + anti-CD28 and PHA are associated with both non-activated and naive $CD4^+$ T cell subsets in HIV-1-infected children on HAART.

Keywords $CD4^+$ T cells children HIV-1 proliferative response viral load

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) causes a progressive breakdown of immunity that can be quantified by several different assays. $CD4^+$ T cell counts, which usually determine the stage of disease, and plasma viral load (VL), which reflects active ongoing viral replication, are used to predict disease progression [1,2]. The impairment of T cell function provides a separate measurement. Impairment occurs early in HIV-1 infection, when $CD4^+$ T cell counts are still within normal ranges, and decreases further as disease progresses [3,4]. The proliferative responses to mitogens, such as pokeweed (PWM) or phytohaemagglutinin (PHA), and to anti-CD3 plus anti-CD28 antibodies are sensitive prognostic markers for AIDS [4,5].

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Activation of the immune system is another major effect of HIV-1 infection. Activation occurs early and increases throughout the course of the infection [6]. The altered expression of lymphocyte surface antigens also reflects the dynamic interaction between the immune system and HIV-1. The surface expression of HLA-DR and CD38 is significantly increased and predicts the progression to AIDS in adults [4,7–11]. However, in children the $CD38^+$ marker is a maturation marker instead, because 75% of all $CD4^+$ T cells in children normally co-express CD38 [12]. Because $CD4^+$ T cells are central to proliferation and are a major target for HIV-1 infection, the phenotypical changes of these cells are of special interest. Furthermore, naive ($CD4^+$ $CD45RA^+$ $CD62L^+$) and memory ($CD4^+$ $CD45RO^+$) T cells differ in several aspects, including proliferative responses, and memory T cells are more sensitive to activation induced by cell death than naive T cells [13]. In addition, memory T cells respond better to recall antigens and exhibit much higher proliferative responses to anti-CD3, whereas naive T cells respond better to mitogenic stimuli such as

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PWM or PHA [14]. However, proliferative responses to alloantigens are similar in both memory and naive CD4$^+$ T cells [14].

On the other hand, highly active antiretroviral therapy (HAART) has had a tremendous impact on the course of HIV infection [15,16], resulting in important suppression of VL, reconstitution of CD4$^+$ T cells [17,18] and a reduction in opportunistic infections [16]. However, complete recovery of CD4$^+$ T cell counts and function is not usually achieved [19–21]. HAART in adults induces a reduction in activation markers on memory T cells [22,23], an increase in memory T cell numbers later on followed by a gradual and sustained increase in naive T cell numbers [19–21].

We have studied the relationship between various CD4$^+$ T cell subsets and the ability of peripheral blood mononuclear cells (PBMC) to proliferate to several stimuli in vertically HIV-1-infected children.

MATERIALS AND METHODS

Patients and control subjects

Twenty-nine vertically HIV-infected infants over 5 years of age were recruited in a cross-sectional study between July 1999 and July 2000 at the Paediatric Department of the General University Hospital 'Gregorio Marañón' in Madrid, Spain. All infants were diagnosed after birth as HIV-infected on the basis of positive results in both DNA-polymerase chain reaction (PCR) and virus culture assays, as described previously [24]. None of these 29 children were breastfed. All children were given HAART with two inhibitors of reverse transcriptase plus an inhibitor of protease (median duration: 12·3 months; range, 6·2–24 months; Table 1). We also studied 16 age-matched HIV-negative children as a control group. The inclusion criterion of an age greater than 5 years was chosen because younger children had major fluctuations in T cell subsets that could result in misinterpretation of data [25]. Clinical classification was based on the 1994 revised guidelines of the Centers for Disease Control (CDC) [26]. It is unresolved whether the CDC immunological category (representing the nadir of an individual subject's CD4 percentage or absolute count) is of relevance to the lymphoproliferative response (LPR). We worked on the hypothesis that the 'current immunological category' (based on the CD4$^+$ T cell percentage at the time of study) accounted for the LPR more than the CDC immunological category. Drugs were prescribed by the treating paediatrician according to CDC guidelines (Table 2) [27] 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.

Quantification of T cell subsets in peripheral blood

Total counts and percentages of CD4$^+$ and CD8$^+$ T cells were analysed by TRUCOUNT™ (Becton-Dickinson Immunocytometry Systems, San José, CA, USA) using whole blood, whereby cells were selected by means of an SSC gate against anti-CD45 [28] following the manufacturer's instructions. The acquisition was carried out in a FACSCalibur cytometer (Becton-Dickinson) using the CELLQuest (Becton-Dickinson) acquisition program immediately after cell staining. TRUCOUNT™ Control Beads were used routinely as a quality control.

The monoclonal antibodies used for the analysis of CD4$^+$ T cell subsets were conjugated with fluorescein-isothyocianate (FITC) (anti-IgG1, anti-HLA-DR, anti-CD45RA, anti-CD38), phycoerithrin (PE) (anti-IgG1, anti-CD45RO, anti-CD62L, anti-HLA-DR) and peridinin chlorophyl protein (PerCP) (anti-CD4). The monoclonal antibodies were obtained from Becton-Dickinson (Becton-Dickinson), except for anti-CD38 which was from Immunotech (Marseille, France). Three-colour phenotypical characterizations of lymphocytes were performed by flow cytometry in whole, lysed and washed blood [29]. Naive CD4$^+$ T cells were defined as CD62L$^+$ and CD45RA$^+$ bright T cells (CD4$^+$ CD45RA^{hi}/CD62L$^+$). Memory cells were defined as CD4$^+$ CD45RO$^+$ or CD4$^+$ CD45RA$^-$CD62L$^+$ T cells. Activated T cells were defined as CD4$^+$ HLA-DR$^+$ and CD4$^+$ HLA-DR$^+$ CD38$^+$ 0. Memory-activated CD4$^+$ were defined as CD4$^+$ CD45RO$^+$ HLA-DR$^+$. CD4$^+$ CD38$^+$ are mainly naive CD4$^+$ T cells [12]. Acquisition was performed in a FACScan (Becton-Dickinson) cytometer using the Lysis II acquisition program (Becton-Dickinson) within 2 h of cell staining. The optimal parameters for acquisition (detector sensitivity, detector amplification and compensation) were determined using Calibrate as a reagent (Becton-Dickinson) and the AutoComp (Becton-Dickinson) program periodically. Five thousand events were compiled using a collection gate for CD4$^+$ T cells. The gate was defined

Table 1. Summary of antiretroviral treatment of all HIV-infected children

	Time on HAART	Basal VL	ΔVL[a]	CDC clinical category			Current immunological category*		
				A	B	C	>25%	15–25%	<15%
2NRTI+Amprenavir	21.5	4.25	–0.25	1	1			1	1
2NRTI+Efavirenz	8.1	4.97	–2.67	1			1		
2NRTI+Indinavir	11.6	2.66	–0.27	1		3	1	2	1
2NRTI+Nelfinavir	5.1	4.43	–0.61	2	6	3	4	4	3
1NRTI+Nelfinavir+ Efavirenz	10.2	4.54	0.69			1			1
1NRTI+Nelfinavir+ Nevirapine	12.7	3.93	–0.11		1	4	4	1	
2NRTI+Ritonavir	20	2.48	–0.18			1	1		
2NRTI+Saquinavir+ Indinavir	22.4	4.74	–2.14	3			2	1	
2NRTI+Saquinavir+ Nelfinavir	11.9	5.73	–0.46		1				1

(a): Change in log viral load on HAART. VL: viral load. CDC: Centers for Disease Control. NRTI: nucleoside analogue HIV-1 reverse transcriptase inhibitor. HAART: highly active antiretroviral therapy. *CD4$^+$ T cell percentages.

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using the low side scatter (SSC) and high expression of CD4 [28,30]. 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. The absolute figures of the different analysed subsets were calculated by multiplying the percentage value by the total number of $CD4^+$ T cells.

Proliferative response of peripheral blood mononuclear cells
PBMC were isolated from blood by Ficoll-Paque density gradient centrifugation (Pharmacia, Uppsala, Sweden). Total PBMC were seeded in 96-well flat-bottom microtitre plates (2×10^5/100 μl per well). PBMC were stimulated with either 1 μg/ml of anti-CD3 (SPV3Tb, kindly provided by Dr J.E. de Vries, DNAX, Palo Alto, CA, USA) plus 1 μg/ml anti-CD28 (Becton-Dickinson), 1 μg/ml of phytohaemagglutinin (PHA) (Murex Biotech Limited, Dartford, UK) or 4 μg/ml of pokeweed (PWM) (Sigma Chemical Co., St Louis, MI, USA). PBMC were cultured for 72 h at 37°C in an atmosphere containing 5% CO_2, after which 1 mCi of [^{3}H]-thymidine was added and after a further 6 h the culture was stopped. The proliferative response was evaluated by incorporation of [^{3}H]-thymidine and cells were harvested in glass fibre filters using an automatic cell harvester (Skatron, Norway). Radioactive incorporation was measured in a liquid scintillation spectrometer (1450 Microbeta Trilux, Wallac, Turku, Finland). The assay was carried out in quadruplicate cultures.

Quantitative viral load assay
Blood samples were collected in EDTA tubes, the plasma was separated within 4 h and stored at –70°C. Plasma viral load was measured in 200 μl plasma using a quantitative reverse transcriptase PCR (RT-PCR) assay (Amplicor monitor, Roche Diagnostic Systems) [31].

Table 2. Summary of the characteristics of HIV-infected children and the healthy non-HIV control group at the time of the cross-sectional study.

Characteristics	Control group	HIV
No. HIV-1 children	13	29
Age (years)[a]	10.6 (5.3–18)	9.7 (5.3; 17.6)
CDC clinical category		
A	–	8 (27.6%)
B	–	8 (27.6%)
C or AIDS	–	13 (44.8%)
Current immunological category		
>25% $CD4^+$	–	13 (44.8%)
15–25% $CD4^+$	–	9 (31.0%)
<15% $CD4^+$	–	7 (24.1%)
Lymphocyte subsets[a]		
% $CD4^+$	40.5 (29.5; 55.4)	23.6 (2; 46.2)
% $CD8^+$	22.1 (13.7; 28.1)	46.6 (32; 86)
$CD4^+$/mm^3	1183 (588; 3123)	558 (86; 2510)
$CD8^+$/mm^3	654 (304; 1641)	1112 (339; 4276)
Virological characteristics[a]		
Log_{10} VL (copies/ml)	–	3.82 (1.3; 5.80)

VL = viral load; AIDS = Acquired immunodeficiency syndrome. [a]Values are expressed as median. (min, max). CDC: Centers for Disease Control.

Statistics
$CD4^+$ and $CD8^+$ counts are expressed as percentages. In all analyses, VL was transformed to log_{10}-scale in order to normalize distribution. The relationship between variables was investigated using a Spearman correlation coefficient. Values range between –1 (a perfect negative relationship) and +1 (a perfect positive relationship), 0 indicates no relationship. The Mann–Whitney *U*-test, a non-parametric analogue of the variance analysis of one factor, was used for comparisons between groups.

Proliferation of PBMC is expressed as stimulation index (SI):

$$s.i. = \frac{\text{proliferation of PBMC with mitogen}}{\text{spontaneous proliferation of PBMC}}.$$

This was chosen to normalize assays. No statistically significant differences of the spontaneous proliferative responses between HIV-1-infected children and healthy controls were found. These SI of HIV-infected children were standardized with respect to a control group of healthy children of similar age range. HIV-infected children were divided into three groups: (a) P1: children with a proliferative response to PWM similar to that of the control group (within 1 standard deviation (s.d.)); (b) P2: children with a lower proliferative response to PWM (lower than 1 s.d.) but similar cell proliferation to PHA than the control group (within 1 s.d.); (c) P3: children with lower proliferative responses to both PWM and PHA than the control group (lower than 1 s.d.). HIV-1-infected children were grouped in this way because it has been reported that there is a progressive loss of lymphoproliferative responses during HIV-1 infection, first PWM and later PHA [3]. Our goal was to describe the values of the different T cell subsets according to lymphoproliferative responses.

According to immunological classification based on the 1994 revised guidelines of the CDC, there was one child in immunological category 1 (IC-1), six children in IC-2 and 22 children in IC-3. Because this classification did not provide much information we divided the children into current immunological categories according to the $CD4^+$ T cell percentage at the time of study.

RESULTS

Proliferative responses of PBMC according to CDC clinical categories and viro-immunological markers
We performed a cross-sectional study in 29 vertically HIV-infected children (mean age 10·4 ± 0·6 years, range 5·2–17·6). Their clinical, immunological and virological characteristics at the time of the cross-sectional study are shown in Table 2. When we stratified these children according to clinical categories, we found a significant loss of PBMC proliferative responses to PWM in clinical categories B and C compared to the control group, but we did not find any differences in the proliferative responses to anti-CD3 + anti-CD28 or PHA (Fig. 1). We used anti-CD3 + anti-CD28 because it is a better stimulus than anti-CD3 alone. CD28 delivers a second signal to T cells that considerably enhances T cell reactivity *in vitro* (approximately eight- to 10-fold). Also, we showed lower variation coefficients for the double stimulation than reactivity to CD3 monoclonal antibody alone (median 5 *versus* 20, respectively). When we stratified children by current immunological category, children with $CD4^+$ T cells ≤15% showed significant decreases in proliferative responses to PWM, anti-$CD3^+$ anti-CD28 and PHA with respect to both the control group and children with $CD4^+$ T cells ≥25% (proliferative responses to PWM,

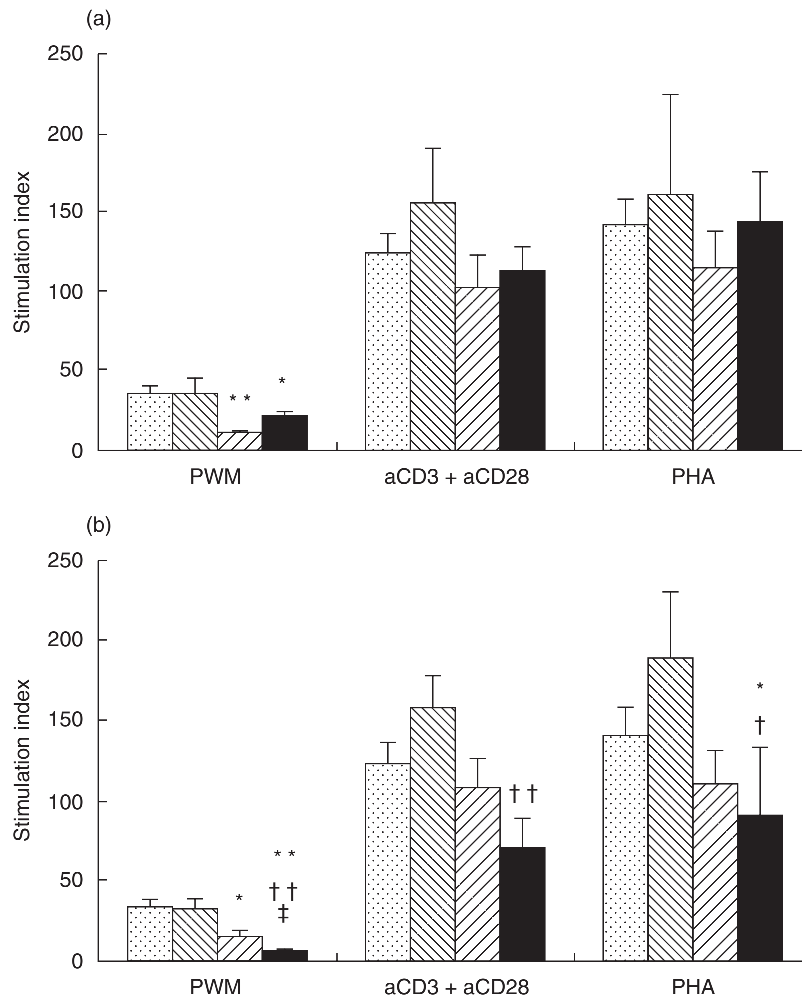

Fig. 1. Proliferative responses to PWM, PHA and anti-CD3 plus anti-CD28 in HIV-1 children and healthy controls. Values expressed as mean ± s.e.m. (min; max) and absolute number (%); CDC: Centers for Disease Control; CIC = current immunological category. aCD3 = anti-CD3; aCD28 = anti-CD28. Statistical differences from the healthy control group ($^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$). Statistical differences with HIV-infected children in clinical category B or immunological category 2 (†$P < 0{\cdot}05$; ††$P < 0{\cdot}01$). Statistical differences with HIV-infected children in clinical category C or immunological category 3 (‡$P < 0{\cdot}05$; ‡‡$P < 0{\cdot}01$). (a) ⬚, Control group; ▧, CDC-A; ▨, CDC-B; ■, CDC-C. (b) ⬚, Control group; ▧, CIC-1 (≥ 25% $CD4^+$); ▨, CIC-2 (15–25% $CD4^+$); ■, CIC-3 (≤ 15% $CD4^+$).

PHA and anti-CD3 plus anti-CD28 in HIV-1 children and healthy controls). Moreover, children with $CD4^+$ T cells 15–25% showed significantly lower proliferative responses than the control group, and significantly higher responses than the children with $CD4^+$ T cells ≤15% (Fig. 1). On the other hand, HIV-children with $CD4^+$ T cells ≤15% or clinical categories C showed higher values of VL than other HIV children ($P < 0{\cdot}05$).

A positive correlation between PBMC proliferative responses to PWM, anti-CD3 + anti-CD28 and PHA with $CD3^+$ T cells/mm^3, $CD4^+$ T cells/mm^3, % $CD4^+$ and CD4/CD8 ratio was found (Table 3). However, a negative correlation between % $CD8^+$ T cells and PBMC proliferative responses to PWM, anti-CD3 + anti-CD28 and PHA was found (Spearman's correlation analysis of $CD3^+$, $CD4^+$ and $CD8^+$ T cell percentages, and viral load with proliferative response). A negative correlation between VL and PBMC proliferative responses to PWM and PHA was also found (Table 3).

Correlation of PBMC proliferative responses with $CD4^+$ T cell subsets and plasma viral load

A positive correlation between PBMC proliferative responses to PWM, anti-CD3 + anti-CD28 and PHA and % $CD4^+$ T cells with naive $CD4^+$ T cells ($CD4^+$ $CD45RO^-$$HLA\text{-}DR^-$, $CD4^+$ $CD45RA^+$ $CD62L^+$, $CD4^+$ $CD45RA^+$), $CD4^+$ $CD62L^+$ was observed. The same trend was observed for the same subsets of cells co-expressing $CD38^+$ ($CD4^+$ $HLA\text{-}DR^-$$CD38^+$, $CD4^+$ $CD38^+$) (Table 4). Therefore, the higher the proliferative response, the higher the proportion of naive T cells.

Conversely, a negative correlation between PBMC proliferative responses to PWM, anti-CD3 + anti-CD28 and PHA and % $CD4^+$ T cells with memory ($CD4^+$ $CD45RO^+$, $CD4^+$ $CD45RO^+$ $HLA\text{-}DR^-$, $CD4^+$ $CD45RA^-$$CD62L^+$, $CD4^+$ $CD45RA^-$$CD62L^-$), memory-activated ($CD4^+$ $CD45RO^+$ $HLA\text{-}DR^+$) and activated ($CD4^+$ $HLA\text{-}DR^+$ $CD38^+$, $CD4^+$ $HLA\text{-}DR^+$ $CD38^-$, $CD4^+$ $HLA\text{-}DR^+$) $CD4^+$ T cells was found (Table 4).

A positive correlation between VL and activated ($CD4^+$ HLA-DR^+ $CD38^+$ and $CD4^+$ HLA-DR^+) and memory-activated ($CD4^+$ $CD45RO^+$ HLA-DR^+) $CD4^+$ T cells was also found (Table 4).

Correlation between PBMC proliferative responses to PWM and PHA and $CD4^+$ T cell subsets numbers

We rated the 29 HIV-infected children based on their proliferative responses to PWM and PHA into three groups (see Statistics). Differences among the three groups of HIV-infected children are shown in Fig. 2. A progressive increase of memory ($CD4^+$ $CD45RO^+$, $CD4^+$ $CD45RA^-$$CD62L^+$, $CD4^+$ $CD45RA^-$ $CD62L^-$), memory-activated and activated $CD4^+$ T cells ($CD4^+$ HLA-DR^+, $CD4^+$ HLA-DR^+ $CD38^+$, $CD4^+$ HLA-DR^+ $CD38^-$) was associated with the loss of PBMC proliferative responses to PWM and to PHA (Fig. 2). A progressive and parallel decrease in naive $CD4^+$ T cells ($CD4^+$ $CD45RA^+$ $CD62L^+$), $CD4^+$ $CD45RA^+$, $CD4^+$ $CD62L^+$, $CD4^+$ $CD38^+$ and $CD4^+$ HLA-$DR^-$$CD38^+$ T cells with the loss of proliferative responses to PWM and PHA in the P3 group was also found (Fig. 2).

Table 3. Spearman's correlation analysis of $CD3^+$, $CD4^+$ and $CD8^+$ T cell percentages and viral load with proliferative response of stimulated PBMC in all HIV-infected children on HAART

	PWM	aCD3 + aCD28	PHA
$CD3^+/mm^3$	0.38*	0.45*	0.47**
$CD4^+/mm^3$	0.78**	0.68**	0.69**
$CD8^+/mm^3$	0.03	0.15	0.26
% CD3	–0.12	–0.14	–0.08
% CD4	0.72**	0.57**	0.53**
% CD8	–0.69**	–0.48**	–0.38*
CD4/CD8	0.70**	0.56**	0.47*
Log_{10} VL (copies/ml)	–0.42*	–0.25	–0.40**

PHA= phytohaemagglutinin; PWM= pokeweed; aCD3= anti-CD3; aCD28= anti-CD28; VL= viral load. Level of significance (*$P < 0.05$; **$P < 0.01$).

DISCUSSION

Although several immune alterations have been identified during HIV-1 infection, only a few studies have examined their interrelationship in HIV-1-infected children on HAART. The impairment of the lymphoproliferative function in HIV-1 infection is particularly interesting because it may be observed earlier during infection than the overt decline of $CD4^+$ T cells and because such changes are reported to be related to disease progression. Furthermore, proliferative responses return to normal levels after the initiation of antiretroviral therapy [4], indicating that those proliferative alterations are induced by HIV-1 infection. In accordance with this, we found that HIV-children with $CD4^+$ T cells ≤15% or clinical category C had higher values of VL than other HIV children, due possibly to a poorer response to HAART. However, analyses of the proliferative capacity of T cells are rarely used for determining the HIV-1 disease stage or monitoring therapy, in part because these assays are more laborious than serological or flow cytometric ones, and must be performed with freshly obtained blood samples. In this study, we found that PWM appears to be a more sensitive stimulus for detecting the impairment of the lymphoproliferative response in HIV-1 infected children on HAART in the B and C clinical categories, in agreement with previous results in adults [4]. This may be explained, at least partially, because PHA acts via both CD2- and TCR/CD3-dependent pathways, while PWM involves primarily TCR/CD3-induced responses. Thus, our results may indicate that the proliferative defect associated with HIV-1 infection mainly alters the TCR/CD3-induced response.

HIV infection also induces a decrease in $CD4^+$ T cell numbers and concomitantly activates the immune system [9,32–34]. This

Table 4. Spearman correlation analysis of $CD4^+$ T cell percentages, viral load and proliferative responses with the different $CD4^+$ T cell subset percentages in all HIV-infected children on HAART included in the study

	PWM	aCD3 + aCD28	PHA	%$TCD4^+$	Log_{10} VL
$CD4^+CD45RO^+DR^+$	–0.84**	–0.70**	–0.72**	–0.73**	0.53**
$CD4^+CD45RO^-DR^+$	0.13	0.32	–0.04	0.12	0.32
$CD4^+CD45RO^+DR^-$	–0.64**	–0.57**	–0.45*	–0.57**	–0.05
$CD4^+CD45RO^-DR^-$	0.77**	0.65**	0.62**	0.70**	–0.22
$CD4^+CD45RO^+$	–0.76**	–0.66**	–0.61**	–0.68**	0.19
$CD4^+CD45RA^+CD62L^+$	0.83**	0.77**	0.70**	0.85**	–0.25
$CD4^+CD45RA^-CD62L^+$	–0.68**	–0.84**	–0.74**	–0.59**	0.28
$CD4^+CD45RA^+CD62L^-$	0.11	0.45*	0.06	0.07	0.21
$CD4^+CD45RA^-CD62L^-$	–0.70**	–0.57**	–0.46*	–0.82**	0.18
$CD4^+CD62L^+$	0.68**	0.51**	0.43*	0.79**	–0.20
$CD4^+CD45RA^+$	0.82**	0.77**	0.69**	0.85**	–0.25
$CD4^+DR^+CD38^+$	–0.68**	–0.50**	–0.66**	–0.50**	0.60**
$CD4^+DR^-CD38^+$	0.70**	0.59**	0.66**	0.86**	–0.31
$CD4^+DR^+CD38^-$	–0.70**	–0.59**	–0.62**	–0.79**	0.37
$CD4^+DR^-CD38^-$	–0.46*	–0.43*	–0.41*	–0.67**	–0.16
$CD4^+DR^+$	–0.79**	–0.62**	–0.71**	–0.67**	0.54**
$CD4^+CD38^+$	0.52**	0.49**	0.48**	0.73**	0.10

PHA= phytohaemagglutinin; PWM= pokeweed; aCD3= anti-CD3; aCD28= anti-CD28; VL= viral load. Level of significance (*$P < 0.05$; **$P < 0.01$).

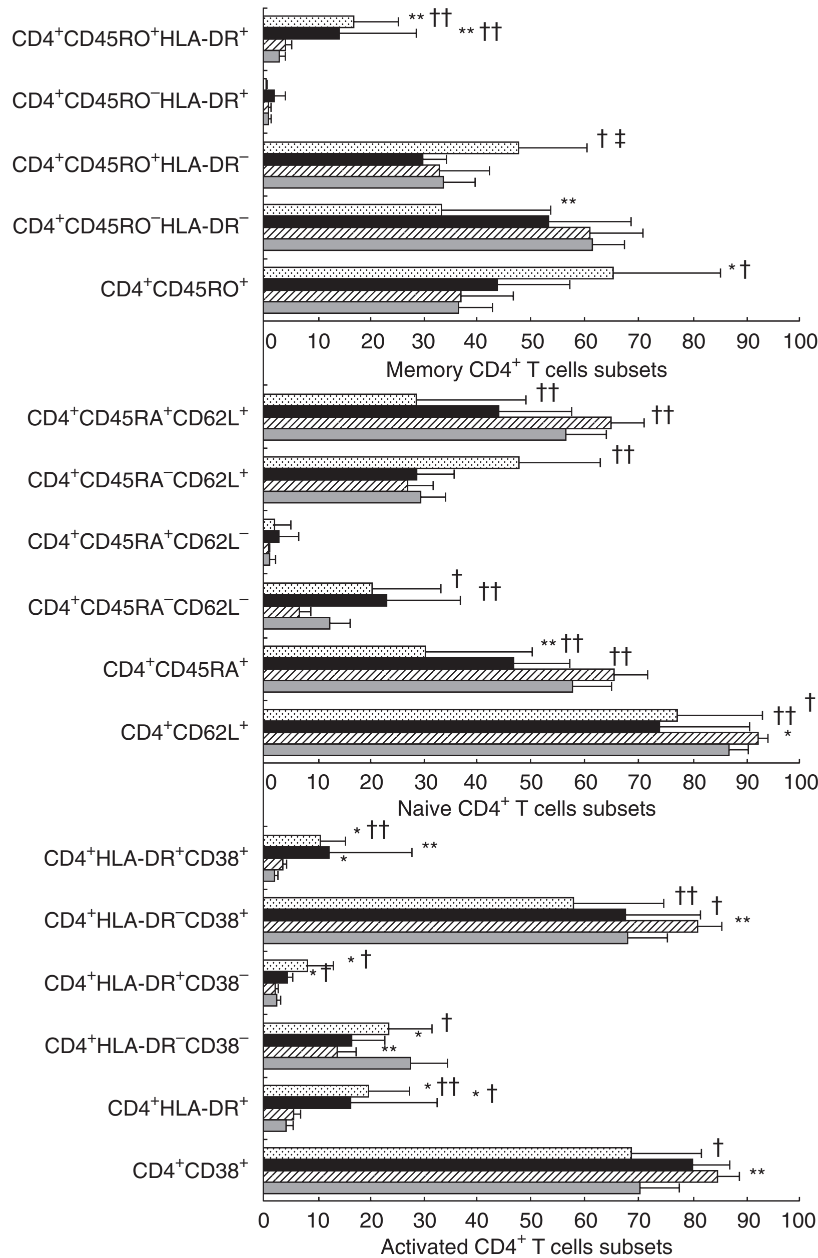

Fig. 2. Summary of the mean percentage values of activated, memory and naive $CD4^+$ T cell subsets stratified according to proliferative responses (see Statistics): (a) P1: children with a normal proliferative response to PWM; (b) P2: children with a lower proliferative response to PWM but normal cell proliferation to PHA; (c) P3: children with lower proliferative responses to both PWM and PHA. (*) Differences with control group (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$). Differences with P1 (†$P < 0{\cdot}05$; ††$P < 0{\cdot}01$). Differences with P2 (‡‡$P < 0{\cdot}01$). ⊡, P_3 = PWM and PHA lower than control; ■, P_2 = PWM lower than, and PHA similar to control; ▨, P_1 = PWM similar to control; ■, control non-HIV.

chronic activation of the immune system has been associated with an increase in expression of activation markers on T cells [9,33,34], i.e. CD38 and HLA-DR, in adults [35]. In our study, we observed a strong positive correlation between activated $CD4^+$ T cells and VL, and a negative correlation between activated T cells ($CD4^+$ $CD38^+$ HLA-DR^+, and $CD4^+$ HLA-DR^+) and % $CD4^+$ T cells and PBMC proliferation to PWM, anti-CD3 + anti-CD28 and PHA. These data suggest that in children, activated T cells ($CD4^+$ $CD38^+$ HLA-DR^+ and $CD4^+$ HLA-DR^+) are associated directly with VL [19]. The relationship between CD38 expression in $CD4^+$ T cells and VL showed a different pattern compared with that between HLA-DR and VL, due probably to the influence of age and cell type on the expression of CD38 [8,36–39]. Contrary to adults, $CD4^+$ $CD38^+$ is not a good disease progression marker in HIV-infected children [11,40–42]. Although we selected children over 5 years of age to minimize the effect of age on CD38 expression, and despite the fact that we found a positive correlation between $CD4^+$ $CD38^+$, % $CD4^+$ T cells and PBMC proliferative responses, we did not find any correlation between $CD4^+$ $CD38^+$ and VL. Therefore, our data indicate that $CD4^+$ $CD38^+$ T cells should not be used as a progression marker in HIV-infected children. However, the expression of CD38 on

CD4+ T cells seems to correlate with a more preserved immune system, as shown by our data rating HIV-infected children according to their loss of PBMC proliferative responses to PWM and PHA.

The loss of reactivity to mitogenic stimuli occurs with the progression of disease [3]. Naive and memory T cells differ in several aspects including cellular proliferative responses. Memory T cells respond better to recall antigens and present much higher proliferative responses to anti-CD3, whereas naive T cells respond better to mitogenic stimuli such as PWM or PHA [14]. We have found that the loss of PBMC proliferative responses to PWM, anti-CD3, anti-CD28 and PHA inversely correlated with memory and memory-activated T cells. Indeed, the P3 group (with diminished responses to both PWM and PHA) showed higher values of memory and memory/activated CD4+ T cells than the P1 and P2 groups.

HAART produces an important suppression of VL and an increase in CD4 T cell numbers [17,18], drastically reducing opportunistic infections [16]. However, the complete recovery of CD4+ T cell counts and function is not usually achieved [19–21]. The reduction of VL in HIV-infected subjects undergoing HAART therapy is accompanied by an increase of PBMC proliferative responses *in vitro* that is due mainly to CD4+ T cells [21]. In agreement with the above results, we found a strong positive correlation between proliferative responses and CD4+ cell numbers. We also found qualitative differences between PWM, anti-CD3, anti-CD28 and PHA-induced proliferation. Although this is a cross-sectional study, the recovery of the proliferative responses to PHA, but not to PWM, may be ascribed to HAART, due probably to its dependence of antigen presenting cells, suggesting that proliferative response to PWM is a more sensitive marker of immunological deterioration.

Naive T cells are crucial for the immune response and their loss have a severe impact in HIV infection [16]. We have observed a positive correlation between naive CD4+ T cells and immunological markers (PBMC proliferation and % CD4+) and a negative correlation with VL. Altogether, this indicates that a preserved immune system, with a high level of naive CD4+ T cells, maintains its capacity to respond to antigens and control viral replication. In agreement with this, the P3 group showed lower levels of naive CD4+ T cells than the P2 and P1 groups. By contrast, activated CD4+ T cells (high HLA-DR expression, co-expression of CD45RO and HLA-DR or CD38 and HLA-DR) are strong and earlier markers of immunological deterioration and viral replication.

In summary, our data suggest that the loss of proliferative responses to PWM is a good and reliable marker of clinical progression during HIV infection. Additionally, naive, memory and activated CD4+ T cell subset responses reflect the status of the immune system, as these subsets correlate differently with CD4+ T cell counts and VL. Moreover, memory and activated CD4+ T cells were associated with the loss of cell proliferation to mitogenic stimuli. Longitudinal studies should be carried out to highlight these correlations between the functional capacity of the immune system and the variations in the different T-cell subsets.

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