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Functional state of CD4+ and CD8+ T lymphocytes and their role in the slow progression of HIV infection in pediatric patients

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Abstract

Objective: To evaluate simultaneously the functional state of CD4+ and CD8+ T lymphocytes from Venezuelan HIV-1-infected pediatric patients.

Methods: Children were assigned to subgroups of rapid progressors (RPs) and slow progressors (SPs), based on clinical features. To determine the degree of CD4+ and CD8+ T-lymphocyte functionality, flow cytometry techniques were used, and diverse parameters of the functionality of these cells were characterized by *ex vivo* tests, such as expression of CD95/Fas and CD127, and frequency of apoptosis. In addition, we determined, in cultured peripheral blood mononuclear cells, HIV-specific proliferation and the production of interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), besides measuring intracellular IFN-γ in CD4+ T cells.

Results: Our results indicate that several molecular and cellular mechanisms of CD4+ and CD8+ T lymphocytes are deteriorated in RPs in comparison with SPs and controls. Indeed, both types of T lymphocytes from RPs exhibited an increased expression of CD95/Fas ($p < 0.01$), a significantly reduced expression of CD127 ($p < 0.01$), and an augmented frequency of apoptosis ($p < 0.01$). Furthermore, T cells from these patients displayed a diminished capacity of mitogen proliferation ($p < 0.05$), a reduced percentage of IFN-γ producing CD4+ T lymphocytes ($p < 0.05$), and a smaller capacity of IL-10, TNF-α and IFN-γ production ($p< 0.01$) in comparison with SP and control patients.

Conclusion: Our findings indicate that the decline of the normal T lymphocyte molecular and cellular responses is related to a rapid progression and a decreased resistance to HIV-1 infection in children.

J Pediatr (Rio J). 2012;88(2):161-8: HIV, progression, pediatric patients, immune function.

Introduction

HIV infection causes high morbidity and mortality in children, accounting for more than 20% of the deaths related to HIV-1 infection in the world.[1] A quarter of vertically infected children born in developed countries progress to serious disease or death in the first year of life without effective antiretroviral therapy, a proportion that rises to a half by 5 years of age.[2] A bimodal clinical behavior has been identified in children infected by vertical transmission. Thus, 10 to 30%

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of patients, which are named rapid progressors (RPs), start presenting AIDS symptoms in the first months of life (within 1 year), whereas a minority group of patients, denominated slow progressors (SPs), remains asymptomatic for several years and can even arrive to the scholar age.[3-5] Although some immunological and virological factors[6,7] associated with progression to disease have been identified, such as clinical symptoms (hepatomegaly, splenomegaly, and lymphadenopathy manifested early in life),[6,8] the cellular and molecular mechanisms underlying non-progression in HIV-1-infected children are not well understood.

The rate of progression to disease in HIV-1 infection varies considerably among individuals, due to a complex interplay between host's genetic and immunological factors and the pathogenic potential of the virus.[9,10] In children, we must consider several factors: relative immunological immaturity, HIV-1-mediated thymic destruction at a time of active thymopoiesis, and human leukocyte antigen class I sharing between mother and infant.[11-13] Few studies have investigated the functional state of CD4+ and CD8+ T lymphocytesin RP and SP patients[11] in order to further establish some links between clinical outcome and immunological features. The relative contribution of host's immunologic factors to delay progression to disease in Venezuelan children has not been examined precisely. Trying to explore these mechanisms, we decided to study a cohort of HIV-1-infected Venezuelan children, classified by clinical features into RP and SP groups, to characterize diverse parameters of CD4+ and CD8+ T cellsby *ex vivo* tests such as expression of CD95/Fas (death receptor) and CD127 (receptor of surviving signals), and frequency of apoptosis. In addition, we determined, in cultured peripheral blood mononuclear cells (PBMCs) of these patients, HIV-specific proliferation and the production of interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), besides measuring intracellular IFN-γ in CD4+ T cells. All these T-cell biological activities were compared against those observed in healthy children from the same hospital. These analyses might yield important information about HIV-1 pathogenesis and the role of protective immunity in HIV-1 infection in infants.

Materials and methods

Patients and study design

We enrolled 15 HIV-1-infected Venezuelan children from the Hospital J.M. de Los Ríos (Caracas, Venezuela), who have been infected *in uterus,* during labor or by breastfeeding from seropositive mothers. All patients were naive to highly active antiretroviral therapy at the beginning of the study. We used 5 mL of whole blood samples to perform all assays. This pediatric group was divided into six SP children and nine RP children, which were monitored in the health center. The SP group had a median of 25.5 months of age (19-36 months) and included patients with or without mild HIV-1 associated signs or symptoms (classified in stages N2 or A2 according to the classification of the Centers for Disease Control and Prevention, CDC). The RP group had a median of 12 months of age (3-26 months) and an onset of severe clinical manifestations (CDC category C) and/or significant immune suppression (CDC category 3) within the first year of life. Eleven HIV-negative infants from the Healthy Children Service of the same hospital participated as controls. The control group had a median of 19 months of age (8-36 months). The hospital's bioethical board approved this study and all children's parents gave a written informed consent.

Viral load measurement

Plasma HIV-1 RNA levels were measured with a commercial quantitative reverse transcriptase polymerase chain reaction kit (Amplicor HIV Monitor Test, Roche Molecular Systems, USA), with a mean detection limit of 2.3 log10 copies/mL.

Cell immunophenotyping

Circulating T cells and their subsets were determined in whole blood samples by standard fluorescence staining, followed by flow cytometry analysis, using fluorescent beads as an internal standard (Beckman Coulter, USA) and commercial monoclonal antibodies (MoAbs) from BD Biosciences, USA. The following MoAbs were used: anti-CD3-PerCP, anti-CD4-APC, anti-CD8-APC, anti-CD45RA-FITC, and anti-CD45RO-PE. Briefly, 100 µL of blood samples were incubated with conjugated MoAbs for 30 min on ice, in the dark, and washed. Red blood cells were lysed with FACS Lysing Solution (BD Biosciences, USA), and samples were immediately applied to a BD FACSCalibur flow cytometer(BD Biosciences, USA). Events were gated on a live lymphocyte region based on forward and side scatter parameters and analyzed using BD CellQuest software(BD Biosciences, USA).

Analysis of expression of Fas (CD95) and IL-7Rα (CD127)

To assess cell surface *ex vivo* expression of Fas (CD95) and IL-7Rα (CD127) in CD4+ and CD8+ T lymphocytes, a direct immunofluorescence staining was used with mouse MoAbs conjugated either with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), respectively. Briefly, 100 µL of whole blood was incubated with anti-CD3-PerCP, anti-CD4-APC or anti-CD8-APC, anti-CD95-FITC, and anti-CD127-PE for 15 min at room temperature in the dark. Later, samples were washed and red blood cells were lysed using FACS Lysing Solution (BD Biosciences, USA). Finally, cells were washed and analyzed immediately in a BD FACSCalibur flow cytometer (BD Biosciences, USA). Isotype-matched

irrelevant mouse antibodies were used as negative controls to determine background fluorescence. Antigen expression was quantified by flow cytometry as the percentage of positive cells in a given cell population.

Detection of apoptosis

Spontaneous apoptosis was measured in fresh whole blood samples. Cells were stained with FITC-conjugated annexin V and propidium iodide using the Annexin V Apoptosis Detection kit I (BD Pharmingen, USA) as recommended by the manufacturer. The extent of apoptosis was quantified by flow cytometry as the percentage of annexin V-positive cells in a given cell population. Both live and apoptotic cells were included in the analysis and cell debris was excluded.

In vitro *lymphocyte proliferation assays*

PBMCs were isolated from ethylenediaminetetraacetic acid-treated blood samples by density gradient centrifugation (Histopaque, Sigma, USA). These PBMCs were then grown in 96-well culture plates at a density of $1x10^5$ cells/well in 0.2 mL of RPMI-1640 (Gibco, Life Technology, USA), supplemented with 10% of heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycinsulfate (all from GIBCO, USA) in the presence of one of the following stimuli: 5 µg/mL of phytohaemagglutinin (PHA, Sigma, USA) and pooled HIV-1 envelope (HIV-1 Env) overlapping 20-mer peptides (each peptide at 1 µg/mL) (BD Biosciences, USA). Non-stimulated cells were used as controls. Lymphocyte proliferation was determined after 72 h of culture by the incorporation of bromodeoxyuridine (BrdU) (Sigma, USA), which was added in the last 4-5 h of culture. Cells were washed, fixed, permeabilized, and stained with FITC-conjugated anti-BrdU, anti-CD3-PerCP and anti-CD4-APC (BD Biosciences, USA) for 30 min. An additional wash step was performed, and cells were resuspended in 1% formaldehyde, applied to a BD FACSCalibur flow cytometer(BD Biosciences, USA), and analyzed using BD CellQuest software (BD Biosciences, USA).

Measurement of cytokine production

Cytokine production was measured in supernatants of PBMCs cultured for 72 h in the presence of PHA or pooled HIV-1 Env overlapping 20-mer peptides, as above described. Supernatants were collected and stored at -80 ºC until analysis. A Cytometric Bead Array kit (BD Biosciences, USA) was used, according to the manufacturer's instructions. Its methodology combines the principles of the "sandwich" immunoassays with the ability of flow cytometry to measure the characteristics of multiple particles, so we were able to measure the level of three human cytokines (IL-10, TNF-α, and IFN-γ) simultaneously in a single supernatant sample.

Intracellular cytokine staining

Production of IFN-γ as an HIV-1-specific CD4+ T cell response was quantified by intracellular cytokine staining. Briefly, 5×10^5 PBMCs were stimulated with either PHA (5 µg/mL) or pooled HIV-1 Env overlapping 20-mer peptides (each peptide at 1 µg/mL) (BD Biosciences, USA). After 72 h, 10 µg/mL brefeldin A (Sigma, USA) was added to prevent export of intracellular cytokines. Following cell wash, cells were fixed, permeabilized, and stained with FITC-conjugated anti-IFN-γ, anti-CD3-PerCP, and anti-CD4-APC (all from BD Biosciences, USA). Cells were further washed and resuspended in 1% formaldehyde. Samples were applied to a BD FACSCalibur flow cytometer (BD Biosciences, USA) and CD4+ T cells were gated for analysis using BD CellQuest software (BD Biosciences, USA).

Statistical analysis

Experimental data are given as mean ± standard deviation. Differences (p values) were evaluated using the two-tailed, nonparametric Mann-Whitney U test and the two-tailed Student's *t* test for normally distributed data. Differences were considered significant for $p < 0.05$.

Results

T cell subsets and viral load in HIV-infected children

T lymphocyte subpopulations and viral loads of the studied groups are summarized in Table 1. A significant decrease in absolute and percentage values of blood CD4+ T cell counts was shown in both groups of HIV-infected patients in comparison to healthy infants ($p < 0.01$). Although SP patients showed a tendency to have higher absolute values of CD4+ T lymphocytes compared with RP patients, the difference was not statistically significant. On the other hand, the percentage of CD8+ T cells of both groups of HIV patients presented higher values than those of the control group, but there were not statistically significant differences. Similarly, it was observed that there was not statistically significant difference in the absolute number of CD8+ T lymphocytes between SP and RP patients. In relation to the viral load, there was not significant difference between both groups of HIV patients, although values were higher in RP patients compared with SP patients (Table 1).

IL-7Rα (CD127) and Fas (CD95) expression in T cell subsets

We studied the *ex vivo* expression of CD95 in CD4+ and CD8+ T lymphocytes, and results are shown in Table 1. HIV-infected patients presented higher values with respect to those of the control group, and differences between RP patients and the control group were statistically significant.

Table 1 - Viral load, lymphocyte subpopulations, and expression of CD95 and CD127 in HIV-1-infected and healthy infants

	CD4+ T cells				CD8+ T cells				Viral load
Group	Cells/µL	%	CD95	CD127	Cells/µL	%	CD95	CD127	(copies/mL)
Control	2,966±1,583	57.3±23.7	18.5±11.4	33.6±10.2	1,033±567	31.7±11.8	9.6±10.0	22.6±20.1	–
SP	598±468*	24.4±17.3*	26.5±16.6	30.1±29.5	1,250±1,053	54.2±14.4	15.3±9.0	21.8±28.0	53,621±39,757
RP	465± 29*	24.8±14.8*	37.8±14.5*	11.6±11.0*	1,011±571	56.9±17.2	38.6±27.7*	9.1±4.3*	355,529±199,47

RP = rapid progressor; SP = slow progressor.
* $p < 0.01$ vs. control.
The absolute, percentage and viral load values are expressed as mean ± standard deviation. All experimental assays were performed once.
Differences (p values) were evaluated using the two-tailed, nonparametric Mann-Whitney Utest.

We evaluated T cell activities such as *ex vivo* expression percentages of IL-7Rαsubunit (CD127) in CD4+ and CD8+ T lymphocytes. It can be seen in Table 1 that receptor expression was significantly diminished in RP patients in relation to the control group, while SP patients presented similar values in comparison to the control group.

Spontaneous apoptosis in CD4+ and CD8+ T lymphocytes from HIV-infected and control patients

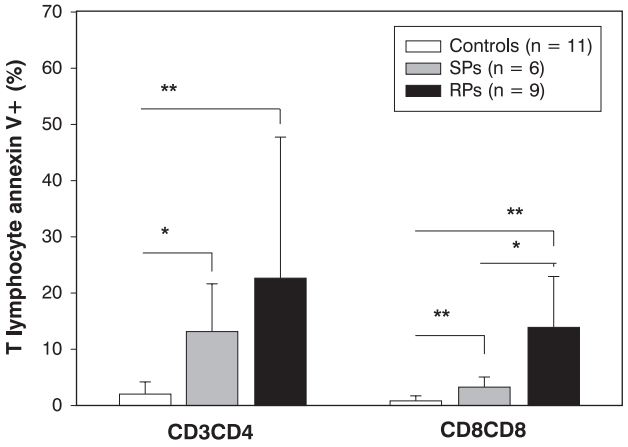
We measured the frequency of cell death associated with spontaneous apoptosis. Both groups of infected patients presented significantly higher values of spontaneous apoptosis of CD4+ and CD8+ T cells compared with the group of healthy children. Noticeably, RP patients presented the highest values of apoptotic cells, even with a higher population of CD8+ T cells compared to SP patients (Figure 1).

Mitogen and viral-specific lymphocyte proliferative responses

Although both groups of patients presented a significant increment in the proliferation of their CD4+ and CD8+ T lymphocytes in the presence of PHA compared to the non-stimulated condition, proliferation responses never reached values of the control group, which were significantly higher (Figure 2). Conversely, when PBMCs were cultured in the presence of viral-specific antigens, no relevant increment in the proliferation of neither CD4+ nor CD8+ T lymphocytes was observed in HIV-infected patients, presenting similar proliferation values than controls (Figures 2A and 2B).

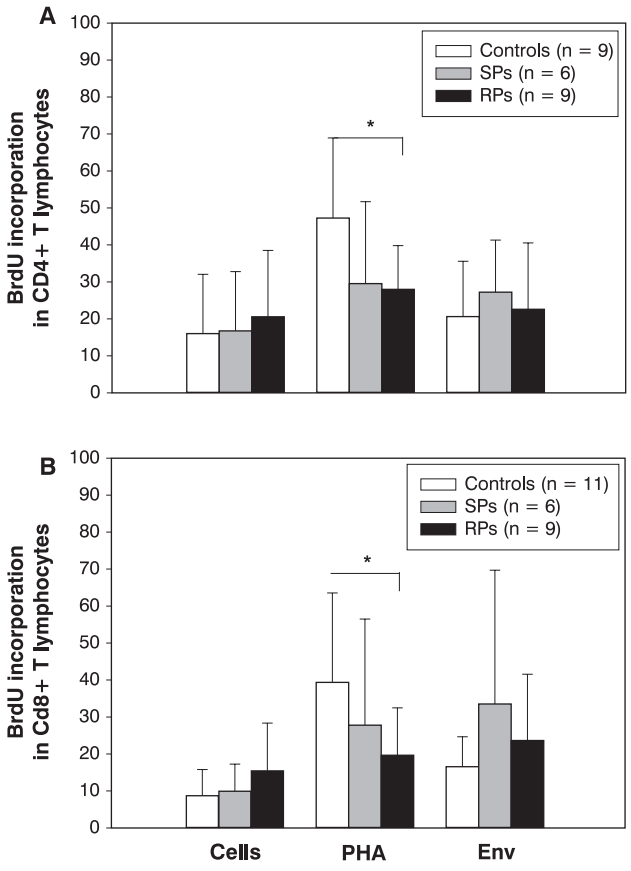
Production of cytokines in PBMCs from HIV patients

PBMCs from the two different HIV- infected groups (SPs and RPs) were cultured for 72 h in different conditions of stimulation. Production of IL-10, TNF-α and IFN-γ was quantified (Figure 3). Results indicated that spontaneous production of IL-10 (non-stimulated cells) was lower in both groups of HIV-infected patients compared to the control group, but differences were not significant. In the presence of PHA, IL-10 production was significantly lower in both groups of patients compared to the control group ($p < 0.01$), while IFN-γ and TNF-α production were significantly lower only in RP patients in comparison to controls ($p < 0.01$). In the presence of Env peptides, the production of these cytokines in both groups of patients was not modified, and their values were similar to those observed in control group and non-stimulated cells.



*p < 0.05 and **p < 0.01 vs. control.
RPs = rapid progressors; SPs = slow progressors.

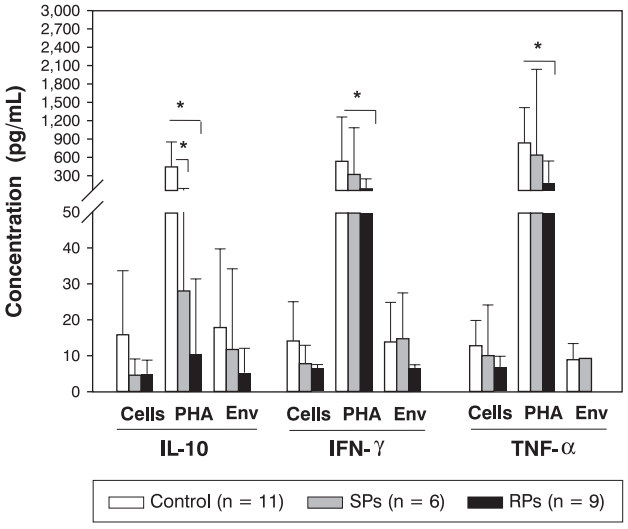
Figure 1 - Spontaneous *ex vivo* apoptosis inCD4+ and CD8+ T cells. Data show the percentage of annexin V^+/PI^- (early apoptotic) cells within gated CD4+ and CD8+ lymphocyte populations. Each value is the mean ± standard deviation. All experimental assays were performed twice in each sample



* p < 0.05 vs.non-stimulated cells (basal activity).

BrdU = bromodeoxyuridine; PBMCs = peripheral blood mononuclear cells; PHA = phytohaemagglutinin; RPs = rapid progressors; SPs = slow progressors.

Figure 2 - Lymphocyte proliferation. PBMCs were cultured for 72 h in the presence of PHA, viral antigens (Env) or medium alone (non-stimulated cells). Proliferation of CD4+ (A) and CD8+ (B)T lymphocytes was measured as the percentage of BrdU incorporation. Each value is the mean ± standard deviation. All proliferation assays were carried out by triplicate



*p < 0.01 vs. non-stimulated cells.

IFN-γ = interferon gamma; IL-10 = interleukin-10; PBMCs = peripheral blood mononuclear cells; PHA = phytohaemagglutinin; RPs = rapid progressors; SPs = slow progressors; TNF-α = tumor necrosis factor alpha.

Figure 3 - Production of cytokines (IL-10, TNF-α, and IFN-γ) (pg/mL) from PBMCs isolated from HIV-infected and controls infants. The quantification of cytokines was performed in supernatants of cultured PBMCs for 72 h in the presence of PHA, viral antigens (Env), or medium alone (non-stimulated cells). Each value is the mean ± standard deviation. All cytokine tests were performed by triplicate

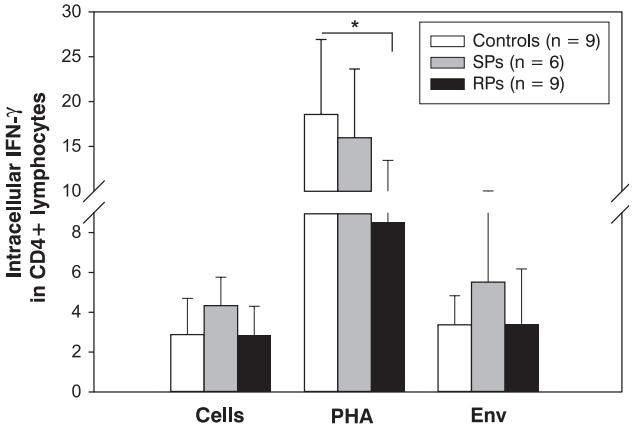
IFN-γ production in CD4+ T from control and HIV-infected children

CD4+ T lymphocytes are the main source of production of IFN-γ, which is one of the most important antiviral cytokines in HIV-1 infection. We evaluated *in vitro* capacity of production of this cytokine by CD4+ T cells in pediatric patients, measured at the intracellular compartment. Results are shown in Figure 4. It was observed that, in cells cultured in the presence of PHA, the production of this cytokine was significantly increased in all studied groups. However, percentages of IFN-γ-producing CD4+ T cells in both groups of HIV-infected patients were lower than those of the control group, being significant different in RPs ($p < 0.01$). The presence of the viral antigen (Env) did not modify the production of this cytokine in all studied groups, showing similar values to those in the absence of antigenic stimuli. However, SP patients displayed a tendency to higher values in comparison with controls.

Discussion

Our research hypothesis is that T cell functionality is relevant to the progression of vertically HIV-infected children, which may explain the distinctive clinical features described between RP and SP patients. Trying to address this hypothesis, we performed a study in which some of the CD4+ and CD8+ lymphocyte biological responses were evaluated simultaneously by means of *ex vivo* and *in vitro* analysis using PHA and viral antigens (Env) to assess the functionality of Tcells from Venezuelan pediatric HIV-infected patients with different clinical status, grouped as RPs and SPs, and to compare these biological activities with those from the T cells of Venezuelan healthy children.

Our results show that RP patients presented greater loss of blood CD4+ T lymphocytes and lower levels of CD8+ T lymphocytes in comparison to SP patients. These changes are consequences of HIV infection,[14,15] which increases the susceptibility of activated T cells to Fas/Fas ligand-mediated apoptosis,[16] an event correlated with progression to disease



* p < 0.05 vs. non-stimulated cells.

IFN-γ = interferon gamma; PHA = phytohaemagglutinin; RPs = rapid progressors; SPs = slow progressors.

Figure 4 - Intracellular cytokine staining of CD4+ T lymphocytes. The intracellular IFN-γ was quantified in CD4+ T lymphocytes following stimulation for 72 h with PHA, viral antigens (Env), or medium alone (non-stimulated cells). Each value is the mean ± standard deviation. All cytokine staining was executed by triplicate

as described.[17] Interestingly, RP patients displayed a greater proportion of apoptotic CD4+ and CD8+ T cells in comparison to the group of SP patients.

To evaluate T cell biological activities, the expression of Fas/CD95 is a relevant apoptosis parameter. Thus, the *ex vivo* expression of Fas/CD95 observed in CD4+ and CD8+ T lymphocytes from our RP patients was higher than that from SP patients. Interestingly, the viral load in both groups of HIVpatients was different, being higher in RP compared to SP patients. It is possible that the lower expression of CD95 in both types of T lymphocytes in SP patients was due to their lower viral load, and consequently displays minor frequency of apoptosis in these cells, which has been described in HIV-infected adults.[18]

IL-7 is an important cytokine for an efficient development and maintenance of T lymphocyte subpopulations. This cytokine binds to the alpha subunit of its receptor (IL-7Rα), located at the surface of T and B lymphocytes and natural killer cells.[19,20] We demonstrated that IL-7Rα expression in both CD4+ and CD8+ T lymphocytes is significantly reduced in the RP group, whereas the SP group presented similar values than the control group. This experimental finding may suggest that the T cells from SP patients have a greater probability to survive, and so these patients are able to maintain their cellular immunity more efficiently than RP patients. To support this assumption, higher levels of CD4+ and CD8+ T lymphocytes and lower frequency of apoptosis in these lymphocytes were found in SP patients. In this sense, our results are consistent with those of previous studies, which show that expression of IL-7Rα is inversely correlated with immune activation and apoptosis, and positively correlated with CD4+ T-cell counts in HIV-infected patients.[21] The loss of IL-7Rα expression induced by immune activation may diminish Bcl-2 and Bcl-xl production, rendering cells more susceptible to apoptosis.[22] Our data support the assumption that a lower expression of IL-7Rα may be related with a higher frequency of apoptosis in these T cells.

Going deeply into more molecular mechanisms, we studied several effector mechanisms of CD4+ and CD8+ T lymphocytes, such as the production of IL-10, TNF-α and IFN-γ by PBMCs, and the proliferative responses of these T lymphocytes to mitogens or viral peptides. According to our analysis, RP patients exhibited a greater deterioration of the functional capacity of CD4+ and CD8+ T cells compared to SP patients. Indeed, we found in RP patients a significant partial loss of PHA proliferative response of CD4+ and CD8+ T lymphocytes, a lower production of IL-10, TNF-α and IFN-γ by PBMCs, and a diminished percentage of IFN-γ producing CD4+ T cells, measured by intracellular cytokine staining. The greater loss of capacity of T cells to proliferate in the presence of PHA in RP patients coincide with results obtained previously showing that non-progressors adults have a greater capacity of T cell proliferation compared to RPs.[23] Interestingly, all these effector responses were completely lost in both groups of patients when their PBMCs were cultured in the presence of the viral antigen (Env). This lack of response could be explained by the inhibitor effect of viral proteins and/or by the loss of HIV-specific clones during the progression of infection,[24,25] or by the fact that the viral infection affected T cell lines at thymus and destroyed the ability to produce anti-HIV clones as described.[11-13] This experimental finding requires further investigation to identify the molecular mechanisms responsible for this lack of responses of HIV-infected children to viral antigen (Env).

Classically, it has been established that, during HIV infection, a change in the Th1/Th2 immunological responses occurs. Th1 response (generally an antiviral response) diminishes as the disease progresses, and Th2 response (associated with a smaller antiviral protection) increases, compromising protection in the HIV-infected patients.[26] Thus, the lower production of IL-10, TNF-α and IFN-γ found in our RP patients may significantly compromise their T cell immunity, since it is well known that IL-10 can induce recruitment, cytotoxic activity and proliferation of CD8+ T cells[27] and that TNF-α and IFN-γ are also required to generate and maintain antiviral responses as described for the CD8+ cytotoxic T lymphocytes, which depend on the presence of these cytokines to generate their effector mechanisms.[28] Similar findings associated with this Th1/Th2 imbalance were described in RP pediatric patients, which may significantly compromise their T cell immunity. Finally, similar findings, such as a gradual loss of IFN-γ expression

in HIV-specific CD8+ T cells, were observed in adults progressing to AIDS,[29] and recent studies[30] indicate that HIV-1 patients having a better management of the disease (known as long-term non-progressors) present significantly increased CD8+ Tcell populations compared to RPs.

In summary, we have found that the functional status of CD4+ and CD8+ T cells are distinct among pediatric HIV-infected Venezuelan patients. Thus, HIV-infected children with rapid progression to disease (RPs) presented more compromised functional immunity than infants whose disease progresses slowly (SPs). The identification of molecular and cellular mechanisms that contribute to the maintenance of protective immune responses is an important hallmark for therapeutic and vaccine development.

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