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Running title: Immunity, HIV RNA and DNA levels

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Abstract

Background: Clinical studies support biologically independent roles of cell-free HIV particles and HIV-infected cells in disease progression. The associations between the level of infected cells and immune markers have been poorly studied, particularly in perinatally infected children.

Objective: We tested the hypothesis that independent roles of cell-free virus and infected cells in HIV pathogenesis should be revealed by different associations between each of them and specific immune markers.

Methods: Levels of HIV RNA and DNA, HIV-specific CD8 T lymphocytes, activated and naïve/memory T lymphocytes were determined in 44 untreated HIV-1-infected children. Pearson's partial correlation coefficients were used to assess associations between the variables.

Results: Here we provide new information, by showing a direct correlation between the percentages of $CD4^+HLA\text{-}DR^+$ lymphocytes and HIV DNA levels. Furthermore, higher HIV-specific CD8 T lymphocyte frequencies were associated with lower HIV DNA levels. In contrast, $CD8^+38^+$ lymphocytes and memory CD4 lymphocytes were correlated only to the HIV RNA level. All correlations were independent of age and CD4 depletion.

Conclusion: Several immune markers were correlated to either the HIV RNA or the HIV DNA level, but never to both of them, supporting the concept that cell-free virus and infected cells play different roles in HIV-1 immunopathogenesis.

Keywords: Perinatal HIV infection; HIV DNA; HIV RNA ; HIV-specific T lymphocytes; immune activation.

Introduction

In infected patients, HIV is present not only as cell-free virions, but also as a component of latently or productively infected cells. In clinical practice, the quantification of cell-free virions in plasma is the gold standard to measure HIV replication. However, the level of cell-associated HIV DNA is another clinically relevant marker, as it is a significant predictor of disease progression [1-5], mother-to-child and heterosexual transmission of the virus [6-10], and treatment efficacy [11-14]. Importantly, in these studies, cell-associated HIV DNA levels had predictive values that were independent of cell-free HIV RNA levels and CD4 cell counts. Thus, several clinical studies support the concept of biologically significant and independent roles for infected cells and cell-free virus in the pathogenesis of the HIV-1 disease.

Biological mechanisms of HIV replication, HIV immunomodulatory properties, and HIV-specific immune responses involve either cell-free virus or infected cells. Three mechanisms of viral spread have been described for HIV: infection of permissive cells by cell-free virions; capture of virions by dendritic cells that transmit infectious virus to a permissive target cell without being productively infected themselves (infection in trans); direct cell-to-cell transmission from an HIV-infected cell to an uninfected target cell without release of virions into the extracellular milieu [15,16]. Multiple mechanisms mediate the immunomodulatory effects of HIV. These involve cell-free viral particles, functional alterations of productively infected cells, and the release of viral proteins and cellular products into the extracellular environment that will induce activation and/or death of bystander cells [17-20]. Finally, the adaptive immune response reduces HIV replication and spread by several mechanisms that block infection by cell-free particles (neutralizing antibodies), destroy productively infected cells (antibody-dependent-mediated cytotoxicity and TcR mediated cytotoxicity), or reduce the production of HIV particles by infected cells through noncytotoxic mechanisms [21,22].

HIV-infected children have a higher level of HIV replication and a higher thymic output than subjects infected during adulthood. Two studies concerning infected children reported associations between an increased HIV DNA level in PBMCs and a higher thymic output that were not observed in adults [23-26]. Information concerning associations between several other immune parameters and cell-associated viremia is lacking for infected children, and data pertaining to adults are still scarce [23,24,27,28]. Furthermore, the relative contributions of cell-free and infected cells *in vivo* have not been established for crucial factors of HIV pathogenesis (replication, immunomodulation, CD4 depletion and immune response) [29]. Our hypothesis is that independent roles of cell-free virus and infected cells in HIV pathogenesis should be revealed by different associations between the levels of virus in these two compartments and major immune parameters of HIV disease.

We report results concerning 44 HIV-1 infected children for whom the HIV RNA level in plasma, the HIV DNA level in PBMCs, the frequency of HIV-specific CD8 T lymphocytes, and the activated and naive/memory T lymphocytes subsets were determined in the absence of HAART. The quantification of HIV RNA in plasma provided a measure of the cell-free HIV. The quantification of total HIV DNA in PBMCs provided a measure of cell-associated HIV. The associations between the two viral markers and T cell function and subsets were examined, using the partial correlation method to control for the effect of age and CD4 lymphocyte depletion. For several immune markers, we observed a correlation to either the HIV RNA or the HIV DNA level, but never to both of them. Our results suggest that cell-free HIV particles and HIV-infected cells are involved in distinct pathways of HIV immunopathogenesis.

Patients and methods

Patients

The patients were followed at the Necker Hospital in Paris. Legal guardians gave their written informed consent before inclusion of the children in the longitudinal follow-up of their immune parameters [30]. This biomedical research was approved by the local ethics committee, and was in accordance with the Helsinki Declaration. For the present study, we selected perinatally infected children who were not receiving HAART, defined by combination therapy including either a protease inhibitor or a non-nucleosidic reverse transcriptase inhibitor. The immunological assessments were performed on fresh blood samples. For 44 children, PBMCs were available to retrospectively quantify the HIV DNA levels. The children received no antiretroviral drug, with the exception of one child that was treated by AZT as part of prophylaxis to prevent mother-to-child transmission of HIV.

Quantification of HIV RNA and DNA levels

The Amplicor HIV-1 Monitor Test v1.5 (Roche, Neuilly, France) was used to quantify HIV-1 RNA in plasma. The cut-off value was 2.6 log (HIV-RNA copies/mL). Total cell DNA was extracted from PBMC pellets by adsorption onto silica membranes (Qiagen, Hilden, Germany). HIV-1 DNA was quantified by real-time polymerase chain reaction, using a 5' nuclease assay in the long terminal repeat (LTR) region [31] and the same primers as those used to amplify HIV RNA [32] . The reaction was performed with an ABI PRISM 7000 (Applied Biosystems, Courtaboeuf, France) using 1 µg of total DNA. HIV-1 DNA was quantified by reference to a standard curve of 8E5 cell DNA. The results were expressed as the number of DNA copies/10^6 PBMCs.

Elispot assay

Elispot assays were performed as previously described, with freshly isolated PBMCs [30,33]. The cell input was $5x10^5$ PBMCs/well. Recombinant Vaccinia virus (rVV) encoding HIV-1 Env, Gag, and Pol proteins from the HIV-1_{IIIB} strain (vAbT489) and the parental

NYCBH strain were obtained from Therion Biologics (Cambridge, MA, USA). rVV were added to PBMC at a final concentration of $2x10^7$ pfu/mL. After overnight incubation at 37°C, enzymatic revelation was performed [33]. Previous experiments showed that most IFN-γ-producing cells were CD8 T lymphocytes [33]. The number of spot forming cells (SFC) was determined with computer-assisted image analysis software (KS-Elispot, Zeiss, Munich, Germany). A minimum of 10 spots/well were retained to calculate the frequencies of IFN-γ producing cells. An assay was considered positive if the spot number obtained in the presence of antigen exceeded the mean plus 3 standard deviations of the spot number obtained with control antigen, and was at least twice the spot number obtained with control antigen. The median value in wells stimulated with the control NYCBH strain was 24 SFC/10^6 PBMCs. The net SFC was calculated as: (SFC obtained with antigen)-(SFC obtained with control antigen). The results were expressed as net SFC/10^6 PBMCs = 10^6 x [(SFC number per well)/(number of cells per well)].

Quantification of major lymphocyte subsets

Percentages of CD4 and CD8 lymphocytes and their activated and naive/memory subsets were measured in fresh whole blood samples by four-colour flow cytometry. The following antibody associations were used. 1: CD45-FITC, CD3-PCy5, CD4-RD1, CD8-ECD; 2: CD4-ECD, CD8-PyC5, CD38-RD1, HLA-DR-FITC; 3: CD4-ECD, CD8-PCy5, CD62L-RD1, CD45RA-FITC. Labelled cells were analyzed on an EPICS-XL MCL flow cytometer (Coulter Electronics Inc., Hialeah, FL, USA). All monoclonal antibodies were from Beckman-Coulter-Immunotech (Paris, France). Using the Cytomics RXP software (Beckman Coulter), cells were gated by forward- and side-scatter for lymphocytes, and lymphocyte subpopulations were analysed using prism-plot. CD4 or CD8 lymphocytes expressing CD38 and/or HLA-DR were considered as activated. The differentiation stage of T lymphocytes was defined by expression of the CD45RA and CD62L molecules, as described [34]. Naive T lymphocytes were defined by the co-expression of CD45RA and CD62L markers.

Lymphocytes that were not co-expressing CD45RA and CD62L markers were considered as memory lymphocytes and the following two subsets were defined: $CD45RA^{-}CD62L^{+}$, $CD45RA^{-}CD62L^{-}$. Due to important age-related changes in lymphocyte counts in the pediatric population, all results were expressed as percentages, as these were less prone to age-related variations than absolute cell counts.

Statistics

For each biological parameter, normality and symmetry of distributions were assessed by Shapiro-Wilk and skewness tests, respectively. Where appropriate, $\log_{10}$ or square-root values were used to normalize data. Quantitative variables from different groups were compared using the ANCOVA test, including age as a covariant. Pearson's correlation coefficients were used to assess associations between quantitative variables. When significant, correlations including HIV RNA level, HIV DNA level, age, and CD4 cell percentage as partial variables were calculated. Partial correlation measures the strength of the relationship between two variables, controlling for the effect of one or more other variables. The significance level was set at 0.05. The analysis was performed with Stata 10 software (Stata Corporation, College Station, TX, U.S.A.).

Results

Patients' characteristics

The study group is comprised of 19 boys and 25 girls. Their geographic origins were European (n=21), sub-Saharan African (n=15), and other (n=8). Twenty-two patients harboured a subtype B virus and 8 a non-clade B virus. The HIV-1 subtype was not documented for 14 children. Thirteen, 17, 8 and 6 children were in CDC stages N, A, B, and C, respectively [35]. The main characteristics of the patients (mean±SD (range)) were as follows: 8.7±5.9 (0.08-18.6) years for age, 4.8±0.75 (3-6.2) $\log_{10}$ copies/mL of plasma for HIV RNA level, 3.2±0.65 (1.9-4.3) $\log_{10}$ copies/10^6 PBMCs for HIV DNA level, 26±9 (6-46) for CD4 cell percentage.

Biological evaluations were performed between the years 2000 and 2003. The absence of any antiviral treatment in the children studied was explained by various factors: the first visit to the hospital for the youngest one, the absence of immunosuppression, the therapeutic interruption after a previous HAART treatment due to treatment failure and/or poor adherence. At the time point selected for the present analysis, 24 children had never received HAART and 20 had previously received HAART but had interrupted their treatment for at least 6 months (mean±SD: 13.8±6.7 months, range: 6.2-32.6). Of the 20 children that had been previously treated by HAART, 7 reached a viral load below 50 copies/mL during a 4 to 13 month period (mean±SD: 11.0±2.9 months). These 7 children with a previously undetectable viral load had a 9 to 24 month period (mean±SD: 14.8±4.8 months) of active replication before biological assessments were performed. Previous HAART treatment, geographical origin, infecting HIV-1 subtype, and sex had no impact on any of the biological markers tested. Thus, data from all patients were analyzed together.

Correlations between major disease parameters

The total HIV-1 DNA in PBMCs was quantified by real-time polymerase chain reaction as described [31]. This method detects both integrated and unintegrated intracellular

DNA. In untreated chronically infected patients, unintegrated HIV DNA levels were 9 to 25-fold higher than integrated HIV DNA levels [36-38]. The HIV DNA level in PBMCs correlated positively with the HIV RNA level in plasma (Figure 1A; Table 1). The HIV RNA level correlated negatively with age, reflecting the high level of viral replication in young children (Figure 1B; Table 1). In contrast, the HIV DNA level was not associated with age (Figure 1C; Table 1). Conversely, we observed a negative correlation between the HIV DNA level and the CD4 cell percentage, but not between the HIV RNA level and the CD4 cell percentage (Figure 1D and 1E). The absence of a correlation between the HIV RNA level and the CD4 cell percentage was probably due to the inclusion of young children with high HIV RNA levels and high CD4 cell percentages. The HIV RNA and DNA levels, age and the CD4 cell percentage were used to calculate partial correlations (Table 1). The multivariate analysis confirmed that (1) the HIV RNA level was positively correlated with the HIV DNA level, independently of age and the CD4 cell percentage; (2) the HIV RNA level was negatively correlated to age, independently of the HIV DNA level and the CD4 cell percentage; (3) the HIV DNA level correlated negatively with the CD4 cell percentage, independently of age and the HIV RNA level. Thus, despite being correlated to each other, the HIV RNA level in plasma and the HIV DNA level in PBMCs were differentially correlated to age and the CD4 cell percentage.

Inverse correlation between the frequency of ex vivo HIV-specific IFN-γ producing CD8 T lymphocytes and the HIV DNA level in PBMCs

HIV-specific CD8 T lymphocytes play a major role in limiting HIV replication, either by lysing infected cells or by producing soluble factors that inhibit viral production. Therefore, we investigated the associations between the HIV RNA level, the HIV DNA level and the HIV-specific CD8 T lymphocyte response. The frequency of IFN-γ producing CD8 T lymphocytes in response to HIV-1 Env, Gag and Pol was quantified using a recombinant Vaccinia based Elispot assay [33]. A significant number of patients were of non-European origin

and were infected by a non clade B HIV-1 isolate. As we used HIV-1 antigens derived from a subtype B virus to quantify HIV-specific CD8 T lymphocytes, we controlled whether the frequencies of HIV-specific CD8 T lymphocytes differed according to the geographic origin and/or the infecting HIV-1 subtype. No significant difference was observed (data not shown). This result is consistent with our previous observations that most HIV-specific responses are cross-reactive [39], and that the CD8 T lymphocyte response against subtype B HIV proteins is not significantly different between subtype B and non-subtype B infected children [40].

The frequency of HIV-specific CD8 T lymphocytes correlated negatively with both the HIV RNA and DNA levels (Table 2; Figures 2A and 2B). In addition, the frequency of HIV-specific CD8 T lymphocytes correlated positively to age (Table 2), in agreement with a previous report [30]. When partial correlations were calculated, the frequency of HIV-specific CD8 T lymphocytes did not correlate with the HIV RNA level in plasma (Table 2). The negative correlation between the HIV RNA level and the frequency of HIV-specific CD8 T lymphocytes was indirect and was probably due to the fact that both parameters were correlated to age (Table 2). In contrast, partial correlation analysis showed that the inverse correlation between the HIV DNA level and the frequency of HIV-specific CD8 T lymphocytes was independent of age and the CD4 cell percentage (Table 2).

The HIV DNA level in PBMCs correlated positively to the percentage of $CD4^+HLA\text{-}DR^+$ lymphocytes, whereas the HIV RNA level in plasma correlated positively to the percentage of $CD8^+CD38^+$ lymphocytes

Next, we analysed the associations the HIV RNA and DNA levels, and T cell subsets expressing the activation molecules HLA-DR and/or CD38. The percentage of $CD4^+$HLA-DR^+ lymphocytes correlated positively with the HIV DNA level, independently of the HIV RNA level, age and the CD4 cell percentage (Figure 3A and 3B; Table 3). Furthermore, the percentage of $CD4^+$HLA-DR^+ lymphocytes was negatively correlated to the CD4 cell percentage, independently of age and HIV RNA and DNA levels (Table 3). The percentage of

$CD4^+HLA\text{-}DR^+$ lymphocytes correlated neither to the HIV RNA level nor to age. The percentage of $CD4^+HLA\text{-}DR^+CD38^+$ lymphocytes had associations with the HIV DNA level and the CD4 cell percentage, similar to those of percentages of $CD4^+HLA\text{-}DR^+$ lymphocytes (Table 3). The percentage of $CD8^+HLA\text{-}DR^+$ lymphocytes was higher than the percentage of $CD4^+HLA\text{-}DR^+$ lymphocytes and was correlated negatively with the CD4 cell percentage, independently of age and levels of both viral markers (Figures 3A and 3E Table 3).

The percentage of $CD8^+CD38^+$ lymphocytes correlated positively to both the HIV RNA and DNA levels, although the correlation to the HIV RNA level was much the stronger of the two (Figure 3G and 3H; Table 3). Partial correlations showed that the percentage of $CD8^+CD38^+$ lymphocytes correlated significantly with the HIV RNA level, independently of age, HIV DNA level and the CD4 cell percentage (Table 3). Similar correlations were observed for the percentage of $CD8^+HLA\text{-}DR^+CD38^+$ lymphocytes (Table 3). The percentage of $CD4^+CD38^+$ lymphocytes was higher than the percentage of $CD8^+CD38^+$ lymphocytes and was correlated to both age and the CD4 cell percentage, independently of the HIV RNA and DNA levels (Table 3, Figure 3C and 3D). In conclusion, the multivariate analysis showed that the percentage of $CD8^+CD38^+$ lymphocytes was associated with the HIV RNA level whereas the percentage of $CD4^+HLA\text{-}DR^+$ lymphocytes was associated with the HIV DNA level in PBMCs.

Total and memory CD4 lymphocytes correlate differentially to the HIV RNA and DNA levels

Finally, we investigated the associations between both viral markers and naive and memory CD4 and CD8 lymphocytes. The HIV DNA level correlated negatively to the percentage of naive $CD4^+CD45RA^+CD62L^+$ lymphocytes (Figure 4B; Table 4). However, partial correlation analysis revealed that the negative correlation between the HIV DNA level and the percentage of naive CD4 lymphocytes was not significant. This result can be explained by the strong correlation between naive $CD4^+CD45RA^+CD62L^+$ lymphocytes and

the total CD4 cell percentage on the one hand (Table 4), and the negative correlation between the HIV DNA level and the total CD4 cell percentage on the other hand (Table 1; Figure 1). No correlation was observed between the HIV DNA level and either memory CD4 lymphocyte subsets or naive/memory CD8 lymphocyte subsets (Table 4, Figures 4D and 4F, and data not shown). The HIV RNA level correlated negatively to the percentages of both $CD4^+CD45RA^-CD62L^+$ and $CD4^+CD45RA^-CD62L^-$ memory lymphocytes, independently of age, HIV DNA level and the total CD4 cell percentage (Table 4, Figures 4C and 4E). No correlation was found between the HIV RNA levels and the percentages of naive/memory CD8 lymphocytes (Data not shown). In conclusion, the amount of HIV DNA correlated negatively with the total CD4 cell percentage, independently of age and the HIV RNA level, whereas the HIV RNA level correlated negatively to the percentages of both $CD4^+CD45RA^-CD62L^+$ and $CD4^+CD45RA^-CD62L^-$ memory lymphocytes, independently of age, HIV DNA level, and the total CD4 cell percentage.

Discussion

Our hypothesis was that independent roles of cell-free HIV virions and HIV-infected cells in HIV pathogenesis should be revealed by different associations between the plasma HIV RNA level, the PBMC-associated HIV DNA level, and major immune parameters of HIV disease. Our study showed that in untreated HIV-1 infected children, the HIV RNA level in plasma and the PBMC-associated HIV DNA levels were associated with different immune parameters. The frequency of HIV-specific CD8 T lymphocytes and the total CD4 cell percentage correlated negatively to the HIV DNA level, whereas the percentage of $CD4^+HLA\text{-}DR^+$ lymphocytes correlated positively to the HIV DNA level; the percentage of $CD8^+CD38^+$ lymphocytes correlated positively to the HIV RNA level whereas the percentages of $CD4^+CD45RA^-CD62L^+$ and $CD4^+CD45RA^-CD62L^-$ memory lymphocytes correlated negatively to the HIV RNA level. All these correlations were independent of age and the CD4 cell percentage. Independent predictive values of cell-free and cell-associated HIV levels have been reported for disease progression, virus transmission, and the response to antiviral treatment [1-14]. Consistent with clinical studies, our analysis supports the concept that *in vivo* cell-free virus and infected cells play independent roles in HIV-1 pathogenesis.

HIV-specific CD8 T lymphocytes recognize peptides presented by MHC-I molecules at the surface of infected cells but do not recognize free virions. They limit viral replication by the lysis of infected cells and by the secretion of soluble factors that inhibit the production of viral particles by a non-cytolytic mechanism. In the latter case, the production of inhibitory factors is not constitutive, but is triggered by antigen-specific stimulation of CD8 T lymphocytes through recognition of an infected cell [21]. Consistent with the mechanisms of both virus recognition and the antiviral action of CD8 T lymphocytes, we observed that a higher frequency of HIV-specific CD8 T lymphocytes was linked to a lower cell-associated viremia, independently of age and the CD4 cell percentage. This observation has never been reported for HIV-infected children, and only two studies concerning untreated infected adults

reported a similar inverse association between the HIV-specific CD8 T lymphocyte response and cellular viremia [27,28]. In contrast, the multivariate analysis showed that the frequency of HIV-specific CD8 T lymphocytes was not correlated to the HIV RNA levels, consistent with reports in chronically infected adults[41]. Altogether these results suggest that the cell-associated viremia is a better correlate of the *in vivo* efficacy of HIV-specific CD8 T lymphocytes than the cell-free viremia is.

Regarding CD4 T lymphocytes that are a major target of the virus, activation is strongly associated with virus replication. Firstly, HIV replicates more efficiently in activated than in resting cells [18]. Secondly, the virus itself drives immune activation, increases the number of susceptible cells and promotes its own replication [20]. Thirdly, a higher level of viral replication will enhance CD4 lymphocyte depletion, which in turn drives homeostatic CD4 lymphocyte activation and proliferation [42]. In this paper, we provide new information showing that the level of HIV-infected cells, reflected by the HIV DNA levels, correlated positively to the percentages of activated HLA-DR^+CD4^+ lymphocytes, independently of the HIV RNA level, age and the CD4 cell percentage. To the best of our knowledge, the association between the CD4 lymphocyte activation and the HIV-DNA level has never been described. The study was performed in untreated patients. Thus, at least 90% of the quantified HIV DNA was unintegrated [36-38] and the HIV DNA level reflected the number of recently infected cells, rather than the number of latently infected ones. Activated CD4 lymphocytes represent the majority of productively infected cells, at least in chronically infected patients [43,44]. Furthermore, in successfully treated patients, the HIV-DNA levels were higher in $CD4^+CD69^+$HLA-DR^+ than in $CD4^+CD69^-$HLA-DR^- T lymphocytes [45]. In contrast to the percentages of HLA-DR^+CD4^+ lymphocytes, the percentages of $CD4^+CD45RA^-CD62L^+$ and $CD4^+CD45RA^-CD62L^-$ memory lymphocytes were not correlated to the HIV DNA level. Our results suggest that the activation status rather than the differentiation status of CD4 T lymphocytes is an important determinant of the level of cellular infection.

We observed a negative correlation between the percentage of $CD4^+HLA\text{-}DR^+$ lymphocytes and the total CD4 cell percentage as well as a positive correlation between the percentage of $CD8^+CD38^+$ lymphocytes and the HIV RNA level. These two correlations are consistent with many reports pertaining to adults, which showed stronger associations between CD8 activation and HIV RNA level on one hand, and CD4 activation and CD4 depletion on the other [46-49]. The CD38 molecule is expressed on both naïve and activated cells, requiring careful interpretation of results in infected children. Indeed, in our study the percentage of $CD4^+CD38^+$ lymphocytes correlated negatively to age, whereas the percentage of $CD8^+CD38^+$ lymphocytes was independent of age and correlated positively to the HIV RNA level as described by others in HIV-1 infected children [50]. These correlations suggest that in infected untreated children, CD38 expression on CD4 cells was mainly driven by the development stage whereas its expression on CD8 lymphocytes was driven by viral replication.

We observed that the HIV DNA level correlated negatively to the total CD4 cell percentage, independently of age and the HIV RNA level. Similar results were reported in infected adult patients [1-3,51]. The HIV RNA level was not correlated to the total CD4 cell percentage. This observation is not surprising as most young children have a persistent high level of viral replication during their first years of life while maintaining high CD4 cell percentages. The level of HIV DNA was not correlated to the naive $CD4^+CD45RA^+CD62L^+$ level. In contrast to our results, De Rossi et al. reported that in untreated children, higher cellular HIV DNA loads correlated with higher TREC levels, independently of age, the CD4+ T lymphocyte count and the plasma HIV RNA level [25]. In treated children, Saitoh et al. reported a positive correlation between the TREC and HIV DNA levels [26]. The divergence between our results and those of these two studies of pediatric patients could be explained by the heterogeneity of naive CD4 lymphocytes regarding their TREC content [52,53]. Furthermore, our patients were older and had probably lower thymic activity than those of the reports cited

above. Indeed, two studies of treated adult patients reported inverse correlations between the HIV DNA level and the naive T cell level [23,24], and two other studies reported the absence of a correlation between the HIV DNA level and the naive T cell level [54,55].

One important issue for the interpretation of our results is the disease progression pattern of patients studied that was determined by historical factors: age at the time HAART became available and age at inclusion in the cohort. The oldest children had survived several years of infection without receiving potent combination therapy and were slow progressors. Indeed, 5 out of the 19 patients more than 10 years old were HAART naive with a CD4 cell percentage higher than 25% at the time of biological assessment, and they could have been classified as long term non-progressors. On the other hand, the study group comprised only two children with the rapid pediatric form of disease progression, i.e. AIDS before two years of age. Thus, the observed associations characterize children with normal or slow HIV disease progression.

The interactions between HIV and the immune system are very complex and only partially understood. We have provided new information about whether each of several immune parameters was more directly related to the level of infected cells or to the level of virus particle production. Our results clearly showed that both viral compartments are associated to distinct immune parameters and are a starting point for further investigation of the level of infected cells in pathophysiological studies.

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Table 1: Correlations between the HIV RNA and DNA levels, age, and the CD4 cell percentage

	Pearson's correlations			Pearson's partial correlations[a]		
	HIV RNA[b]	HIV DNA[c]	Age[d]	HIV RNA	HIV DNA	Age
HIV DNA						
r	**0.548**			**0.454**		
p	**0.0001**			**0.003**		
Age						
r	**-0.498**	-0.15		**-0.496**	0.280	
p	**0.0007**	0.34		**0.001**	0.08	
CD4[e]						
r	-0.170	**-0.347**	-0.267	0.006	**-0.328**	0.018
p	0.27	**0.03**	0.08	0.98	**0.04**	0.92

[a] The HIV RNA and DNA levels, age, and the CD4 cell percentage were included as partial variables. Bold characters indicate results with p values of <0.05

[b] Expressed as $\log_{10}$ copies/mL of plasma

[c] Expressed as $\log_{10}$ copies/10^6 PBMCs

[d] Expressed in years

[e] Expressed as % of lymphocytes

Table 2: Correlations between the frequency of HIV-specific CD8 T lymphocytes, the HIV RNA and DNA levels, age, and the CD4 cell percentage

	Pearson's correlations				Pearson's partial correlations[a]			
	HIV RNA[b]	HIV DNA[c]	Age[d]	CD4%[e]	HIV RNA	HIV DNA	Age	CD4%
HIV-specific CD8 T lymphocytes[f]								
r	**-0.458**	**-0.403**	**0.650**	0.149	-0.004	**-0.386**	**0.402**	0.000
p	**0.002**	**0.007**	**0.00005**	0.34	0.98	**0.02**	**0.02**	0.99

[a] The HIV RNA and DNA levels, age, and the CD4 cell percentage were included as partial variables. Bold characters indicate results with p values of <0.05

[b] Expressed as $\log_{10}$ copies/mL of plasma

[c] Expressed as $\log_{10}$ copies/10^6 PBMCs

[d] Expressed in years

[e] Expressed as % of lymphocytes

[f] Frequency of Env, Gag and Pol-specific IFN-γ producing cells, expressed as net SFC/10^6 PBMCs

Table 3: Correlations between the percentages of CD4 and CD8 lymphocytes expressing HLA-DR and/or CD38 molecules, the HIV RNA and DNA levels, age, and the CD4 cell percentage

	Pearson's correlations				Pearson's partial correlations[a]			
	HIV RNA[b]	HIV DNA[c]	Age[d]	CD4%[e]	HIV RNA	HIV DNA	Age	CD4%
$CD4^+HLA\text{-}DR^{+f}$								
r	0.109	**0.457**	0.061	**-0.652**		**0.425**		**-0.598**
p	0.50	**0.003**	0.71	**0.00005**	na[g]	**0.008**	na	**0.0005**
$CD4^+CD38^{+f}$								
r	**0.468**	0.048	**-0.609**	**0.468**	0.150		**-0.591**	**0.438**
p	**0.002**	0.77	**0.00005**	**0.002**	0.35	na	**0.005**	**0.005**
$CD4^+HLA\text{-}DR^+CD38^{+f}$								
r	0.304	**0.480**	-0.162	**-0.531**		**0.343**		**-0.508**
p	0.06	**0.002**	0.31	**0.0003**	na	**0.03**	na	**0.001**
$CD8^+HLA\text{-}DR^{+f}$								
r	0.22	0.150	-0.090	**-0.385**				**-0.371**
p	0.16	0.34	0.57	**0.01**	na	na	na	**0.02**
$CD8^+CD38^{+f}$								
r	**0.686**	**0.376**	**-0.598**	0.208	**0.488**	0.192	-0.166	
p	**0.00005**	**0.02**	**0.00005**	0.192	**0.002**	0.25	0.32	na
$CD8^+HLA\text{-}DR^+CD38^{+f}$								
r	**0.575**	0.301	**-0.352**	-0.278	**0.379**		-0.075	
p	**0.0001**	0.07	**0.02**	0.08	**0.02**	na	0.65	na

[a] The HIV RNA and DNA levels, age, and the CD4 cell percentage were included as partial variables. Bold characters indicate results with p values of <0.05.

[b] Expressed as $\log_{10}$ copies/mL of plasma

[c] Expressed as $\log_{10}$ copies/10^6 PBMCs

[d] Expressed in years

[e] Expressed as % of lymphocytes

[f] Expressed as % of CD4 or CD8 lymphocytes. Values of $CD4^+HLA\text{-}DR^+$, were $\log_{10}$ transformed in order to normalize their distribution. The square root of $CD8^+HLA\text{-}DR^+CD38^+$

lymphocytes percentage was used in order to normalize the distribution. HLA-DR and CD38 expression on CD4 and CD8 subsets were available for 41 of the 44 patients.

[g] not applicable: the partial correlation was not calculated when Pearson's correlation was not statistically significant.

Table 4: Correlations between the percentages of naive and memory CD4 lymphocytes, the HIV RNA and DNA levels, age, and the CD4 cell percentage

	Pearson's correlations				Pearson's partial correlations[a]			
	HIV RNA[b]	HIV DNA[c]	Age[d]	CD4%[e]	HIV RNA	HIV DNA	Age	CD4%
$CD4^+RA^+CD62L^+$ [f]								
r	-0.054	**-0.316**	-0.269	**0.858**		-0.158		**0.840**
p	0.73	**0.04**	0.09	**0.00005**	na[g]	0.34	na	**0.0005**
$CD4^+RA^-CD62L^+$[f]								
r	**-0.329**	-0.149	-0.032	**0.515**	**-0.312**			**0.478**
p	**0.03**	0.34	0.84	**0.0005**	**0.05**	na	na	**0.002**
$CD4^+RA^-CD62L^-$ [f]								
r	**-0.366**	-0.097	0.088	0.083	**-0.415**			
p	**0.02**	0.54	0.58	0.60	**0.009**	na	na	na

[a] The HIV RNA and DNA levels, age, and the CD4 cell percentage were included as partial variables. Bold characters indicate results with p values of <0.05.

[b] Expressed as $\log_{10}$ copies/mL of plasma

[c] Expressed as $\log_{10}$ copies/10^6 PBMCs

[d] Expressed in years

[e] Expressed as % of lymphocytes

[f] Expressed as % of lymphocytes. Values of $CD4^+CD45RA^-CD62L^-$ were $\log_{10}$ transformed in order to normalize their distribution. Values of naive/memory CD4 subsets were available for 42 of the 44 patients.

[g] not applicable: the partial correlation was not calculated when Pearson's correlation was not statistically significant.

Figure 1: Correlations between the HIV RNA and DNA levels, age and the CD4 cell percentage. For 44 HIV-1 infected children, the following biological parameters are presented: the HIV RNA level in plasma and the HIV DNA level in PBMCs (A), the HIV RNA level in plasma and age (B), the HIV DNA level in PBMCs and age (C), the HIV RNA level in plasma and the CD4 cell percentage (D), the HIV DNA level in PBMCs and the CD4 cell percentage (E). As indicated on above the figure, different symbols are used according to the age of children and regression curves for the whole group are shown.

Figure 2: Correlations between the frequency of HIV-specific CD8 T lymphocytes, the HIV RNA and DNA levels. The frequency of HIV-specific IFN-γ producing CD8 T lymphocytes is presented as a function of the HIV RNA level in plasma (A) or the HIV DNA level in PBMCs (B). As indicated on above the figure, different symbols are used according to the age of children and regression curves for the whole group are shown.

Figure 3: Correlations between the percentages of CD4 and CD8 lymphocytes expressing HLA-DR or CD38, the HIV RNA and DNA levels. The percentages of $CD4^+HLA\text{-}DR^+$, (A, B), $CD4^+CD38^+$ (C, D), $CD8^+HLA\text{-}DR^+$ (E, F), $CD8^+CD38^+$ (G, H) are presented as a function of the HIV RNA level in plasma (A, C, E, G) or the HIV DNA level in PBMCs (B, D, F, H). Results were expressed as % among the CD4 or CD8 subset. As indicated on above the figure, different symbols are used according to the age of children and regression curves for the whole group are shown.

Figure 4: Correlations between the percentages of naive and memory CD4 lymphocytes, the HIV RNA and DNA levels. The percentages of naive $CD45RA^+CD62L^+$ (A, B), memory $CD45RA^-CD62L^+$ (C, D) and memory $CD45RA^-CD62L^-$ (E,F) CD4 lymphocytes are presented as a function of the HIV RNA level in plasma (A, C, E) or the HIV DNA level in PBMCs (B, D, F). For CD4 subsets, results were expressed as percentages among total lymphocytes. As indicated on above the figure, different symbols are used according to the age of children and regression curves for the whole group are shown.