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Tumor necrosis factor-alpha and nitric oxide in vertically HIV-1-infected children: implications for pathogenesis

Abstract

We performed a cross-sectional study to investigate the plasma TNF-alpha and nitric oxide (NO) production in 44 vertically HIV-1-infected children, and the relationship with immunological status and viral replication. As a control group, 36 healthy, uninfected children were studied. Plasma TNF-alpha and NO levels were determined by ELISA. Viral load was quantified using standard assays. Cell

proliferation, apoptosis and viral replication were evaluated in vitro by incorporation of (3H)-thymidine, flow cytometry and p24 antigen, respectively. Higher plasma TNF-alpha and NO levels were observed in HIV-1-infected children compared with healthy controls. We found a very strong correlation between plasma TNF-alpha and NO levels in HIV-1-infected children ($r = 0.98$; $p < 0.001$). Moreover, HIV-1-infected children with higher viral load (> 4.7 log10) showed higher TNF-alpha and NO levels than those with viral load below this threshold. Interestingly, we detected inducible nitric oxide synthase (iNOS) mRNA in T-lymphocytes from HIV-1-infected children. To address their possible patho-physiological significance, we tested the in vitro effects of NO and TNF-alpha in HIV-1 replication. Addition of TNF-alpha and NO donors to mitogen-activated, HIV-1-infected PBMC cultures produced a significant increase in viral replication. Moreover, HIV-1 replication in mitogen-stimulated, PBMC cultures was partially inhibited by iNOS specific inhibitors, and a neutralising, anti-TNF-alpha monoclonal antibody. Our results indicate that TNF-alpha and NO correlated with high viral load in HIV-1-infected children and favoured HIV-1 in vitro replication. These data suggest a detrimental role of NO in HIV-1 infection, and that NOS inhibitors may have some therapeutic benefit in HIV-1-infection.

Full text

INTRODUCTION

Nitric oxide (NO) is a free-radical gas produced by many cell types, which has a diverse repertoire of important functions [1-5], including its role as a neurotransmitter and regulator of blood pressure or vasodilatation. In addition, NO has antiplatelet, tumoricidal, and microbicidal activities. On the other hand, high NO production has also been associated with several important pathological situations [5-7]. NO is synthesized from L-arginine by a family of three nitric oxide synthase (NOS) proteins: neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3), and inducible NOS (iNOS or NOS2) [8]. Cytokines such as TNF-alpha and other proinflammatory stimuli induce this latter enzyme [9].

More recently, NO has been shown to modulate immune functions, although contrasting effects have been described [3, 10-12]. Thus, in one report, NO has been shown to increase lymphocyte proliferation and glucose uptake by T-lymphocytes [13], whereas other reports have shown that NO inhibits T cell

activation [14-16]. NO also has controversial effects on cytokine synthesis. It has been described that the synthesis of TNF-alpha by human peripheral blood mononuclear cells (PBMC) [13] and in lipopolysaccharide-stimulated neutrophil preparations [17], is increased by exogenous NO.

On the other hand, HIV-infected patients often show elevated circulating levels of proinflammatory cytokines, particularly TNF-alpha [18, 19], which has been associated with the pathogenesis of HIV infection. TNF-alpha is able to increase viral replication *in vitro* through activation of the nuclear factor NF-kappaB [19-21]. In turn, infected PBMC secrete higher TNF-alpha levels, which may contribute in an autocrine or paracrine manner to maintain or increase viral replication [20, 22].

The importance of NO production in HIV infection has already been established. HIV infection has been associated with accumulation of NO metabolites, nitrates and nitrites, in patients with central nervous system symptoms [23]. Other reports have shown an association between high plasma viral load (VL) levels and increased production of NO in serum of HIV-infected adult patients [24, 25]. Recently, NO production and iNOS expression induced by HIV infection in macrophages [26, 27] has been described. However, neither high NO levels nor inhibition of NO synthesis seems to modify the extent of virus replication in macrophages [28]. We have also shown that HIV induces iNOS expression and infection of neuroblastoma cells [29]. However, the role of NO in HIV-infected T cells remains unclear. The aim of the present study was to analyse the production of TNF-alpha and NO in HIV-infected children, and their relation to immunological status and viral replication in order to address their possible pathogenic role. We measured *in vivo* plasma TNF-alpha and NO levels, peripheral $CD4^+$ T-lymphocytes and VL. Furthermore, we analysed the production and influence of TNF-alpha and NO in an *in vitro* activated HIV-infected T cell model.

PATIENTS AND METHODS

Patients

A cross-sectional study was conducted on 44 HIV-infected children born to HIV-infected mothers at the General University Hospital "Gregorio Marañón" in Madrid, Spain. All infants were diagnosed as HIV-infected on the basis of positive

results in both DNA PCR and virus culture assays, as previously described [30]. None of these 44 children had been breastfed, or had history of haemophilia or blood transfusion. In addition, exclusion criteria included the absence of symptoms of concomitant opportunistic infections, in order to avoid interference with the interpretation of results. As a healthy control group, 36 seronegative, healthy children with an equivalent age range were studied. Written informed consent was obtained for all the paediatric patients. The Ethics Committee of the Hospital also gave consent. Clinical classification was based on the 1994 revised guidelines of the Center for Disease Control and Prevention (CDCP) [31].

Reagents

Recombinant human IL-2 was purchased from Cetus Co; recombinant human TNF-alpha (10^7 U/mg) was purchased from Promega Co (Madison), L-NG-monomethyl-arginine (L-NMMA); D-NG-monomethyl-arginine (D-NMMA) were purchased from Calbiochem-Behring Corp. (La Jolla, CA, USA). L-N^6- (1-iminoethyl)-lysine hydrochloride (L-NIL) was from Alexis Biochemicals, Laufelfinger, Switzerland. Sodium nitroprusside (SNP), S-nitroso-N-acetyl-penicillamine (SNAP), phorbol myristic acetate (PMA), phytohemaglutin (PHA) as purchased from Sigma Chemical Co (St. Louis, Mo, USA). The neutralising anti-TNFalpha monoclonal antibody (mAb) B13.2, was developed in our laboratory. It was used in purified form obtained from ascitic fluid.

Quantitative HIV-1 RNA assay

Blood samples were collected into EDTA tubes, separated within 4 hours and plasma stored at - 70° C. Viral load was measured in 200 mul plasma using a quantitative reverse transcriptase PCR (RT-PCR) assay (Amplicor-HIV MonitorTMTest, Roche Diagnostic Systems, Branchburg (New Jersey), USA) [30].

Determination of iNOS mRNA

Determination of iNOS mRNA was carried out by reverse transcription (RT)-PCR. Cells (10^6/ml) were incubated in RPMI 1640 supplemented with 5% (vol/vol) FCS, in the presence or absence of HIV-1NL4.3. At the indicated times, cells were washed in phosphate-buffered saline, and the pellet was frozen at - 70° C until

further analysis. The mRNA from 10^5 cells was isolated using oligo(dT)-coated magnetic beads and by subsequent RT analysis (PolyAtract series 9600 mRNA isolation and cDNA synthesis system; Promega), according to the manufacturer ´s instruction. PCR analysis was carried out with an automatic thermal cycle (GeneAmp PCR system 9600; Perkin Elmer).

For amplification of the desired cDNA, the following gene-specific primers were used: iNOS sense (5'CGGTGCTGTATTTCCTTACGAG- GCGCGA-AGAAGG-3') and iNOS antisense (5'GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC-3'). The reaction mixture contained 5 mul of cDNA (1/6 of the isolated cDNA), 1 mul sense and antisense primers, 200 muM deoxynucleotide triphosphates, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer) in a final volume of 50 mul. The cycle program was set to denature at 94° C for 45 sec, to anneal at 60° C for 45 sec, and to extend at 72° C for 2 min for a total of 40 cycle. Electrophoresis of the PCR products was performed with 1.5% agarose gels containing ethidium bromide 1 mg/ml. A 100-bp DNA ladder (GIBCO BRL) was used as a molecular weight marker. Glyceraldehyde-3-phosphate dehydrogenase mRNA was amplified as a control.

Quantification of T cell subsets in peripheral blood

T lymphocyte subsets in peripheral blood were quantified by direct immunofluorescence using monoclonal antibodies of the T series and flow cytometry (FACScan, Becton-Dickinson, San José, C.A., USA) as previously described [30].

Cytokine and NO production assays and proliferation

Plasma TNF-alpha level was determined by ELISA (INNOGENETICS N.V. Haven, Zwijnaarde, Belgium). NO production was evaluated using the Griess reaction (Bioxytech Nitric Oxide Assay; Portland, USA) [32]. TNF-alpha and NO concentrations were assayed in duplicate.

Cell cultures

PBMC (10^6/ml) from healthy, HIV seronegative donors were isolated from whole blood by Ficoll Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden), by centrifugation, and resuspended in RPMI-1640 medium (BIOCHROM) supplemented with 10% fetal calf serum (FCS), basically as described [30]. T

cells were purified from PBMC by removal of adherent cells on plastic Petri dishes, followed by passage through a nylon wool column. Phenotypic analysis of purified T cells by flow cytometry show-ed that the T cell population contained > 98% $CD3^+$; < 1% $CD11b^+$ and < 1% $CD20^+$ cells. For *in vitro* infections, the cells were infected or mock infected with 0.03 m.o.i. $HIV\text{-}1_{NL4.3}$, in the presence of different concentrations of SNP, SNAP, L-NIL, L-NMMA, D-NMMA or anti-TNF-alpha monoclonal antibody where indicated. The cultures were incubated for 3 days at 37° C and maintained in a humidified atmosphere containing 5% CO_2. None of the reagents affected the viability of the cells at the concentrations used, as indicated by the trypan-blue dye exclusion test.

Cell proliferation was evaluated by incorporation of $[^3H]$-thymidine. Cells were harvested in glass fibre filters using an automatic cell harvester. Radioactive incorporation was measured in a liquid scintillation spectrometer. The assay was carried out in triplicate cultures.

To measure apoptosis, cells were harvested and washed with phosphate-buffered saline (PBS). Then, the cells were fixed with 2% paraformaldehyde (Sigma) for 20 min at 4° C. Later, cells were washed with 0.5% PBS-Tween 20 and incubated with 50 mug/ml of propidium iodide (Sigma) and 20 mug/ml of Rnase A (Boehringer Mannhein), for 45 min at room temperature, in the dark. Finally, intracellular DNA content was analysed by flow cytometry (Becton Dickinson). Those cells with a sub G_0/G_1 DNA content were considered as apoptotic.

HIV-1-p24 antigen assay

Culture supernatants harvested at different post-infection times (usually 3, 6 and 9 days) were assayed for viral Ag p24 content using an antigen capture immunoassay (INNOTEST HIV Antigen mAb INNOGENETICS N.V. Haven, Zwijnaarde, Belgium).

Statistical analysis

$CD4^+$ and $CD8^+$ counts are expressed as percentages. In all analyses, VL was transformed to log_{10}-scale in order to normalise the distribution. The Pearson correlation coefficient was used to measure the association between different variables. Values of both range between - 1 (a perfect negative relationship) and + 1 (a perfect positive relationship). A value of 0 indicates no linear relationship.

The differences between the HIV-infected group and uninfected children were evaluated by multiple regression analysis adjusted by age.

RESULTS

Clinical, immunological and virological characteristics of the HIV-infected children

We performed a cross-sectional study in 44 vertically HIV-infected children, whose demographic, clinical, immunological and virological characteristics are summarised in Table 1. As a healthy control group, 36 healthy, uninfected children were studied (Table 1). HIV-infected children showed significantly higher plasma TNF-alpha and NO levels than the uninfected, healthy control group. We also found a very strong correlation between plasma TNF-alpha and NO levels ($r = 0.98$; $p < 0.001$) in the HIV-infected children, but not in the healthy control group.

*Relationship between viral load and plasma TNF-*alpha *and NO levels*

HIV-infected children were divided according to the VL values, using the cut-off level of 4.7 $\log_{10}$ copies/ml, previously described in our laboratory as an indicator of progression of HIV infection [33]. The TNF-alpha and NO levels in both HIV-groups were higher than the healthy control group. However, HIV-infected children with VL > 4.7 $\log_{10}$ copies/ml had higher TNF-alpha and NO levels than HIV-infected children with VL < 4.7 $\log_{10}$ copies/ml (Table 2).

*Plasma viral load, TNF-*alpha *and NO levels in HIV-infected children in different clinical and immunologic categories*

Table 3 shows plasma TNF-alpha and NO levels according to the immunological and clinical category of the infected children. We found that HIV-infected children in immunological category IC-2 (15-25% CD4) had significantly higher TNF-alpha and NO levels than children in IC-1 and -3. We also found statistically significant differences in the TNF-alpha and NO levels between HIV-infected children in immunological category IC-1 (CD4 T cells > 25%) and the healthy control group. Notably, children in clinical category A, despite being asymptomatic, had statistically significantly higher TNF-alpha and NO levels

than the healthy controls. As expected, children in IC-3 or clinical category C had significantly higher VL levels than children in clinical categories A or B.

Effects of NO on HIV infection and replication in activated PBMC

The above results show a very strong correlation between plasma TNF-alpha and NO levels ($r = 0.98$; $p < 0.001$) in HIV-infected children. We found a relationship between plasma NO and VL levels, suggesting that HIV could induce the production of NO *in vivo* or *vice versa*. Moreover, the HIV-infected children with the lowest % $CD4^+$ T cells also had significantly lower TNF-alpha and NO levels. These data indicate a relationship between production of TNF-alpha and NO, and T cells exist in HIV infection.

In order to understand the relationship between TNF-alpha, NO and VL, a series of *in vitro* studies was carried out. Firstly, we isolated T cells from 8 HIV-infected children with very different plasma VL, TNF-alpha and NO levels, and activated them with PHA (Figure 1). We did not detect production of NO by the Greiss reaction in any of the cultures analysed at days 3, 6 and 9, not even in the presence of excess L-arginine (not shown). However, we detected mRNA of iNOS in T-lymphocytes by RT-PCR upon stimulation, which is a more sensitive technique, in 5 out of the 8 children (Figure 1). Interestingly, iNOS was detected in those children having higher VL and higher plasma TNF-alpha and NO levels. iNOS mRNA could not be detected in unstimulated T cells (not shown).

In order to test if the *in vivo* alterations in plasma TNF-alpha and NO levels had any physiological significance, we assessed the effect of TNF-alpha and NO on HIV replication *in vitro*. Firstly, we tested the effect of L-arginine the physiological substrate of NOS. PHA-activated T-cells were infected *in vitro* with $HIV_{NL4.3}$, and viral replication was monitored 3 days later by measuring HIV p24 viral Ag-production. As shown in Figure 2A, the HIV replication in PBMC was strongly dependent on the concentration of L-arginine in the culture medium.

Next, we analysed the activity of two different NO generating compounds, SNAP and SNP. Although the results varied from donor to donor, the addition to the culture of both NO donors at low or moderate concentrations had a significant, enhancing effect on HIV replication. Figure 2B shows the average p24 Ag production from 4 independent donors. A similar enhancing effect was observed after TNF-alpha supplementation of the cultures. Furthermore, this effect of NO

donors, SNP and SNAP, was observed in HIV-infected T cells cultures at any time after infection (not shown). This increase in viral replication in PHA-stimulated T cells was neither associated with a significant increase in apoptosis of T cells (Figure 2C) nor with a significant change in cell proliferation (not shown). It is worth mentioning that concentrations of NO donors greater than 100 muM resulted in a significant decrease in cell viability. Addition of NO donors after 2 days of HIV-1 infection had no effect on Ag p24 at day 3 (data not shown).

The above results suggest that NO affected HIV replication. Thus, in order to study whether endogenously produced NO played a role in HIV replication, we assayed the effect of two specific and structurally unrelated iNOS inhibitors on HIV replication in T cells. The results shown in Figure 3 demonstrate that HIV replication was partially prevented, in a dose-dependent manner, by the specific NOS inhibitor L-NMMA. As a control the D-enantiomer, D-NMMA, which is unable to inhibit the NOS, had no effect (Figure 3). More interestingly, L-NIL, a highly specific inhibitor of the inducible NOS enzyme, was also a good inhibitor of HIV replication. Similar levels of inhibition were observed in the presence of a neutralising anti-TNF-alpha monoclonal antibody, in agreement with previously published results [20]. The decrease in HIV replication due to iNOS inhibitors was not due to a toxic effect since they did not affect cell proliferation in the same cultures (Figure 3).

DISCUSSION

In this study, we have detected increased circulating TNF-alpha and NO levels in HIV-infected children. We found higher TNF-alpha and NO plasma levels in children with higher VL (> 4.7 $\log_{10}$ copies/ml), which is the best virological marker for HIV disease progression in children [34-36]. Our results are in agreement with previous results in HIV-infected adult patients describing a positive correlation between VL, TNF-alpha [37, 38], and NO [24, 25] levels.

We observed higher TNF-alpha and NO levels in children from the initial stages of HIV infection (clinical category A and IC-1) than in healthy controls. We found that as the infection progresses to clinical category B and IC-2, TNF-alpha and NO levels increase, suggesting that in these children NO and TNF-alpha probably already had a pathogenic role. Finally, NO and TNF-alpha levels decrease in children with AIDS probably either as a consequence of treatment or because those patients had low % $CD4^+$ T cell levels (< 15%). Since TNF-alpha is

synthesised by T cells after activation [39, 40] or HIV infection [21], it is possible that the TNF-alpha found in plasma of HIV-infected children is mostly of T cell origin and not derived from macrophages.

The correlation between serum NO levels and the % of $CD4^+$ T cells indicates a direct or indirect contribution of these lymphocytes to the production of NO in HIV-infected children. In this regard, it has been shown that murine CD4 T lymphocytes, which are predominantly T helper type-1 cells, express iNOS during antigen stimulation [41], but such data are not available for human T lymphocytes. This view is supported by our observation that iNOS is expressed in activated PBMC from HIV-infected children. In apparent contrast with these results, Hermann *et al.* did not find increases in nitrate accumulation (a measure of NO production) upon HIV infection of PBMC or macrophages [27, 28]. We have not detected NO in the supernatants of HIV infected cultures either. However, it is well known that human cells are poor producers of NO as compared to murine cells [9] and RT-PCR is a more sensitive technique. Our data indicate that besides human monocyte-macrophages [26], astrocytes and neurons [29, 42], previously described as expressing iNOS upon HIV-1 infection, T lymphocytes from HIV-infected children also express iNOS. Although our results clearly indicate a role for HIV-1 infected T cells in NO production, it is likely that other cells, as macrophages or endothelial cells, may be an important *in vivo* source of the increased NO levels found in the plasma of HIV-infected children.

Interestingly, our *in vitro* studies have shown that several NO generating compounds, at moderate to low concentrations, were able to activate HIV replication in PBMC. Although, variations from donor to donor of PBMC existed, we always observed stimulation of HIV replication. The concentrations of the NO donors used seem to be important, since higher concentrations inhibited HIV replication (data not shown), probably because they cause apoptosis, which is in agreement with a previous report [28]. More interestingly, two structurally unrelated inhibitors of iNOS, L-NIL and L-NMMA, partially abrogated HIV-replication induced by PHA. This indicates that endogenous NO produced as a consequence of T cell activation is required for HIV replication.

Since TNF-alpha is a good inducer of iNOS [9], the observed correlation of NO with TNF-alpha levels in HIV- infected children, even in early and in asymptomatic stages, suggests that this cytokine may induce an NO increase. In addition to TNF-alpha, HIV or the gp120 envelope protein may also directly contribute to

induction of iNOS in T cells similarly to that shown in other cell types [43]. We, and others, have previously shown that in T cells, HIV replication is dependent on autocrine TNF-alpha production [20, 44]. In this regard, increased TNF-alpha production has been described upon HIV infection of several cell types [45] and after exposition of monocytes to HIV [46]. On the other hand, NO donors induce TNF-alpha secretion [13], and HIV-infection or gp120 envelope protein alone increase the production of TNF-alpha [47]. Since TNF-alpha augmented HIV replication in our cultures, it was theoretically possible that induction of TNF-alpha by NO donors may contribute to their enhancing effect on HIV replication.

Altogether, our data clearly indicate that there is an association between TNF-alpha and NO, and VL levels in HIV-infected children that might explain the rapid course of perinatally acquired HIV infection. TNF-alpha may increase VL, while HIV infection may subsequently induce TNF-alpha and NO secretion, that in turn activates HIV replication leading to a vicious circle that accelerates the disease. The impact of NO production on HIV-infection was difficult to predict. Thus, NO can inhibit the replication of certain viruses [48], but can also activate NF-kappaB, and increases the production of TNF-alpha [13, 17], both events favouring viral replication.

It is somewhat surprising that NO enhanced HIV replication, since it is generally accepted that NO and related species have antiviral activity [49, 50]. However, those antiviral effects have been shown to take place at higher NO concentrations than those used here. In contrast, our results indicate that NO promotes HIV replication and suggest that NO neutralisation may be a potential target for fighting HIV infection. Moreover, NO induced directly by HIV infection or by HIV products such as gp120, or indirectly through HIV- induced cytokines, such as TNF-alpha, has previously been implicated in AIDS pathogenesis, being considered a leading cause of AIDS dementia [43]. In addition to these effects, we have shown here that autocrine NO may also be detrimental in HIV infection by increasing HIV replication in T cells. Taken together, these findings indicate that NO may play a deleterious role in HIV-infected children, and suggest that NOS inhibitors may have some therapeutic benefit in HIV infection, most likely in combination with antiretroviral drugs.

CONCLUSION

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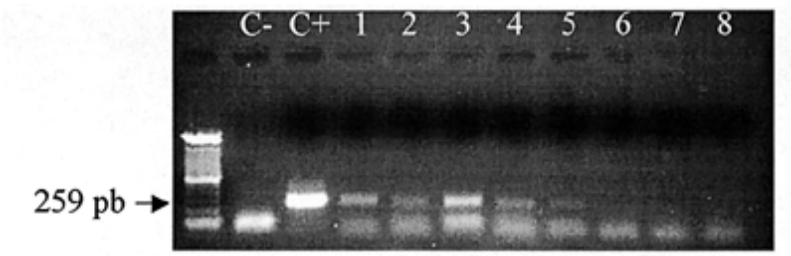
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Figures



Child	1	2	3	4	5	6	7	8
TNF-α (pg/ml)	48	47	36	42	32	10	9	7
NO (nM)	531	509	443	436	326	115	106	54
Log_{10} VL (copies/ml)	6.1	5.1	4.6	4.7	5.1	3.9	3.7	3.8

Figure 1

Induction of iNOS mRNA in PHA-activated PBMC from HIV-infected children: correlation with plasma TNF-alpha, NO and VL levels.

T cells from 8 HIV-infected children were activated with PHA. Lane C- healthy negative control; lane C+, healthy positive control; lanes 1-8, activated PBMC from each child. Two days after PHA stimulation, iNOS mRNA was detected by RT-PCR, with specific primers. Plasma TNF-alpha, NO and VL levels for each child are shown below each lane.

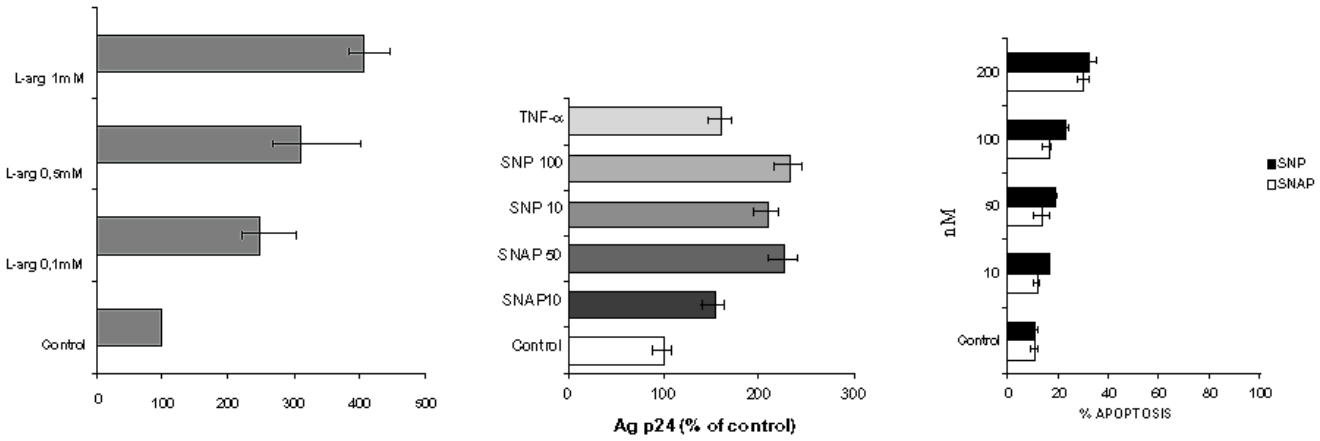


Figure 2

Effect of NO on HIV replication in PHA-activated T cells.

A: Effect of L-arginine on viral replication. **B:** Effects of NO donors, SNAP (10 muM, 50 muM) and SNP (10 muM and 100 muM) or TNF-alpha were added to the cultures as indicated. **C:** Effect of NO donors on cell viability (apoptosis). Human PBMC cells were stimulated with PHA (1 mug/ml) and infected with $HIV_{NL4.3}$ (0.03 moi.) in the presence of the indicated compounds. Three days later

cultures were assayed in triplicate for Ag p24 viral antigen content in the supernatant by using an antigen capture immuno-assay. Proliferation was measured by [^{3}H] thymidine incorporation during the last 16 hours or the 3 day culture and apoptosis was evaluated by flow cytometry. Data shown are the means ± SD of independent experiments from 4 blood donors.

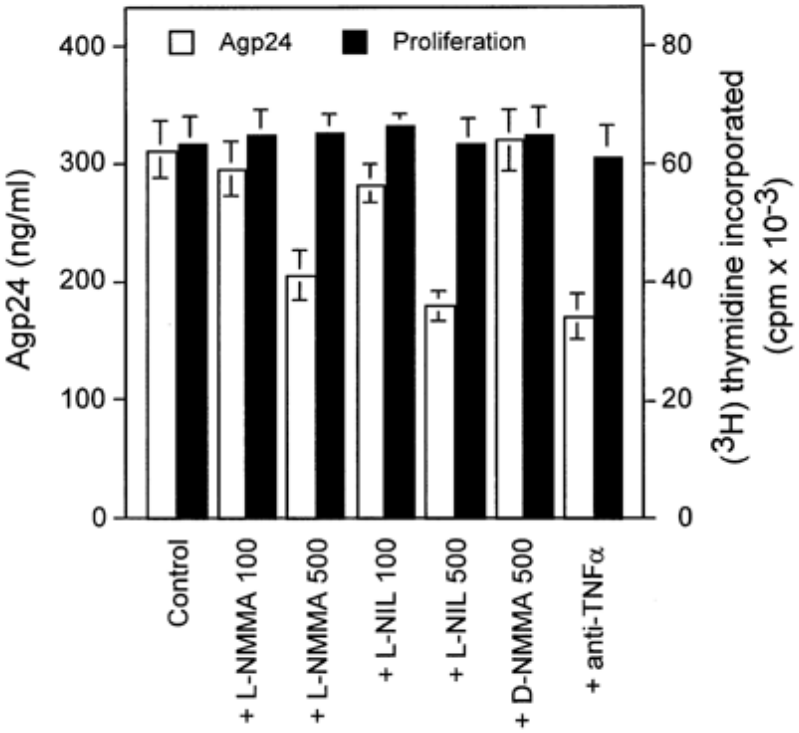


Figure 3
Effect of NOS inhibitors on HIV replication in PHA-activated T cells.

Human PBMC cells were stimulated with PHA (1 mug/ml) and infected with HIV. L-NIL (100 muM and 1,000 muM), L-NMMA (500 muM), D-NMMA (500 muM) and anti-TNF-alpha (10 mug/ml) were added to the culture as indicated. Three days after infection, cultures were assayed in triplicate for Ag p24 viral antigen (mug/ml x 2 x 10^6 cells)

content in the supernatant, using an antigen capture immuno-assay. Proliferation was measured by [^{3}H] thymidine incorporation during the last 16 hours or the 3 day cultures. Data shown are the mean ± SD of 4 independent experiments from 4 blood donors.

Table 1
Immunological and virological characteristics of the 44 HIV-infected children and 36 healthy controls

Characteristics	HIV	Healthy control group
No. of children	44	36
Gender (males/females)	15/29	14/22
Age (years)	4.99 ± 0.58 (0.08-13)*	2.89 ± 0.49 (0.08-12)
Clinical category (CDCP)		
A	14 (31.8%)	–
B	15 (34.1%)	–
C	15 (34.1%)	–
Immunological category (CDCP)		
IC-1 (≥ 25% CD4+)	15 (34.1%)	–
IC-2 (15-25% CD4+)	8 (18.2%)	–
IC-3 (≤ 15% CD4+)	21 (47.7%)	–
Lymphocyte subsets		
% CD4+ T cells	20.28 ± 1.89 (1-41)*	42.68 ± 1.38 (27-57)
% CD8+ T cells	33.18 ± 2.08 (4-56)*	18.60 ± 0.92 (8-29)
CD4+/mm^3 T cells	801 ± 145 (8.6-5,368)*	2,387 ± 230 (1,028-4.975)
CD8+/mm^3 T cells	1,128 ± 156 (1-4,945)	986 ± 88 (426-2,150)
CD4/CD8	0.68 ± 0.07 (0.03-2)*	2.57 ± 0.17 (1.08-5.33)
Virological markers		
Log_{10} CV (copies/ml)	4.48 ± 0.15 (2.3-6.81)	–
Plasma levels		
TNF-alpha (pg/ml)	26.93 ± 2.96 (0-103)*	5.14 ± 0.96 (0-22)
NO (nM)	284.75 ± 29.92 (0-958)*	50.23 ± 10.87 (0-214)

Values expressed as mean ± SEM, range (min; max) and absolute numbers (%). VL = viral load; CDCP = Center for Diseases Control Prevention. Statistical differences from healthy control group (* $p < 0.05$).

Table 2
Differences in plasma TNF-alpha and NO levels in HIV-infected children and healthy control according to viral load

	Healthy control group	VL > 4.7 $\log_{10}$ copies/ml	VL < 4.7 $\log_{10}$ copies/ml
No. of children	36	30	14
TNF-alpha (pg/ml)	5.14 ± 0.96 (0-22)	34.07 ± 4.64[a] (0-64)	23.60 ± 3.66[a, b] (0-103)
NO (nM)	50.23 ± 10.87 (0-214)	370.29 ± 48.25[a] (0-644)	244.83 ± 35.88[a, b] (0-958)

Values expressed as mean ± SEM, range (min; max); VL = Viral load. Statistical differences from healthy control group (a, $p < 0.05$) and between both groups of HIV-infected children (b, $p < 0.05$).

Table 3
Differences in plasma TNF-alpha and NO levels in HIV-infected children and healthy controls according to clinical and immunological categories

	No.	TNF-alpha (pg/ml)	NO (nM)	Log_{10} VL (copies/ml)
Healthy controls	36	5.14 ± 0.96 (0-22)	50.23 ± 10.87 (0-214)	–
Clinical category				
A	14 (31.8%)	26.5 ± 7.1[a] (0-103)	263 ± 68.20[a] (0-958)	3.97 ± 0.25[c] (2.30-5.10)
B	15 (34.1%)	29.40 ± 3.01[a] (9-57)	318.47 ± 33.94[a] (106-617)	4.25 ± 0.21[c] (2.81-5.81)
C	15 (34.1%)	24.86 ± 5.11[a] (0-64)	271.33 ± 52.51[a] (0-644)	5.20 ± 0.24 (3.77-6.81)
Immunological category				
IC-1 (≥ 25% TCD4+)	21 (47.7%)	25.6 ± 3.1[a, b] (0-56)	272.9 ± 34.1[a, b] (0-612)	4.24 ± 0.24[c] (2.3-6.81)
IC-2 (15-25% TCD4+)	8 (18.%)	44.50 ± 10.05[a, c] (7-103)	455.8 ± 95.8[a, c] (54-958)	4.43 ± 0.44[c] (2.81-6.3)
IC-3 (≤ 15% TCD4+)	15 (34.1%)	19.4 ± 4.2[a] (0-64)	210 ± 42.1[a] (0-644)	4.87 ± 0.19 (3.68-6.48)

Values expressed as mean ± SEM, range (min; max) and absolute number (%); VL = viral load; IC = immunological category. Statistical differences from the healthy control group (a, $p < 0.05$). Statistical differences with HIV-infected children in immunological category 2 (b, $p < 0.05$). Statistical differences with HIV-infected children in clinical category C or immunological category 3 (c, $p < 0.05$).

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microsatellites, interleukin-10 promoter, multiplex-PCR, Caucasian, African.

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