Cytokine gene expression and T-cell proliferative responses in lymph node mononuclear cells from children with early stage human immunodeficiency virus infection

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Background and Objectives. The immunologic events taking place in secondary lymphoid tissue from children with early stage human immunodeficiency virus (HIV) infection are poorly understood. The aim of this study was to investigate cytokine gene expression and proliferative responses in lymph node (LN) biopsies from five children with early stage HIV infection, in the context of LN morphology and viral load.

Design and Methods. The design of the study was approved by the local Ethical Committee. Cytokine gene expression was studied in LN biopsies and in paired peripheral blood (PB) samples from HIV-infected children by reverse transcriptase-polymerase chain reaction. T-cell proliferation was assessed by 3H-thymidine incorporation. Viral burden in germinal centers was assessed by video densitometric analysis following immunohistochemical staining for HIV p24.

Results. Interleukin (IL)-2, IL-4 and IL-5 mRNA were not detected in any LN or PB sample from HIV-infected children. Interferon (IFN)-γ mRNA was found only in CD8+ cells. IL-12 p35, IL-10, transforming growth factor-(TGF)-β1, regulated on activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, MIP-1β and IL-16 transcripts were detected in all samples. Proliferation of LN and PB mononuclear cells to polyclonal mitogens and soluble (recall and HIV-related) antigens was impaired as compared with the responses in a group of age-matched healthy controls.

Interpretation and Conclusions. Changes in cytokine gene expression and T-cell proliferative responses are already detectable in lymph nodes from HIV-infected children at an early stage of disease.

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Key words: pediatric HIV infection, lymph nodes

Pediatric human immunodeficiency virus (HIV) infection has peculiar epidemiology, mode of transmission and clinical features. Most cases are perinatally acquired, incubation is usually shorter than in adults and the majority of clinical manifestations are related to opportunistic infections. In these patients, central nervous system involvement is common and the overall incidence of neoplasia is 130-fold higher than that in non-immunosuppressed children. Progression of HIV infection is generally faster in children than in adults and most pediatric patients suffer from HIV-related manifestations at school age.

Although the reasons for fast progression are not well understood, the age-related immunologic status of the neonate and of the young child may contribute to the evolution of disease. The knowledge of the immunologic events that occur in peripheral lymphoid tissues of HIV-infected children at early stages of their disease should help elucidate the mechanisms underlying disease progression.

Studies in adults have shown that progression of HIV infection is associated with changes in the patterns of cytokines produced by CD4+ cells, both in lymph nodes (LN) and in peripheral blood (PB). Shifts from a T-helper (Th)-1 type pattern, characterized by interleukin (IL)-2 and interferon (IFN)-γ production, to a Th2 or Th0 pattern, whereby CD4+ cells produce IL-4 and IL-5 or IL-2, IFN-γ, IL-4 and IL-5, respectively, have been demonstrated in short-term cultured PB mononuclear cells (MC) or T-cell clones from HIV-infected patients. Such changes in cytokine profiles may facilitate HIV spreading since the virus replicates better in Th0 and Th2 than in Th1 cells.

LN biopsy is not a common practice in HIV-infected children. Therefore, information on patterns of cytokine gene expression and T-cell reactivity to specific antigens in LN from pediatric patients is limited. The present study was...
Five children (age range 2-11 years) with early-stage HIV infection, four at A1 and one at B1 CDC stage, underwent diagnostic biopsy of a single enlarged LN, either cervical or axillary. Concomitantly, PB was drawn by venipuncture and subdivided into two aliquots: one was heparinized and used as the source of MC, the other was allowed to coagulate to obtain serum (see below). LN from 6 age-matched children who underwent biopsy for diagnostic purposes due to HIV-unrelated inflammatory disorders were studied as controls. All the latter LN samples displayed follicular hyperplasia, thus matching the histologic pattern consistently detected in the HIV-infected LN herein investigated (see Results section). As controls for the experiments with PB MC from HIV-infected patients, 3 PB MC samples from age-matched healthy children were tested.

The main clinical and immunologic parameters of HIV-infected children are summarized in Table 1. At the time of study, two patients (Pt 1 and Pt 5) had been receiving treatment with azidothymidine (AZT) for six and five years, respectively, whereas the other three had not received antiretroviral therapy. All patients had absolute CD4+ counts higher than 500/mm3 and, in all cases, the CD4/CD8 ratio was higher in LN MC than in paired PB MC (Table 1). In both compartments, the CD4/CD8 ratio was lower than that detected in age-matched, non-HIV-infected individuals. In particular, the CD4/CD8 ratio in LN MC from six age-matched children with HIV-unrelated reactive lymphadenopathies ranged from 1.85 to 2.8. The CD4/CD8 ratio in PB MC from 20 healthy, age-matched children ranged from 1.1 to 1.8. In HIV-infected children, the percentage of CD20+ B-cells ranged from 9 to 30% in LN MC and from 7 to 20% in paired PB MC.

Histology and immunohistochemistry
LN were fixed for 2 h with 10% formalin containing 6% HgCl2 and 1% CH3COONa (B5 fixa-

<table>
<thead>
<tr>
<th>Pt.</th>
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<th>Age at biopsy</th>
<th>Stage</th>
<th>CD4 cells/mm3</th>
<th>LN</th>
<th>CD4/CD8 ratio</th>
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<td>F</td>
<td>11</td>
<td>B1</td>
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<td>1.13</td>
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<tr>
<td>2</td>
<td>F</td>
<td>2</td>
<td>A1</td>
<td>1426</td>
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<tr>
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<td>M</td>
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<tr>
<td>4</td>
<td>F</td>
<td>1</td>
<td>A1</td>
<td>1412</td>
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<tr>
<td>5</td>
<td>F</td>
<td>8</td>
<td>A1</td>
<td>520</td>
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</table>

The study, conducted at the Department of Pediatrics, University of Brescia, Brescia, Italy, was approved by the local Ethical Committee. Written informed consent was obtained from the parent or the legal guardian of each child.
and paraffin embedded. In some experiments, LN fragments were snap-frozen in a cryotube plunged into isopentane pre-cooled in liquid nitrogen and stored at –80°C until cryostat sectioning. Paraffin sections 4 µm thick were placed on gelatin-coated slides, whereas cryostat sections were air-dried at room temperature for 6 h and fixed with cold acetone for 5 min. Sections were treated with pronase (Sigma Chemical Company, St. Louis, MO, USA) and incubated overnight with the primary monoclonal antibody (mAb). The reaction was revealed using a two-step alkaline phosphatase anti-alkaline phosphatase (APAAP) method. The following mAbs were used: anti-IgM (R1/69), anti-IgG (A57H), anti-κ (A8B5) or anti-λ (N10/2) Ig light chains, anti-CD21 (1F8), and anti-HIV gp24 (Kal-1). In addition, CD4+ and CD8+ cells were also identified in frozen sections using mAb MT310 (anti-CD4) and C8/144B (anti-CD8) (Dako). Controls were performed by incubation of sections with the secondary antibody alone, or with an irrelevant primary mAb of the same isotype.

Recombinant biotinylated HIV gp120, produced in the baculovirus expression system, was purchased from Intracel (Cambridge, MA, USA) and used at 1:20 dilution in Tris-buffered saline. Sections were incubated at 4°C overnight with the gp120 antigen followed by extravidin-alkaline phosphatase (Sigma) and APAAP for an additional 30 min. Biotinylated rabbit antimouse Ig was added for 30 min, followed by another extravidin step for 30 min and subsequent incubation, for 30 min, with the APAAP solution. Sections were rinsed with Tris-buffered saline after each incubation step. The chromogenic substrate (naphthol-AS-TR phosphate and fast red TR salt, Sigma) was applied on sections for 20 min. The appropriate controls are briefly described in the Results section.

Quantitative analysis of the viral load in LN germinal centers (GC).

Paraffin sections (7 µm) from lymph nodes fixed with B5 were treated with pronase and stained with anti-HIV p24 mAb and the APAAP protocol, as described in the preceding section. Immunohistochemical images were acquired using an Optimas 4.0™ (Bioscan, Edmonds, WA, USA) analyzer connected with a Dialux 20EB microscope (Leitz, Wetzlar, Germany) and a high resolution Sony XC75/CE camera. The staining area (A(area)) and a measure of the pixel grey value (ArLIGV) were calculated. Technical details of these image analyses are provided in reference #29.

Electron microscopy

Tissues were fixed with 2.5% glutaraldehyde (Polyscience, Warrington, PA, USA) in 0.1 M cacodylate buffer, pH 7.3, and post-fixed with 1% OsO4 (Polyscience) in the same buffer. Following en bloc staining with 1% uranyl acetate and dehydration with ethanol and propylene oxide, samples were embedded in LXX12 (Polyscience). Grey silver sections were stained with uranyl acetate and lead citrate, and observed with a Zeiss EM 10C electron microscope.

Cell separation and culture

PB MC were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, washed twice with Hank's solution and resuspended in complete RPMI 1640 medium at 2×10⁶ cells/mL. Hank's solution and RPMI 1640 were from Flow (Irvine, Scotland). RPMI 1640 was supplemented with 100 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10⁻⁵ M 2-mercaptoethanol and 5% human AB serum.

LN biopsies were minced in Hank's solution with sharp scissors. Fragments were forced through a fine mesh stainless steel screen. LN MC were washed once, resuspended in complete RPMI 1640 medium prior to functional and molecular analyses. An aliquot of cells (2×10⁶/mL) was cultured overnight in RPMI 1640 in the absence of stimuli. Culture supernatants were harvested by centrifugation and stored at –80°C until tested for HIV p24. Viral antigen p24 was tested in serum and LN MC culture supernatants using a commercial ELISA kit (Abbott, Abbot Park, IL, USA) (in collaboration with G. Gabutti, Department of Health Sciences, University of Genoa).

CD4+ T-cells were isolated from 4 HIV-infected lymph nodes by immunomagnetic separation with Dynabeads according to the instructions of the manufacturer (ImmunoTech, Marseille, France). CD8+ lymphocytes were positively selected from the CD4+ cell fraction by the same method. The remaining cells that did not express CD4 or CD8 were defined as CD4–,CD8– cell fractions.

PB MC or LN MC were dispensed in flat-bottom microtiter plates (4×10⁵ cells in 0.2 mL medium) in the absence of or in the presence of antigens or polyclonal mitogens (see below). Plates were incubated, pulsed on day 4 with 0.5 µCi 3H-thymidine (specific activity 5 Ci/mmole) (Amersham, UK) and harvested 18 h later with a Micromate 196 apparatus after addition of 50 µL 1N NaOH to inactivate HIV. The filters were dried in a microwave oven and counted with a Matrix 96 β-counter (Packard-Canberra, Meriden, CT, USA) in the absence of scintillation flu-
Polyclonal stimuli were recombinant (r)IL-2, from Eurocetus (Amsterdam, The Netherlands) tested at the final concentration of 50 IU/ml; a CD3 mAb, kindly donated by Dr. Elisabetta Cosulich and tested at the final concentration of 1 µg/mL and PHA-P (Gibco, Paisley, Scotland), that was used at the final concentration of 1 µg/mL.

Antigens were tetanus toxoid (TT) purchased from Connaught Laboratories (London, Ontario, Canada), PPD from Statens Seruminstitut (Copenhagen, Denmark), HIV-1 gp160 from Microgenics (Meredith, CT, USA). Candida albicans (Ca) was grown overnight in complete RPMI 1640 and autoclaved for 20 min after washing in Hanks solution. HIV-1 peptides were synthesized as described: 20 mer peptides overlapping by 10 residues encompassing HXB2 HIV strain gp120 or reverse transcriptase (RT) or p24 were synthesized, pooled and tested at 1 µg/mL final concentration, as previously reported.31-33 Pso-ralen-plus UV-inactivated HIV-1 HXB2 viruses were prepared and tested as described elsewhere.44 The stock preparation contained 10^10 particles/mL.

The results of cell proliferation assays were expressed as a stimulation index (SI), i.e. the ratio between counts per minute (cpm) in stimulated and unstimulated (control) cultures. The SI for PB MC from 35 age-matched healthy children and from LN cells from 6 age-matched children (see above) were >10 for PHA, >10 for CD3 mAb and >5 for IL-2, TT, Ca and PPD in 90% of the individuals tested. These SI values were considered as the minimum threshold to define a positive response.

Immunophenotypic analysis
PB and LN MC were stained with the following mAbs: OKT3 (CD3), OKT4 (CD4) and OKT8 (CD8) (Ortho, Raritan, NJ, USA) and CD20 (Dako) Cells were analyzed by indirect immunofluorescence using a FACScan (Becton-Dickinson).

Reverse transcription-polymerase chain reaction (RT-PCR) for cytokine mRNA detection
The expression of cytokine (IL-2, IFN-γ, IL-4, IL-5, IL-12, IL-10 and TGF-β1 and chemokine (MIP-1α, MIP-1β, RANTES and IL-16) genes was investigated using the RT-PCR procedure. MC isolated from fresh PB or LN and subsequently fractionated into CD4+, CD8+ and CD4–, CD8– cells were used for total RNA extraction.35 RNA was then reverse transcribed into cDNA for PCR amplification using a cDNA Synthesis kit (Clontech Laboratories Inc., Palo Alto, CA, USA). The cDNA was diluted to 100 µl and 3 µl of the diluted samples were amplified with primers specific for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) housekeeping gene to check the efficiency of reverse transcription.

Qualitative RT-PCR
Two microliters of cDNA from each sample were amplified in a total volume of 50 µl using 25 pmoles of each cytokine specific primer and 2 U of Taq polymerase (Clontech Laboratories Inc., Palo Alto, CA, USA) by 36 cycles of denaturation at 94°C for 1 min, annealing at 55°C (IL-2), 62°C (IFN-γ), 70°C (IL-10) for 2 min or 50°C (MIP-1α and IL-16), 60°C (IL-4, IL-5, MIP-1β and RANTES), 65°C (TGF-β1 and IL-12) and 40°C (p40) for 1 min and extension at 72°C for 1 min. Primers specific for IL-2, IL-4, IL-5, IL-10, RANTES and MIP-1α were synthesized according to published sequences,35-38 whereas the following primers were prepared in our laboratory: IFN-γ sense ATG CAG GTC ATT CAG ATG TAG, IFN-γ antisense GTC ATC CGA ATT AGT C, TGF-β1 sense GCC CTG GAC ACC AAC TAT TGC, TGF-β1 antisense GCA GGA GCG CAC GAT CAT GT, IL-16 sense CAG CCA GTG ATG TTT CTG T, IL-16 antisense ATC GTG ACA GTT CCA TCA G, MIP-1α sense TGC TGC CCT TGC TGT CCT C, MIP-1α antisense GAA GAG GTA GCT GTG GAC GTC.

PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining.

Quantitative RT-PCR
In order to quantify the absolute amount of IL-10 and TGF-β1 mRNA in the individual samples, a previously reported competitive PCR procedure was used.39 Competitors were designed by introducing a 30 bp long sequence into the product of amplification to distinguish the cDNA product from the internal standard. The competitive PCR experiments were carried out as follows: five reactions with a decreasing concentration from 1.6 × 10^-3 to 2.56 × 10^-6 attomoles and 1 µl of cDNA were performed. The amplification profile, the number of cycles, the experimental conditions and the primers were used, as for qualitative PCR. Fifteen microliters of each reaction product were resolved on a 12% polyacrylamide gel, stained with ethidium bromide, recorded by computer scanning and analyzed using the NIH Image 1.49 software. The concentrations of cytokine mRNA were calculated as previously reported.39
Results

Morphologic and immunocytochemical characterization of LN from HIV-infected children

In all patients, florid LN follicular hyperplasia with large germinal centers (GC), reduction of the mantle zone and focal follicular lysis were observed (not shown).

Immunocytochemical analyses showed the following: i) deposits of IgM were detected in the GC interdendritic spaces (Figure 1a); these are presumably the secretory product of IgM plasma cells that are present within the GC and stain predominantly for Igλ chains (not shown);28 ii) the GC network of follicular dendritic cells (FDC), shown by anti-CD21 mAb, was focally disrupted in areas where infiltrates of small, non-centrocytic lymphocytes were detected (Figure 1b); iii) Staining for CD8 shows a heavy infiltrate of CD8+ cells into both the mantle zone and the GC in a secondary lymphoid follicle of a lymph node from an HIV-infected child. Note that these cells are absent from secondary lymphoid follicles from normal lymph nodes. M = follicular mantle; GC = germinal center of secondary lymphoid follicles. a, ×900; b, ×600; c, ×900.

Controls to prove the specificity of gp120 binding to lymph node GC were the following: i) pre-treatment of the sections with anti-λ antibodies completely prevented the binding of gp120; ii) lymph nodes from rheumatoid arthritis adult patients which displayed IgM-λ producing plasma cells and abundant extracellular Ig deposits in the GC failed to bind biotinylated gp120.28

The presence of HIV in the interdendritic spaces was demonstrated by ultrastructural analyses, that also showed mitochondrial damage in the FDC (Figure 3).

One possibility raised by the above observa-
tions is that the large number of CD8+ T-cells, putative cytotoxic T-lymphocytes, present in the LN GC destroy FDC (see Figure 1b, right-hand side).

Staining for HIV p24 was positive in all samples, with a distribution similar to that of CD21. Wide variations existed for the $V_{\text{VEM}}$ (mean values between 2.43±0.65 and 10.62±0.48) and ArLIGV (mean values between 0.129±0.07 and 0.355±0.17). Ample variations in the viral burden were found between different lymph nodes and between GC in the same lymph node section. There was no association between p24 and CD21 staining.29

The above data were supported by the p24 immunoassay run on the same samples (Figure 4). With the exception of patient #2, p24 concentrations were consistently higher in LN supernatants than in sera.

Taken together, these results suggest that, similarly to that observed in adults, LN MC are a major reservoir for the virus in HIV-infected children.

Cytokine and chemokine gene expression in LN and PB MC from HIV-infected children

Freshly isolated LN and PB MC were tested for cytokine and chemokine gene expression by RT-PCR. All of the LN and PB MC samples consistently expressed IFN-γ mRNA (Figure 5). In contrast, IL-2, IL-4 or IL-5 transcripts were never detected in any sample (not shown). IL-12 p35 mRNA was ubiquitously expressed (not shown), whereas IL-12 p40 mRNA was detected in 3/5 LN MC samples and in 2/5 PB MC samples (Figure 5). Notably, the 2 cases lacking IL-12 p40 mRNA in LN MC (patients #2 and #3) expressed it in PB MC, whereas the opposite was observed for the 3 remaining cases (Figure 5). IL-10, TGF-β1, RANTES, MIP-1α, MIP-1β and IL-16 transcripts were detected in all samples (Figure 5).

Control experiments were performed with LN MC from 6 children with HIV-unrelated reactive lymphadenopathies and with PB MC from 3 age-matched healthy children. The transcripts of IL-2, IL-4, IL-5, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TGF-β1, RANTES, MIP-1α, MIP-1β and IL-16 were found in every sample (not shown).

Next, the expression of IFN-γ, IL-10, TGF-β1, RANTES, MIP-1α, MIP-1β and IL-16 mRNA was investigated in CD4+, CD8+ and CD4−,CD8− cell fractions isolated from the LN of 4 HIV-infected children. Due to the limited number of cells available, similar experiments were not carried out with PB MC. IFN-γ mRNA was expressed exclusively by CD8+ cells, whereas all the other cytokine transcripts were detected in CD4+, CD8+ and CD4−,CD8− cells. Table 2 shows one
representative experiment out of the four carried out with similar results. These findings indicate that, in the early stages of pediatric HIV infection, IFN-γ expression in CD4+ LN T-cells is impaired, whereas expression of chemokines that inhibit HIV infection is consistently detected in all LN MC subsets.

Impaired proliferative responses of LN and PB MC from HIV-infected children to stimuli that signal through the CD3 T-cell receptor (TCR) complex: potential role of the TGF-β1 and IL-10 immunosuppressive cytokines

In subsequent experiments, LN and PB MC from HIV-infected and control children were tested for proliferative responses to: 1) low dose rIL-2, that expands preferentially in vivo activated T-cells, as well as the PHA and CD3 mAb polyclonal T-cell mitogens; 2) recall antigens (TT, Ca and PPD) and 3) a panel of HIV antigens. The stimuli mentioned in 1) trigger proliferation of both CD4+ and CD8+ T-cells, whereas soluble antigens, either recall or HIV-related, selectively stimulate CD4+ T-cell proliferation.

The responses detected in these experiments were defined as positive or negative on the basis of minimum threshold values obtained from the study of 35 age-matched children and detailed in the Design and Methods section as well as in the legend to Figure 6.

Response of MC to rIL-2 was found in 2/5 PB and in 0/5 LN samples (Figure 6, panel a). Reactivity to PHA was detected in all LN and in 4/5 PB samples, whereas proliferative responses to CD3 were absent in all LN and in 3/5 PB samples (Figure 6, panel a). Proliferation to TT was detected only in 1/5 LN and 1/5 PB samples (Figure 6, panel b). Responses to Ca were observed in 2/5 PB and in 3/5 LN samples, whereas PPD specific proliferation was detected in 3/5 PB samples but not in any LN sample (Figure 6, panel b). Responses to HIV antigens were variable, but altogether low, in both LN and PB samples (Figure 6, panel c).

The proliferative responses of LN and PB MC from the individual HIV-infected children to recall and HIV antigens were as follows. In patient #1, PB MC responded to Ca, PPD, gp160, gp120 peptides, RT peptides and whole inactivated HIV virions, whereas LN MC responded to TT and Ca, but not to any of the HIV-derived antigens. PB MC from patient #2 did not respond to any recall antigen, whereas a response to HIV virions was detectable; in contrast, LN MC were responsive to Ca and, weakly, to RT peptides and HIV. In patient #3, no response was detected in PB or LN MC to recall or to HIV antigens. In patient #4, PB MC responded to PPD, but not to any other antigen; LN MC responded to Ca and to p24 peptides. Patient #5 had PB MC which responded to TT, Ca, PPD and to all of the HIV antigens in the absence of response to whole virions. In contrast, no response of LN MC to any recall or HIV antigen was detected (Figure 6).

The above experiments show that LN and PB MC from HIV-infected children express various cytokine transcripts, including those of IL-10 and TGF-β1. The latter molecules mediate immunosuppressive effects in several experimental systems. Furthermore, IL-10 has been identified as a contributory factor to T-helper cell dysfunction in asymptomatic HIV-infected adults.

We, therefore, investigated by quantitative RT-PCR the expression of IL-10 and TGF-β1 mRNA in paired LN and PB MC samples from the 5 HIV-infected children. As shown in Figure 7, IL-10 mRNA concentration of TGF-β1 mRNA was 4-10 fold higher than that of IL-10 mRNA. A similar TGF-β1/IL-10 mRNA ratio was detected in four paired PB MC samples (#1, 2, 4 and 5), whereas in patient #3 this ratio was reversed.

In conclusion, all of the stimuli that signal through the CD3-TCR complex (such as recall antigens, HIV antigens and the CD3 mAb) were poorly effective at inducing T-cell proliferation from both PB and LN MC of HIV-infected children. The results obtained in the experiments with soluble antigens indicate that CD4+ cells from both compartments had severe impairment of antigen-specific responses. In addition, as shown by quantitative PCR, TGF-β1, more than IL-10, may play a role in T-cell immunosuppression.

Discussion

The aim of this study was to investigate patterns of cytokine gene expression and proliferative responses to antigens and polyclonal mito-

Table 2. Cytokine gene expression in fractionated mononuclear cell subsets from lymph nodes of HIV-infected children.

<table>
<thead>
<tr>
<th>Cytokine</th>
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Samples from 4 different patients were tested with identical results. One representative experiment is shown. + and - indicate the presence or absence of specific cytokine transcripts.
gens in LN MC from HIV-infected children with lymphadenopathy at an early stage of their disease, in the context of LN morphology and viral load. As for the latter issue, our immunocytochemical and ultrastructural investigations show that LN represent an early viral reservoir, with a predominant HIV localization within the interdendritic spaces of GC. The LN is also the site of a specific anti-HIV response due to the production of antibodies, predominantly IgM λ, by plasma cells that are located within the GC. All of these events occur when infectious HIV is poorly detectable in the PB.

Within the LN, disease progression in adult patients is accompanied by a shift from a Th1 to a Th0/Th2 pattern of cytokine gene expression associated with downregulation of specific anti-HIV immunity and faster viral replication.6-10 In this study, neither freshly isolated LN MC nor paired PB MC expressed IL-2, IL-4 or IL-5 genes, whereas IFN-γ gene expression was detected in all samples and found to be restricted to CD8+ cells.8 In this respect, it is of note that MC freshly isolated from LN of children with non-HIV related inflammatory lymphadenopathies or from PB of age-matched healthy children consistently displayed IL-2, IL-4, IL-5 and IFN-γ transcripts, thus highlighting pro-

Figure 6. Proliferative responses of LN and PB MC from HIV-infected children to: a) polyclonal stimuli, i.e. IL-2, PHA and a CD3 mAb; b) recall antigens, i.e. TT, Ca and PPD and c) HIV antigens, i.e. gp160; pooled peptides (pp) of gp120, RT and p24, respectively; inactivated whole virions (HIV). Cells were cultured for 4 days with or without stimuli, and subsequently harvested. Cell proliferation was determined by 3H-thymidine incorporation. Results are expressed as a proliferation index, i.e. the ratio between cpm measured in the stimulated versus the corresponding unstimulated (control) cultures. Round symbols indicate LN MC, whereas square symbols are PB MC. Dashed lines in each histogram indicate the threshold values that define a positive response. Such values were obtained in 90% of MC samples from PB of 35 age-matched healthy children and from LN of 6 age-matched children with HIV-unrelated inflammatory lymphadenopathies (see Study design and Patients paragraph of the Design and Methods section).

Figure 7. Quantitative RT-PCR analysis of IL-10 and TGF-β1 gene expression in freshly isolated, paired LN and PB MC samples. Results are expressed as cDNA attomoles x10^-1.
found alterations in the patterns of Th1/Th2 cytokine gene expression that occur in early pediatric HIV infection.

Since IL-12 plays a pivotal role in the induction of Th1 cell differentiation, we investigated IL-12 gene expression in LN and PB MC from our patients. IL-12 p40 mRNA, which is selectively expressed in antigen-presenting cells and correlates with the production of the biologically active heterodimeric cytokine, was detected in 3/5 LN and 2/5 PB samples that were mutually exclusive, likely reflecting individual differences in the recirculating ability of IL-12-expressing cells. Thus, although IL-12 production is impaired in HIV-infected adults with advanced disease, this may not be the case in the early stages of pediatric HIV infection. It is conceivable that additional factors beside IL-12 deficiency contributed to the inability of CD4+ cells to express the IFN-γ gene in our patients. For example, the product of the HIV nef gene downregulates in vitro IL-2 and IFN-γ production by CD4+ cells.

A previous study addressed the issue of cytokine patterns in freshly isolated PB MC from a large number of HIV-infected children. Constitutive expression of TNF, IFN-γ and IL-10 mRNA was found to be increased, whereas that of IL-12 was decreased. No IL-2 or IL-4 transcripts were detected. Although these findings are largely consistent with our results, it should be emphasized that patients enrolled in Tham’s study were heterogeneous as far as concerns disease stage and that no experiments were addressed towards the investigation of cytokine gene expression in LN cell suspensions.

Transcripts of RANTES, MIP-1α, MIP-1β and ILL6, which have been shown to inhibit in vitro HIV entry into target cells, were detected in LN and PB MC from HIV-infected patients and control subjects, as well as in CD4+CD8+ and CD4+CD8− LN cell subsets from the former group. These findings may suggest that M C from HIV-infected children with early stage disease retain the ability to express the above chemokine genes. In this connection, it is of note that a direct relationship between decreased chemokine production and AIDS progression has been reported in various studies.

The proliferative responses of LN and PB MC to recall antigens, HIV antigens or CD3 mAb were severely impaired. In contrast, PHA-induced proliferation was measurable in all patients, consistent with the notion that PB MC from HIV-infected adults retain PHA reactivity until advanced stages of disease.

Helper T-cell responses of PB MC from HIV-infected children to recall antigens (influenza A virus, tetanus toxoid), alloantigens or PHA were the subject of a previous study which identified four patterns of T-cell reactivity: i) response to all stimuli; ii) response to alloantigens and PHA, but not to recall antigens; iii) response to PHA but not to recall antigens or alloantigens and iv) no response to any stimulus. These results had prognostic value since children with functional helper T-cell defects showed a history of more numerous opportunistic and bacterial infections in comparison with patients with intact helper T-cell function.

Several mechanisms contribute to T-helper cell dysfunction in HIV-infected patients, such as HIV-related CD4+ cell death or anergy, defective production of immunostimulating cytokines, cytokine-mediated immunosuppression and impaired thymic generation of T-lymphocyte precursors caused by HIV infection. These mechanisms are not mutually exclusive and presumably operate in concert to determine progressive immune deficiency in HIV-infected individuals.

HIV-related CD4+ cell death may be the result of a direct cytopathic effect of the virus on cycling infected cells or of bystander mechanisms, such as binding of the viral gp120 to the CD4 surface molecule of HIV infected cells. Defective production of immunostimulating cytokines is exemplified by low to absent IL-2, IL-4 and IL-12 gene expression in freshly isolated PB MC of HIV-infected individuals, as well as by severely impaired stimulus-induced IL-2 production resulting in decreased antigen-specific T-cell proliferation.

TGF-β1 and IL-10 are potent immunosuppressive cytokines in different experimental systems. In the case of IL-10, it has been shown that this cytokine participates in the impairment of antigen-specific T-helper cell responses in HIV-infected patients. In this study, the transcripts of IL-10 and TGF-β1 were detected in all patients’ LN and PB MC, but the concentration of TGF-β1 mRNA was 4-10 fold higher than that of IL-10 mRNA, suggesting that TGF-β1 may play a more relevant role than IL-10 in immune suppression associated with pediatric early stage HIV infection. Notably, TGF-β1 and IL-10 appear to suppress or decrease HIV replication in human macrophages and/or lymphocytes. A final hypothesis that may help explain T-cell hyporesponsiveness to antigen-specific stimulation in our patients is that perinatally acquired HIV infection of the thymus influences the process of intra-thymic T-cell selection and differentiation leading to developmental defects of the T-cell lineage.

In conclusion, this study demonstrates that children with early stage HIV infection display deranged patterns of Th1/Th2 cytokine gene expression and severely impaired T-cell proliferative responses to soluble antigens both in the
LN and in the PB compartments. Higher concentrations of infectious virus are detected in the former site since the lymph node serves as an HIV reservoir and the local microenvironment supports viral replication.

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IA and FG had primary responsibility for planning and performing the RT-PCR studies. AF was responsible for histologic and immunohistochemical analyses. CT and CG planned and performed electron microscopy studies. DS, PF, GP, GLiP, DF and FM were responsible for cell handling, separation and culture, as well as for flow cytometric analyses and the p24 assay. AP and MD cared for the patients and designed the clinical study. CEG, EC, AP and VF drafted the article and revised it critically. VP and CEG prepared the final version of the manuscript that was approved by all the Authors.

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Potential implications for clinical practice
- The above data may have important clinical implications in terms of better knowledge of the natural history of pediatric HIV infection and planning novel therapeutic trials.

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