Characterization of Quantitative and Functional Innate Immune Parameters in HIV-1–Infected Colombian Children Receiving Stable Highly Active Antiretroviral Therapy

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Background: The immunological benefits of highly active antiretroviral therapy (HAART) in HIV-1–infected children include reconstitution of CD4+ T-cell count and functional activity. The effect of HAART on innate immune cells has not been well established.

Aim: To characterize innate immune responses in HAART-treated HIV-1–infected children.

Patients and Methods: 23 HIV-1–infected children on stable HAART and 23 uninfected children were evaluated. The frequency of innate immune cells in peripheral blood was determined by flow cytometry and functional activity was evaluated using Toll-like receptor agonists.

Results: Compared with uninfected children, HAART-treated HIV-1–infected children exhibited a significant decrease in the frequency of plasmacytoid dendritic cells and natural killer and T-cell receptor (TCR)-invariant CD1d-restricted T cells. This deficiency of innate immune cells was observed mainly in children with detectable viral load. We also compared the magnitude of the quantitative restoration of those cells comparing HIV-1–infected children with HIV-1–infected adults and found a partial effect of HAART on immune restoration that was independent of age. In both pediatric and adult subjects Toll-like receptor agonists induced expression of costimulatory molecules and production of proinflammatory cytokines by dendritic cells. Peripheral blood mononuclear cells of HIV-1–infected children produced significantly reduced amounts of interferon-α compared with uninfected children.

Conclusions: HAART administration to HIV-1–infected children does not lead to a complete increase of circulating innate immune cells, particularly in patients with incomplete suppression of HIV.

Key Words: innate immune cells, HAART, pediatric HIV-1 infection

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INTRODUCTION

Although vertical transmission of HIV-1 infection has decreased considerably, particularly in developed countries, in 2006, 2.3 million children were living with HIV-1 infection worldwide and 0.38 million have died as a consequence of clinical AIDS.[1] In contrast to adults, HIV-1 infection in children is characterized by a higher level of viral replication and more rapid and severe clinical evolution, including chronic and recurrent infections with non opportunistic microorganisms.[2] As a result, early administration of highly active antiretroviral therapy (HAART) in HIV-1–infected children is necessary to reduce patient mortality.[3] The effects of HAART on inhibiting HIV-1 replication and decreasing the plasma viral load have led to significant reductions in HIV-1–related morbidity and mortality and are a highly cost-effective medical intervention.[4–6]

The benefits of HAART have been attributed to improvements in immune function, mainly those associated with reconstitution of the CD4+ T-cell count and functional activity.[7–9] For example, it was reported that those individuals who effectively control HIV-1 replication with HAART exhibit an increase in the frequency of peripheral blood CD4+ T cells and an improvement in the in vitro lymphoproliferative responses to several antigens. This pattern of immune reconstitution was particularly evident when therapy was initiated early during HIV-1 infection.[8,9] However, several studies have shown that HAART (administered in chronically HIV-1–infected individuals) induces only a partial immune reconstitution, without complete normalization of immune parameters such as immune activation or persistent impairment of the anti–HIV-1-specific T-cell responses.[10,11] Nevertheless, the majority of studies evaluating HAART-associated immune reconstitution have been performed mainly in adults and have evaluated only adaptive immune parameters while few studies have

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investigated restoration of the innate immune response in HIV-1–infected children. However, one of the few studies addressing this issue has reported a greater impact of HAART on myeloid dendritic cells (mDCs) than that observed on plasmacytoid dendritic cells (pDCs) in both HIV-1–infected adults[12] and in pediatric cohorts.[13]

Recent observations emphasize the dual role that different components of the innate immune response play during HIV-1 infection; several components have demonstrated strong anti–HIV-1 activity[14] but some innate immune cells are also targets for viral infection.[15–17] Indeed, HIV-1–infected individuals have quantitative and functional abnormalities in innate immune cells, which correlate with the level of viral replication.[18,19] It is postulated that these abnormalities in the innate immune response during chronic HIV-1 infection may enhance the immunodeficiency caused by the progressive lost of CD4+ T cells. Consequently, HAART administration that is effective in controlling HIV-1 replication should be associated with improvement in innate immune parameters and might explain the decreased morbidity in HIV-1–infected patients receiving suppressive HAART.[4,20]

The impact that HAART has on the reconstitution of innate immunity in HIV-1–infected children has not been extensively studied. To explore the status of innate immune parameters in HIV-1+ children on HAART at least for 1 year, we performed a cross-sectional quantitative and qualitative study of the innate immune system in HIV-1–infected children receiving stable HAART compared with uninfected age- and gender-matched children. The frequencies of peripheral blood mDCs and pDCs, monocytes, natural killer (NK) cells, and T-cell receptor (TCR)-invariant CD1d-restricted T cells (iNKT cells) and functional responses of pDCs and mDCs after in vitro stimulation with Toll-like receptor (TLR) agonists were evaluated. Despite HAART being effective in inhibiting HIV-1 replication in this group of HIV-1–infected children, some quantitative and functional alterations were still found in the cells involved in innate immunity.

MATERIALS AND METHODS

Study Population

Twenty-three chronically HIV-1–infected children were recruited through the outpatient service of the ESE Hospital La María and the AIDS Pediatric Foundation, Sí Futuro, in Medellin, Colombia, and 23 chronically HIV-1–infected adults were recruited from the outpatient service of the Hospital Universitario San Vicente de Paul in Medellin, Colombia. Ninety-five percent of the HIV-1–infected children (22/23) acquired the infection by vertical transmission; in 1 child the source of infection is unknown. All patients were on stable HAART for at least 1 year; the treatment was initiated at the time of HIV-1 diagnosis and no other antiretroviral therapy was initiated before HAART (demographic and clinical information in Table 1). The pediatric HIV-1–infected patients were additionally classified according to the effect of HAART on control of viral replication, and 2 groups were identified: patients with undetectable viral load (VL <400 copies/mL) and patients with detectable viral load (VL >400 copies/mL). In addition, 23 age- and sex-matched HIV-uninfected healthy children and 23 healthy adults without known risk factors for HIV-1 infection were evaluated; their HIV-1 status was confirmed by a negative serum enzyme-linked immunosorbent assay (ELISA).

This study was approved by the University of Antioquia Review Board. A clear explanation of the objectives and the implications of the results were given to each participant. An institution-approved informed consent was subsequently signed by the adult individuals or children's parent/legal guardians, according to the Colombian Government resolution 00843 of 1993's legislation.

Reagents

Fluorochrome-labeled mouse monoclonal antibodies (mAbs) against human molecules CD3, CD4, CD8, CD11c, CD14, CD16, CD25, CD45RA, CD56, CD69, CD80, CD86,

TABLE 1. Demographic and Clinical Information of Studied Individuals

	Age in Years, Median (Range)	Gender, Male/Female	Immunologic Category* 1/2/3	Clinical Category* A/B/C	Years on HAART, Median (Range)	CD4+ T-Cell Count, Median (Range)	CD4+ T-Cell Percentage, Median (Range)	Viral Load, Median (Range)
HIV-1+ children total (n = 23)	7 (2–14)	10/13 (43%/57%)	10/11/2 (43%/48%/9%)	10/10/3 (43%/43%/13%)	4 (1–7)	721 (294–2157)	25.64 (8.10–49.79)	12,200 (<400 to 253,000)
HIV-1+ children VL <400 (n = 11)	7 (2–12)	6/5 (55%/45%)	5/5/1 (45%/46%/10%)	5/4/2 (46%/36%/18%)	3 (1–5)	785 (426–2157)	30.18 (19.85–49.79)	400 (<50 to <400)
HIV-1+ children VL >400 (n = 12)	7 (3–14)	4/8 (33%/67%)	5/6/1 (42%/50%/8%)	4/7/1 (33%/59%/8%)	4 (1–9)	703 (294–949)	22.68 (8.10–42.70)	12,200 (2390 to 253,000)
Uninfected children (n = 23)	7 (2–14)	10/13 (43%/57%)	NA	NA	NA	1346 (525–2039)	36.84 (27.99–48.83)	NA
HIV-1+ adults (n = 23)	41 (21–60)	21/5 (78%/22%)	1/2/20 (4%/9%/87%)	2/4/17 (9%/17%/74%)	4.6 (2–12)	272 (31–816)	18.08 (6.48–41.19)	71,800 (<400 to 1,160,000)
Uninfected adults (n = 23)	38 (20–60)	13/10 (57%/43%)	NA	NA	NA	768 (446–1515)	54.68 (42.90–67.72)	NA

NA: not applicable.
*According with the Centers for Disease Control and Prevention classification system.[21]

CD123, HLA-DR, lineage markers (Lin-1, a mixture of anti-CD3, CD14, CD16, CD19, CD20, and CD56), the CDR3 region of the invariant TCR a chain (clone 6B11), and the corresponding isotype control antibodies were from Becton Dickinson-Pharmingen (San Jose, CA). Fcγ-receptor–blocking reagent was from Miltenyi Biotec (Bergisch Gladbach, Germany).

The Class A CpG ODN 2216 (sequence 5′-ggGGGACGATCGTCgggggG- 3′; stock at 1 μg/mL) was kindly provided by Coley Pharmaceutical Inc. (Wellesley, MA) and had no detectable endotoxin. Lipoteicoic acid (LTA) was from InvivoGen (San Diego, CA; stock at 2 μg/mL).

Culture of Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole-blood, by density-gradient centrifugation using lymphocyte separation medium (BioWhittaker, Walkersville, MD). Viability of PBMCs was determined by trypan blue exclusion. PBMCs (1×10^6/mL) were suspended in complete culture media (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 U/mL penicillin (Sigma, St Louis, MO), 100 mg/mL streptomycin (Sigma), and 2 mM of L-glutamine (Sigma) and stimulated with/without LTA at 0.2 mg/mL or Class A CpG ODN 2216 at 4 mg/mL, for 24 hours at 37°C/5% CO_2. Culture supernatants were collected and stored at −70°C until cytokine determination. All samples were processed within 2 hours of sample collection.

Flow Cytometry

The frequency and phenotype of pDCs (defined as Lin−/CD123+/HLA-DR+), mDCs (Lin−/CD11c+/HLA-DR+), monocytes (CD14+/CD3−), NK cells (CD3−/CD16+/CD56+), iNKT cells (6B11+/CD3+), and iNKT subpopulations (according to the expression of CD4 and/or CD8 molecules) in whole blood was determined by flow cytometry. Briefly, 100 mL of anticoagulated blood was incubated with the specific mAbs for 20 min/room temperature in the dark. The erythrocytes were lysed by incubating for 10 min with 2 mL of 1X fluorescence-activated cell sorting lysing solution (Becton Dickinson, San Jose, CA); then, cells were washed twice with 2 mL of cold phosphate-buffered saline (PBS) at 250*g*/5 min and fixed with 250 mL of 2% formaldehyde.

To evaluate surface molecule expression on cells after culture, PBMCs collected from wells were washed with cold PBS; nonspecific mAb binding was controlled by blocking Fcg receptors with 20 mL of blocking reagent (Miltenyi Biotec) for each 1×10^7 cells (20 min/4°C). PBMCs were washed with PBS and resuspended in cytometry buffer (PBS, 0.5% bovine serum albumin and 0.1% NaN_3) at 1×10^7/mL, and 100 mL of this cell suspension was incubated with the required combination of mAbs for 20 min/RT in the dark. Finally, PBMCs were washed with cytometry buffer, fixed with 2% formaldehyde, and stored at 4°C until analysis.

Appropriate isotype-matched control antibodies were included. Due to the low frequency of innate cells in PBMCs, 1 to 5×10^5 total cells were analyzed. Dead cells were gated out by forward and side scatter. Flow cytometry was performed using the Becton Dickinson FACSort instrument and analyzed with BD CellQuest Pro software. The absolute number of the different leukocyte subpopulations was calculated on the basis of manually determined total and differential peripheral blood cell counts.

Cytokine Determination

Interleukin-12 (IL-12p70), IL-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α concentration was determined by flow cytometry using the Becton Dickinson Cytometric Bead Array (Human Inflammatory Kit, Becton Dickinson Biosciences Pharmingen, San Diego, CA) and following manufacturer's recommendations. An ELISA kit was used to measure interferon-α (IFN-α) following the manufacturer's instructions (PBL Biomedical Laboratories, Piscataway, NJ).

Statistical Analysis

Statistical comparison between 2 groups was performed using the unpaired nonparametric Student *t* test (Mann–Whitney *U* test), while comparisons among 3 or more groups were performed using the analysis of variance nonparametric Kruskal–Wallis test. P value <0.05 was considered to be statistically significant. All the statistical tests were performed using the Graph-Pad Software version 5.00.

RESULTS

Decreased Frequency of pDCs in HIV-1–Infected Children

When we compared the frequency of innate immune cells with the antigen-presenting ability between HIV-1–infected children and uninfected controls, we observed that the infected children had a significantly decreased absolute number (median: 5.54 cells/μL, range min–max: 2.26–21.55 vs. 10.24 cells/μL, 2.79–40.95, respectively, Fig. 1A) and also a decrease in the percentage of peripheral blood pDCs (0.15%, 0.07%–0.51% vs. 0.29%, 0.11%–0.79%, respectively. $P = 0.0086$; graphic data for percentage not shown). The absolute number and percentage of peripheral blood mDCs and monocytes was similar between HIV-1–infected children and uninfected controls (Figs. 1B, C; graphic data for percentage not shown).

When the HIV-1–infected children were classified according to their level of plasma viral load, we observed that the absolute number of pDCs were significantly decreased in both the HIV-1–infected children with undetectable (VL <400 copies/mL; 6.39, 3.03–21.55) and detectable (VL >400 copies/mL; 3.35, 2.26–10.59) viral load compared with uninfected children (10.24, 2.79–40.95). A similar finding was obtained when the frequency of pDCs was determined as percentage: The HIV-1–infected children with undetectable (VL <400 copies/mL; 0.23, 0.10–0.51) or detectable viral load (VL >400 copies/mL; 0.13, 0.06–0.33) had a lower percentage of pDCs compared with uninfected children (0.28, 0.11–0.79). However, the HIV-1–infected children with detectable viral load had a significantly lower number of pDCs compared with HIV-1–infected children with undetectable viral load (Fig. 1D). There were no significant differences in the absolute numbers or percentages of peripheral blood mDCs and monocytes when we compared both subgroups of

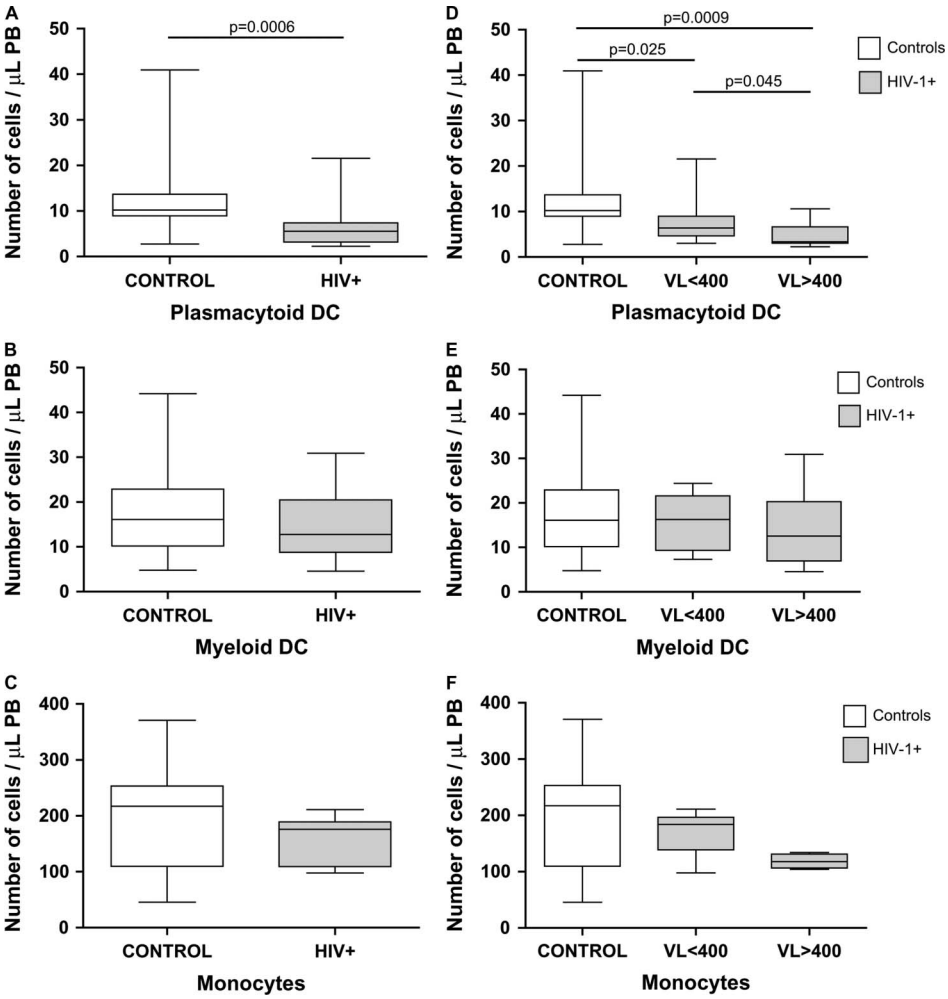

FIGURE 1. Quantitative evaluation of peripheral blood innate immune cells of myeloid lineage. Absolute number of innate immune cells with antigen-presenting ability per milliliter of peripheral blood was determined in HIV-1+ children (n = 23) and uninfected controls (n = 23, but monocytes n = 13, based on sample availability), establishing their frequency by flow cytometry and adjusting according with the manually determined total and differential peripheral blood cell counts (A, B, C). To establish the quantitative effect of controlling viral replication, on innate immune cells HIV-1+ children were additionally grouped according to their plasma viral load (D, E, F). Results are presented as median, 25%, and 75% percentiles and range. Statistical comparison between 2 groups was performed using the unpaired Student *t* test (Mann–Whitney *U* test), whereas comparisons among 3 or more groups were performed using the analysis of variance nonparametric Kruskal–Wallis test, with a confidence level of 95%. Significant differences are indicated on the top of the figure. PB: peripheral blood; DC: dendritic cell.

HIV-1–infected children (with detectable or undetectable viral load) and uninfected controls (Figs. 1E, F; graphic data for percentages not shown).

Decreased Frequency of NK and iNKT Cells in HIV-1–Infected Children

When we compared the absolute number of peripheral blood innate immune cells of lymphoid lineage between HIV-1–infected children and uninfected controls, we observed that the infected children had a significantly decreased number of NK cells (216 cells/μL, 12.95–491 vs. 371 cells/μL, 6.76–894, respectively; Fig. 2A) and iNKT cells (1.83 cells/μL, 0.48–6.95 vs. 3.72 cells/μL, 0.42–57.66, respectively; Fig. 2B). These differences were also observed when we compared the percentage of NK cells (8.84%, 0.33%–18.15% vs. 11.54%, 0.16%–20.17%; $P = 0.0313$) and iNKT cells (0.06%, 0.02%–0.19% vs. 0.14%, 0.01%–1.12%; $P = 0.0160$; graphic data for percentages not shown).

In addition, we observed a significant decrease in the absolute number of peripheral blood NK cells in HIV-1–infected children with undetectable viral load compared with uninfected controls (217 cells/μL, 74.54–491 vs. 371 cells/μL, 6.76–894; Fig. 2C) and of iNKT cells in both HIV-1–infected children with undetectable (1.46 cells/μL, 0.66–6.96 vs. 3.72 cells/μL, 0.42–57.66) and detectable viral load (2.07 cells/μL, 0.49–4.62 vs. 3.72 cells/μL, 0.42–57.66; Fig. 2D), when compared with uninfected controls.

Subsets and Phenotype of iNKT Cells in HIV-1–Infected Children

Peripheral blood iNKT cells were phenotypically and functionally classified based on their expression of surface molecules, such as CD4 and CD8.[22] The frequency of the different subsets of iNKT cells was similar between HIV-1–infected children and uninfected controls (Fig. 3A):CD4+ iNKT cells: 30.59% (8.33%–59.3%) vs. 31.42% (10%–57.14%), respectively; CD8+ iNKT cells: 9.16% (2.85%–40.28%) vs. 15.81% (0.0%–29.47%), respectively; CD4+/CD8+ (double-positive) iNKT cells: 2.85% (0.0%–8.33%) vs. 4.94% (0.0%–26.5%), respectively; and CD4−/CD8− (double-negative) iNKT cells: 53.41% (30%–78.57%) vs. 46.25% (10.26%–5.71%), respectively. When we compared the frequency of these iNKT cell subpopulations among healthy controls and both subgroups of HIV-1–infected

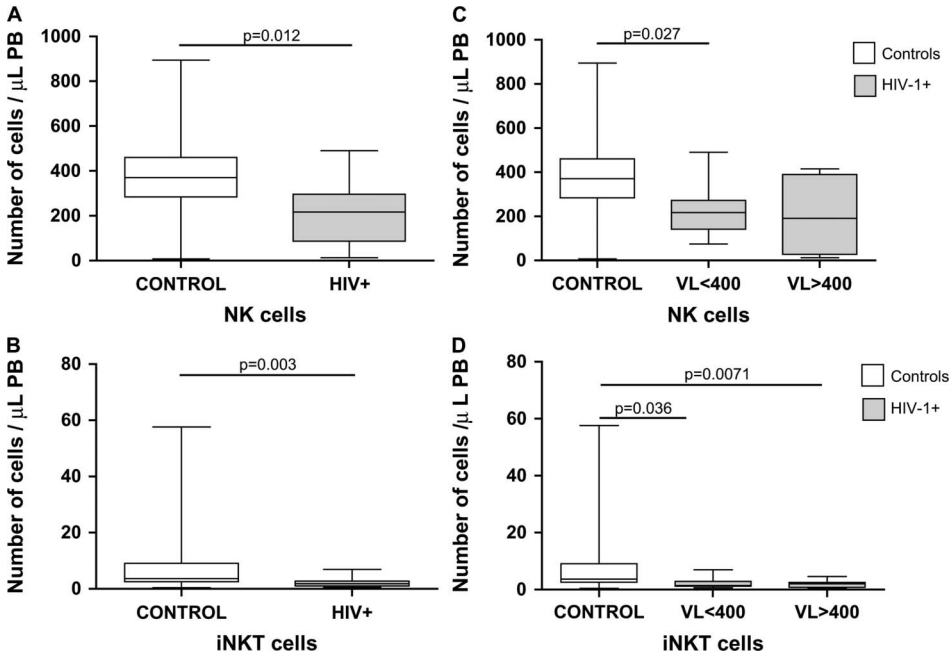

FIGURE 2. Quantitative evaluation of peripheral blood innate immune cells of lymphoid lineage. Absolute number of innate immune cells of lymphoid origin per milliliter of peripheral blood was determined in HIV-1+ children (n = 23) and uninfected controls (n = 23, but monocytes n = 13) establishing their frequency by flow cytometry and adjusting according with the manually determined total and differential peripheral blood cell counts (A, B). To establish the quantitative effect on innate immune cells of controlling viral replication, HIV-1+ children were additionally grouped according to their plasma viral load (C, D). Results are presented as median, 25%, and 75% percentiles and range. Statistical comparison between 2 groups was performed using the unpaired Student *t* test (Mann–Whitney *U* test), whereas comparisons among 3 or more groups were performed using the analysis of variance nonparametric Kruskal–Wallis test, with a confidence level of 95%. Significant differences are indicated on the top of the figure. PB: peripheral blood.

children (based on their viral load), we did not observe any significant difference (data not shown).

In addition, HIV-1–infected children had a significantly higher percentage of iNKT cells expressing HLA-DR (65.79%, 8.75%–87.95% vs. 25.4%, 3.59%–100%) and CD45RA+ (60.71%, 3.06%–84.94% vs. 23.11%, 0.99%–79.07%) when compared with uninfected children (Fig. 3B). The frequency of peripheral blood iNKT cells expressing CD16, CD25, CD56, and CD69 was similar in HIV-1–infected children and healthy controls (Fig. 3B).

The Partial Immune Restoration Associated With HAART Is Similar in Chronically HIV-1–Infected Children and Adults

In most of the HIV-1–infected patients, control of HIV-1 replication by HAART is associated with quantitative and qualitative restoration of immune responses, including an increase in the absolute number of different leukocyte subpopulations in peripheral blood.[23,24] The degree of HAART-associated immune restoration is influenced by several host and viral factors, such as age and level of thymic activity, the magnitude of lymphoid tissue damage dependent on HIV-1 infection, viral resistance, drug toxicity, and poor adherence to medications.[25,26] Children and adults have been shown to differ in their immune reconstitution to several diseases.[27,28] Therefore, we compared the magnitude of the quantitative restoration of innate immune cells between our group of HIV-1–infected children and a group of HIV-1–infected adults on stable HAART for similar periods of time (demographic and clinical information in Table 1). We also established the proportion (ratio) of the absolute number of each innate immune cell subpopulation between each HIV-1–infected subject (child or adult) and his/her corresponding uninfected control. As shown in Table 2, the magnitude of the quantitative reconstitution was similar in HIV-1+ children and HIV-1+ adults for pDCs (51% vs. 53%, respectively; $P = 0.9475$), mDCs (78% vs. 50%; $P = 0.0506$), monocytes (83% vs. 97%; $P = 0.6081$), NK cells (54% vs. 78%; $P = 0.0727$), and iNKT cells (35% vs. 48%; $P = 0.8951$), indicating a similar partial effect of HAART on immune restoration in HIV-1–infected individuals independent of their age.

Normal Upregulation of CD80 and CD86 in DCs From HIV-1–Infected Children

Surface expression of CD80 and CD86 molecules on both dendritic cell subpopulations was evaluated after incubation of PBMCs with TLR agonists. The stimulation with a Class A CpG ODN (agonist of TLR9, expressed by pDCs) upregulated the expression of CD80 and CD86 on pDCs from both uninfected controls and HIV-1–infected children (Fig. 4A), whereas the incubation with LTA (agonist of TLR2, expressed on mDCs) upregulated the expression of CD80 on mDCs (Fig. 4B). Because mDCs spontaneously upregulate the expression of CD86 after overnight cell culture,[29] the expression of this molecule on mDCs after the stimulation with LTA was similar to that observed in unstimulated cell cultures (Fig. 4B).

When we compared the net increase (level in TLR agonist–stimulated cells minus level in unstimulated cells) utilizing mean fluorescence intensity for CD80 or CD86 expression on pDCs or mDCs, we did not find any significant difference between HIV-1–infected children and uninfected

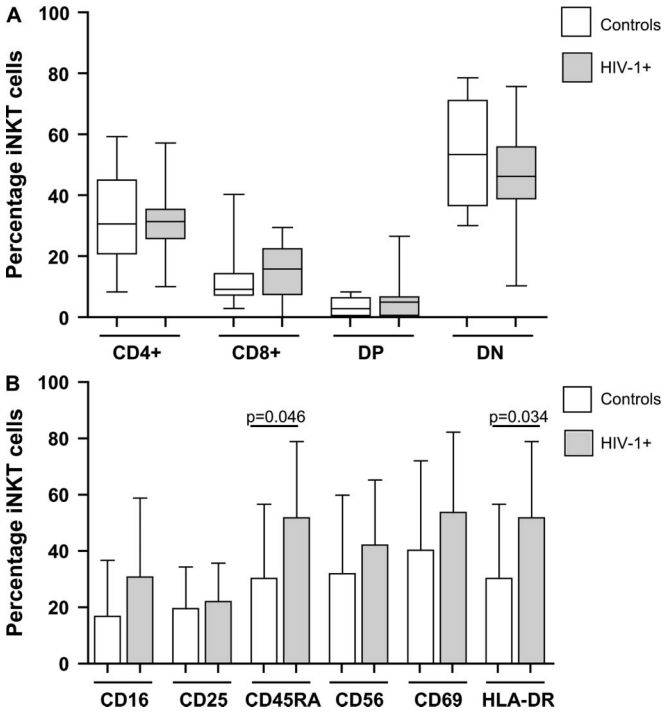

FIGURE 3. Phenotypic evaluation of iNKT subpopulations. A, Frequency of different iNKT subpopulations (based on CD4 or CD8 expression) was determined in HIV-1+ children (n = 16) and uninfected controls (n = 16) by flow cytometry. Results are presented as median, 25%, and 75% percentiles and range. B, The percentage of iNKT cells expressing surface phenotypic markers (CD16, CD25, CD45RA, CD56, CD69, and HLA-DR) was evaluated in healthy controls (n = 15) and HIV-1–infected children (n = 15) by flow cytometry. Data are presented as mean and standard error. Significant differences are indicated on the top of the figure. Statistical comparison between the 2 study groups for each marker was performed using the Mann–Whitney *U* test, with a confidence level of 95%. DP: double-positive (CD4+/CD8+); DN: double-negative (CD4−/CD8−).

controls (Fig. 4C). Also, the net increase of CD80 and CD86 expression was similar among healthy controls and both subgroups of HIV-1–infected children (based on viral load; data not shown).

Reduced Secretion of IFN-α in HIV-1–Infected Children

The concentration of IFN-α was evaluated in the supernatants of PBMCs stimulated with or without Class A CpG ODN. A significantly reduced net concentration (concentration in stimulated minus concentration in unstimulated supernatants) of IFN-α was found in supernatants from HIV-1–infected children (164.2 pg/mL, 3.5–1768) when compared with that from healthy controls (441.5 pg/mL, 3.5–1199; Fig. 5A). When we classified the HIV-1–infected children with respect to their level of viral load, no significant differences were found between infected children with or without detectable viral load. There was a significantly reduced IFN-α concentration in supernatants from infected children with detectable viral load (64 pg/mL, 3.5–1768) compared with uninfected children (441.5 pg/mL, 3.5–1199, Fig. 5B). Despite that, HIV-1–infected children with undetectable viral load also had a lower concentration of IFN-α, and this difference was not significant when compared with uninfected controls ($P = 0.0535$; Fig. 5B).

Normal Production of Proinflammatory Cytokines and IL-10 in HIV-1–Infected Children

The concentration of proinflammatory cytokines (IL-1β, IL-6, IL-12p70, TNF-α) and IL-10 was determined in supernatants from PBMCs stimulated with or without LTA. Although this stimulation was more efficient for inducing the secretion of IL-1β, IL-6, and TNF-α, a similar net concentration of all the cytokines analyzed was found in supernatants from HIV-1–infected children and healthy controls (data not shown). Also, when we classified the HIV-1–infected children with respect to their level of viral load, the net concentration of IL-6, IL-10, IL-12p70, and TNF-α was similar in both subgroups of HIV-1–infected children and uninfected controls (data not shown). However, there was a significantly lower net concentration of IL-1β in supernatants from HIV-1–infected children with detectable viral load (535 pg/mL, 397–1137) compared with uninfected children (1159 pg/mL, 228–4990, Fig. 5C). A similar concentration of IL-1β was produced by PBMCs from HIV-1–infected children irrespective of their plasma viral load (877 pg/mL, 627–1320, for children with undetectable vs. 535 pg/mL, 397–1137, for children with detectable viral load; Fig. 5C).

TABLE 2. Comparison of the Magnitude of Immune Reconstitution After HAART Between Pediatric and Adult HIV-1–Infected Individuals

	HIV+ Children, n = 23*	Healthy Children, n = 23*	Ratio HIV+/HIV−†	HIV+ Adults, n = 23*	Healthy Adults n = 23*	Ratio HIV+/HIV−†
Number of pDCs/μL	5.54	10.24	0.51	2.82	5.42	0.53
Number of mDCs/μL	12.75	16.09	0.78	9.23	15.41	0.50
Number of monocytes/μL	176	217	0.83	359	311	0.97
Number of NK cells/μL	217	371	0.54	122	225	0.78
Number of iNKT cells/μL	1.84	3.72	0.35	0.78	2.64	0.48

*The absolute number of innate immune cells is presented as median value.

†The ratio of the absolute number of each innate immune cell subpopulation between each HIV-1+ subject and his/her corresponding uninfected control was determined; the data presented are the median of these ratios.

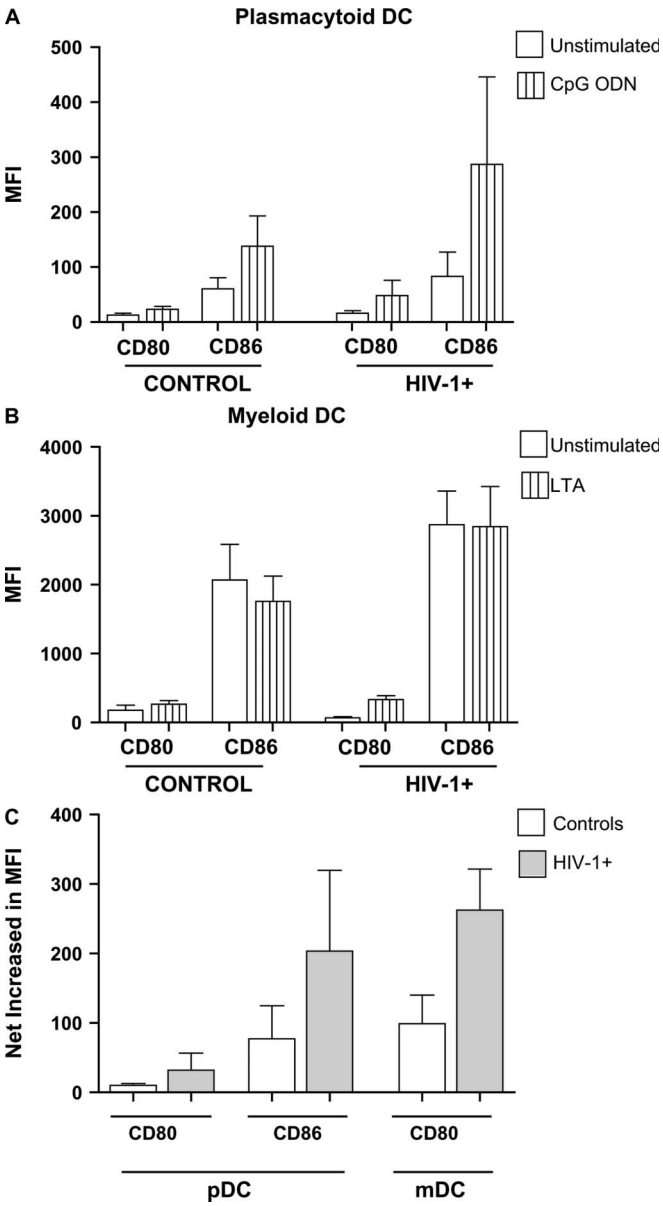

FIGURE 4. Modulation by CpG ODN and LTA of CD80 and CD86 expression on DCs. In HIV-1–infected patients (n = 23) and uninfected controls (n = 23), the expression of CD80 and CD86 on pDC and mDC (absolute; mean fluorescence intensity (MFI) in A and B, or net MFI in C) was determined by flow cytometry after the incubation of PBMCs with/without TLR agonists (Class A CpG ODN 2216 or LTA). The net increase in MFI expression correspond to the MFI for each molecule in cells stimulated with TLR agonist minus the MFI in unstimulated cells. Results are presented as mean and standard error. Statistical comparison between the 2 study groups was performed using the Mann–Whitney *U* test, with a confidence level of 95%. CpG ODN: oligodeoxyribonucleotides with CpG motifs.

DISCUSSION

Although previous studies have shown that HIV-1–infected individuals have quantitative alterations in innate immunity,[30–32] the characterization of innate immune cells in

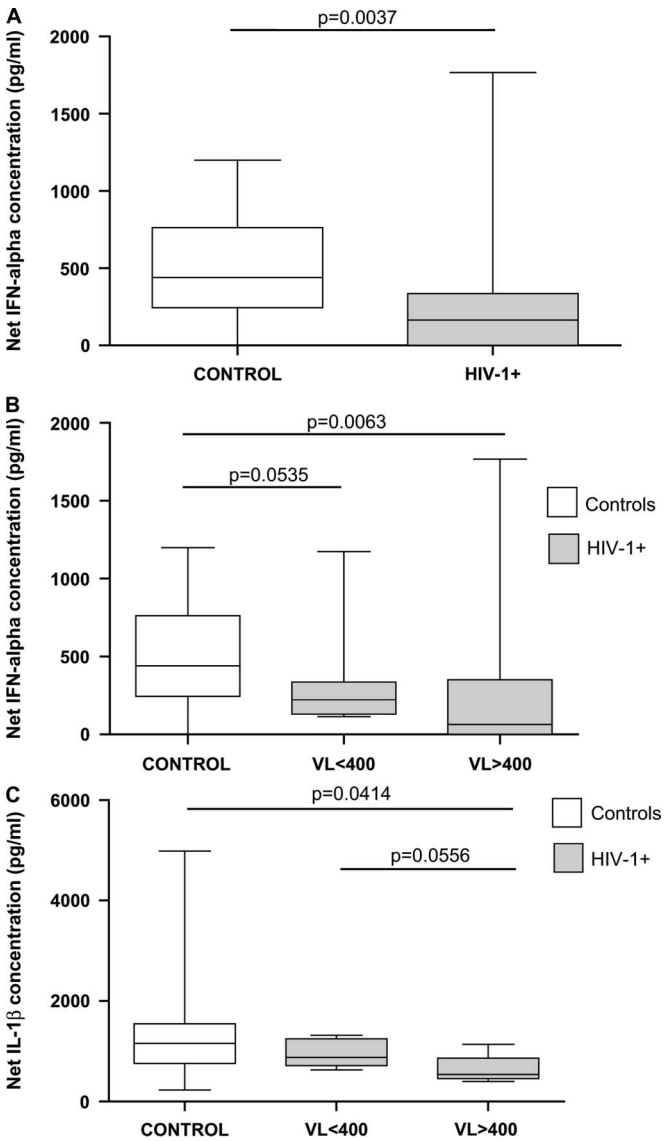

FIGURE 5. Secretion of cytokines by TLR agonist–stimulated PBMCs. A, The concentration of IFN-α was measured by ELISA in supernatants of PBMCs from HIV-1–infected children (n = 23) and uninfected controls (n = 23) incubated with/without Class A CpG ODN 2216. B, To establish the effect of viral replication control on the production of IFN-α, HIV-1+ children were additionally grouped according to their plasma viral load. C, The concentration of IL-1β was measured by flow cytometry [Cytometric Bead Array (Human Inflammatory Kit), Becton Dickinson Pharmingen] in supernatants of PBMCs from HIV-1–infected children (n = 10) and uninfected controls (n = 10) incubated with/without LTA. Results are graphed as net secretion, indicating the concentration of each cytokine in supernatants from LTA-stimulated PBMCs minus the concentration in supernatants from unstimulated cells. Results are presented as median, 25%, and 75% percentiles and range. Statistical comparison between 2 groups was performed using the unpaired Student *t* test (Mann–Whitney *U* test), whereas comparisons among 3 or more groups were performed using the analysis of variance nonparametric Kruskal–Wallis test, with a confidence level of 95%. Significant differences are indicated on the top of the figure. CpG ODN: oligodeoxyribonucleotides with CpG motifs.

HAART-treated HIV-1–infected children has not been completely defined. In this investigation, we evaluated the frequency and functional response of peripheral blood innate cells in HIV-1–infected children receiving stable HAART during a mean period of 4 years, to determine the status of those parameters and their correlation with the capacity of HAART to effectively inhibit viral replication. In addition, we compared the extent of the immune reconstitution achieved by infected children and infected adults after HAART treatment. We found that HIV-1–infected children had alterations in the frequency of several circulating innate immune cells, in the expression of functional markers on iNKT cells and in the IFN-α and IL-1β production after PBMC stimulation with TLR agonists. However, the level of innate immune cell reconstitution achieved with HAART was similar between HIV-1–infected children and adults.

Similar to previous reports in HIV-1–infected adults,[12] the pDC frequency was significantly lower in our HIV-1–infected children receiving HAART; this decrease was more significant in children with detectable viral load than in HIV-1–infected children with control of viral replication. In contrast, a previous study in HIV-1–infected children reported a normal percentage of pDCs,[33] although the absolute number was not determined, making it difficult to compare both studies. In addition, in the study by Azzoni et al the population of HIV-1+ children had a mean age of 11 years, whereas the mean age of our HIV-1+ children was 7 years. Considering that the antiretroviral therapy is started very early in HIV-1–infected children, the age could be interpreted as an indicator of the time that the patients have been receiving HAART; this fact suggests that the HIV-1+ children from our study have been taking HAART for less time, raising the possibility that the reconstitution of the pDC population was not complete in our study.

The fact that pDCs, but not mDCs, were significantly decreased in our HIV-1+ children can be due to their differential susceptibility to HIV-1 infection.[34] Although both dendritic cell subsets are target cells for HIV-1, mDCs were found to be preferentially infected by CCR5-using HIV-1 isolates, whereas pDCs were also susceptible to CXCR4 isolates.[34] Also, pDCs are directly activated by HIV-1 in a TLR7-dependent manner, whereas mDCs have only a bystander activation and maturation induced by cytokines released by HIV-1–activated pDCs.[35] All these differences might be responsible for the greater loss of the pDC pool before the initiation of antiretrovirals and for the lower restoration in response to HAART. In fact, a previous report in HIV-1–infected treated adults indicated normal reconstitution of mDCs but not pDCs.[13]

We did not find any significant difference in the expression of CD80 and CD86 or in the secretion of proinflammatory cytokines among the different groups. However, we found significantly diminished production of IFN-α in HIV-1–infected children (both with detectable and undetectable viral load) and of IL-1β in HIV-1+ children without control of HIV-1 replication, when compared with uninfected controls. The decreased production of IFN-α might be a consequence of the quantitative alteration of pDCs (main producers of IFN-α)[31,36] and may also be related to a persistent functional impairment of pDCs, which is present irrespective of viral suppression, probably due to alterations in the signaling pathways involved in IFN-α production in response to TLR9 agonists as previously reported.[31,36,37]

The absolute number of peripheral blood NK cells was significantly diminished in our HIV-1+ children. Although few studies have evaluated NK cells in pediatric HIV-1–infected subjects, these studies reported a normal frequency of this cell population.[33,38] Differences in the reported units and in the methodological approach used to characterize peripheral blood NK cells might explain the contrasting results. Casanova et al[38] identified the NK cells using only anti-CD56 but did not use anti-CD3 nor anti-CD16; this strategy identifies a subpopulation that includes CD56+ leukocytes other than NK cells and does not include the CD16++/CD56− NK cells. Also, both studies reported percentage instead of absolute numbers of circulating NK cells. Similarly, we found a significant decrease in the absolute number of peripheral blood iNKT cells in our HIV-1+ children. This finding was previously reported in a similar pediatric population.[39] Interestingly, the decreased frequency of circulating iNKT cells in our HIV-1+ children was independent of the level of inhibition of viral replication achieved with HAART.

The peripheral blood iNKT cells have been phenotypically and functionally classified based on their expression of surface molecules, such as CD4 and CD8.[22] When we evaluated the HIV-1–infected children, they had the same distribution of iNKT subpopulations (double-negative>CD4+ >CD8+ >double-positive) that was found in uninfected children; this result differs to that observed in HIV-1+ adults, which have significantly decreased CD4+ iNKT cells.[32] This finding suggests that despite our HIV-1–infected children having a deficient quantitative reconstitution of circulating iNKT cell number in response to HAART, their thymic functional activity is adequate to generate a normal proportion of CD4+ and other subsets of iNKT cells. This last hypothesis is consistent with the higher percentage of iNKT cells expressing CD45RA that we observed in our patients, a finding probably associated with higher thymic activity and immune reconstitution. Additionally, the phenotypic characterization of circulating iNKT cells performed in this study allowed us to demonstrate that the HIV-1+ children had a higher percentage of circulating iNKT cells expressing the activation marker HLA-DR, which indicates a phenotype of chronic activation.[37]

Several studies in HIV-1–infected children and adults reported the quantitative increase of circulating innate and adaptive immune cell numbers after suppressive HAART.[13,40] The low number of peripheral blood pDCs, NK cells, and iNKT cells observed in our HIV-1+ children receiving HAART can be the consequence of an irreversible damage in bone marrow and lymphoid organs[41,42] developed before starting HAART, which limited the production and maturation of several leukocyte subpopulations. Also, the persistent immune activation observed in HIV-1+ patients despite HAART may be associated with alterations in the pattern of leukocyte recirculation and with a shorter survival dependent on accelerated activation-induced cell death.[43]

In addition, impaired hematopoeisis is also a characteristic of chronic HIV-1 infection and AIDS, with patients exhibiting abnormalities in one or more blood cell lineages

resulting in neutropenia, anemia, and thrombocytopenia.[41,44–46] Several studies have demonstrated decreased in vitro growth of bone marrow progenitor cells from seropositive patients.[46,47] The effects of HIV-1 on cells and the ability of this virus to impact the proliferation and differentiation of progenitor cells has been proposed to cause the hemopoietic abnormalities observed in AIDS patients.[48–51] In fact, infection and replication of HIV-1 in CD34+ progenitor cells[49] and a direct inhibitory effect of this virus on the proliferation of progenitor cells from normal human bone marrow have been demonstrated.[48–52] In our study, we observed a decrease in the absolute number of pDCs, NK cells, and iNKT cells, all cells from lymphoid lineage, despite the fact that these children were taking HAART and that this therapy was effective in inhibiting viral replication. This finding is possibly related to the HIV-1–dependent and TNF-α–mediated inhibitory effects on lymphopoiesis[53]; particularly, increased production of TNF-α has been demonstrated in HIV-1–infected children.[54]

HAART-associated immune restoration could be influenced by several host and viral factors, such as age, level of thymus activity, magnitude of lymphoid tissue damage dependent on HIV-1 infection, viral resistance, drug toxicity, and poor adherence to antiretrovirals.[25,26] To have a better idea on the level of innate immune reconstitution achieved with HAART, we determined and compared the magnitude of the quantitative restoration of innate immune cells between our HIV-1–infected children and a group of HIV-1–infected adults under HAART for a similar period of time; we did not find any significant difference between the degree of innate reconstitution observed in both groups of HIV-1+ subjects presented in children compared with adults. These findings suggest that the innate immune reconstitution associated with HAART is partial and has a magnitude that is apparently independent of age.

HAART has dramatically modified the clinical course and prognosis of chronically HIV-1–infected individuals by causing a drastic inhibition of viral replication and by allowing partial recovery of immune competency, which leads to a reduction of opportunistic infections and a better clinical outcome.[4–6] Therefore, it is suggested that stable administration of HAART is effective in controlling HIV-1 replication and is associated with improvements in adaptive and innate immune parameters. Our findings indicated that administration of HAART to chronically HIV-1–infected children does not lead to a complete recovery of peripheral blood innate immune cells, particularly in children without control of viral replication. Additional prospective studies will be necessary to clarify the significance of this subclinical immunodeficiency. These results highlight the importance of achieving suppression of viral replication with HAART and suggest developing strategies to augment the partial immune increases achieved with HAART.

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