Dendritic and Natural Killer Cell Subsets Associated with Stable or Declining CD4\(^+\) Cell Counts in Treated HIV-1–Infected Children

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**Background.** Natural killer (NK) cells and plasmacytoid and myeloid dendritic cells (DCs) are depleted, and their function impaired, in advanced adult human immunodeficiency virus (HIV)–1 infection. Studies in perinatally infected children are lacking.

**Methods.** Percentages of NK cells and plasmacytoid and myeloid DCs were evaluated by flow cytometry. Forty children with perinatal HIV-1 infection were compared with 11 age-matched, uninfected children. Plasma- and myeloid DC function was evaluated by activation-induced cytokine secretion.

**Results.** Virally suppressed children had normal levels of circulating plasmacytoid and myeloid DCs and total NK cells but had sustained depletion of a mature (CD3\^-/161^+156^+/161^+) NK cell subset and decreased interferon-\(\alpha\) secretion by plasmacytoid DCs. Despite similar viral loads, percentages of myeloid and plasmacytoid DCs and mature NK cells were significantly lower in viremic children with a history of decreasing CD4\(^+\) cell percentages, compared with children with stable CD4\(^+\) cell counts.

**Conclusions.** Children achieve partial reconstitution of myeloid and plasmacytoid DCs and NK cells during viral suppression; irrespective of viral load, a clinical history of decreasing CD4\(^+\) cell percentage is associated with greater depletion of these subsets. We hypothesize that the evaluation of selected innate-immunity effector cells may serve as a marker of CD4\(^+\) cell loss in pediatric HIV-1 infection.

Recent reports on HIV-1 immunopathogenesis have increasingly focused on the role of the innate immune system, particularly numbers and function of dendritic cells (DCs) and NK cells [1–3]. DCs initiate T and B cell responses [4], produce interleukin (IL)–12 and interferon (IFN)–\(\alpha\), and participate in the innate immune response against pathogens [5, 6]. Two distinct circulating subpopulations of DCs are recognized in humans—myeloid and plasmacytoid [6]—and both are depleted in adult patients with advanced HIV-1 disease [7–10], but only myeloid DCs are fully recovered after successful antiretroviral therapy (ART). Together with CD4\(^+\) cell counts, decreased IFN-\(\alpha\) production and numbers of plasmacytoid DCs [7, 8, 11, 12] are independent predictors of clinical progression and the onset of opportunistic infections [13]. An inverse association between plasmacytoid DC–mediated IFN-\(\alpha\) secretion and opportunistic infections indicate a potential role for IFN-\(\alpha\)–mediated activation of NK-mediated responses in asymptomatic patients [13–15]. NK cells lyse HIV-1–infected cells [16, 17] and contribute to the control of viral infections, as has been established in murine models [18]. Past work has demonstrated the ability of Lin\(^-\)/HLA-DR\(^+\) DCs to activate NK cells to lyse target cells [19]. Furthermore, IFN-\(\alpha\) produced by HLA-DR\(^+\) cells has been shown to be necessary for NK cell–mediated lysis of virally infected cells [20, 21]. Most studies, including ours [22], have shown a decrease in NK cytotoxicity in viremic HIV-positive adult patients in association with the depletion of mature cytotoxic NK cell subsets [23–25], identified on the basis of coexpression of CD161 with CD56 and/or CD16 cells [22, 26]. A partial recovery of functionally mature NK sub-
sets is observed after ART-mediated viral suppression in adults [22, 27], whereas, in children, aggressive combination ART results in CD4+ cell reconstitution [28–30], but little is known about innate immune reconstitution.

CD4+ cell counts and HIV RNA load predict the risk of disease progression and opportunistic infections in adults, independently [31–33] or in combination [34, 35]. In children, CD4+ cell percentage is considered to be a better predictor of clinical progression than serum HIV RNA load [36], with a sharp increase in the risk for AIDS indicated by CD4+ cell percentages of <15%, which highlights a need for additional parameters predictive of future CD4+ cell loss. We established, in earlier studies [37], that children with stable CD4+ cell counts and partial viral suppression (viral load <50,000 copies/mL) while receiving ART had higher levels of lymphoproliferative responses to HIV-1 antigens, reduced expression of activation markers (CD38 and HLA-DR), and higher percentages of CD8+/CD28+ cells, compared with viremic children who had shown a >15% decline in CD4+ cell percentages during the preceding year. We report the results of a cross-sectional study of 40 HIV-1–infected children that identified pediatric-specific outcomes of innate immune reconstitution after successful ART-mediated viral suppression and identified novel innate immune parameters that are affected to a greater extent in HIV-1–positive viremic children who had a history of decreasing CD4+ cell percentage (change in CD4+ cell percentage of >15% during the preceding year), compared with children who had stable CD4+ cell counts.

**SUBJECTS, MATERIALS, AND METHODS**

**Patient population.** Forty children with HIV-1 infection and 11 age- and sex-matched, HIV-negative control children were recruited at the Children’s Hospital of Philadelphia (CHOP). Each blood sample was evaluated for viral load and CD4+ cell percentage as part of routine clinical monitoring. The viremic group was further subdivided into 2 groups: children who had maintained their CD4+ cell percentage over the previous year of follow-up (stable CD4+ cell percentage) and those who had a decrease of >15% in CD4+ cell percentage over the same period (declining CD4+ cell percentage) [37]. The use of CD4+ cell percentage as a prognostic indicator in children has been endorsed by the Working Group on Antiretroviral Therapy (http://www.aidsinfo.nih.gov/guidelines/) and the HIV Paediatric Prognostic Markers Collaborative Study Group [36]. The study was approved by the Institutional Review Board of CHOP. Informed consent was obtained by the parents or legal guardians, in accordance with institutional review board guidelines for the protection of human subjects.

**Flow cytometric analysis.** Immunofluorescence staining was performed by use of 4-color flow cytometry; briefly, 200 μL of whole blood was incubated for 15 min at room temperature with each of the following monoclonal antibody (mAb) combinations (all antibodies from BD Biosciences, except where indicated): NK cells, CD56 fluorescein isothiocyanate (FITC), CD16 phycoerythrin (PE), CD3 peridinin-chlorophyll-protein complex (PerCP)–Cy5.5, and CD161 allophycocyanin (APC); myeloid DCs, Lin-1 FITC, CD11c PE, HLA-DR PerCP-Cy5.5, CD86, and CD95 APC; and plasmacytoid DCs, Lin-1 FITC, CD123 PE, HLA-DR PerCP-Cy5.5, CD86, and CD95 APC (Caltag). Samples were fixed, lysed to eliminate erythrocytes, and then analyzed on a FacsCaliber Flow Cytometer (BD Biosciences). Electronic gates were created, on the basis of forward- and side-scatter parameters, to include lymphocytes (NK cell stainings) or all live peripheral blood mononuclear cells (PBMCs; DC stainings); compatibly with the sample size, 200,000 gated events were collected in each run and were analyzed by use of CellQuest software (BD Biosciences).

**Cytokine assays.** PBMCs were isolated by Ficoll/Hypaque density gradient centrifugation and were cultured for 18 h (10⁶ cells/mL) in 1 mL of RPMI 1640 with medium alone (control), cell-free viral UV cross-linked supernatant (5HA Influenza-PR8; 10 hemagglutinin units/mL), or CpG-2216 (10 μg/mL; Integrated DNA Technologies). Cell-free supernatants were harvested and tested in duplicate for IFN-α by use of a commercial ELISA, according to the manufacturer’s instructions (Endogen). Absorbance was measured on an automatic plate reader. Sensitivity of the assays was ~10 pg/mL. Recombinant cytokine standards were used in ELISA assays. “Per cell” IFN-α production was calculated by dividing the amount of IFN-α produced in each test by the number of CD123+ plasmacytoid DCs (PDCs) present in the test by use of the formula IFN-α/[(%PDC/100) × 10⁶].

**Statistical methods.** Data are expressed as median and interquartile range (IQR). Nonparametric tests (Mann-Whitney U test and Kruskall-Wallis analysis of variance) were used to compare between-group distributions, stratified by viral load and CD4+ cell percentage. Correlations between variables were evaluated by use of the Spearman rank correlation test. For all tests, 2-sided P < .05 was considered to be significant, unless otherwise stated. All tests were performed by use of the statistical software Analyse-it or JMP (SAS Institute). In all graphics, brackets indicate significant differences between groups.

**RESULTS**

**Patient cohort.** Our cohort was composed of 40 children: 18 girls and 22 boys; 29 were black, 7 white, and 4 Hispanic. All children had been previously treated with combination ART, and 37 children were undergoing treatment at the time of analysis. The mean age of our HIV-positive patient group was 11.1 years; this was matched to the control uninfected group. In the HIV-positive group, 19 children had CD4+ cell percentages of >30%, 15 had percentages of 20%–30%, and 5 had percentages of <20%.
Of the 40 HIV-infected children, 22 had undetectable serum HIV RNA loads (<400 copies/mL), whereas the median viral load of viremic children was 31,000 copies/mL (IQR, 116,550 copies/mL). Of the 14 viremic children for whom complete clinical histories were available, 8 had stable CD4+ cell percentages and 6 had had a >15% decline in the CD4+ cell percentage during the preceding year. The age of children in the 3 resulting groups was not significantly different (suppressed [≤400 copies/mL], median, 10 years [IQR, 6 years]; viremic with stable CD4+ cell count, median, 11 years [IQR, 6 years]; viremic with declining CD4+ cell count, median, 13.5 years [IQR, 5.5 years]). The mean CD4+ cell percentage of all viremic children was 22%, whereas that of fully suppressed children was 34%. As expected, in viremic children, the CD4+ cell percentage was inversely correlated to the viremia level (figure 1A). However, HIV RNA loads (figure 1B) did not segregate children with different histories for change in CD4+ cell count during the preceding year; no significant difference in HIV load was observed between viremic children who had a history of declining (median HIV RNA load, 27,500 copies/mL), stable CD4+ cell counts (median HIV RNA load, 12,000 copies/mL [IQR, 222,098 copies/mL]).

Selective depletion of NK cell subsets in viremic HIV-infected children. To determine whether HIV infection resulted in the depletion of any NK cell subset, as has been observed in adults [22], we studied the expression of CD161, CD56, and CD16 on CD3+ lymphocytes in viremic and ART-suppressed (<50 copies/mL) children (2 children with missing viral loads were excluded from this analysis). Levels of CD3+CD56- NK cells in whole blood were significantly lower (P < .05) in viremic children (median, 1.05% [IQR, 1.24%]) than in uninfected control children (median, 1.89% [IQR, 1.92%]). Within this mature NK population, we observed a sustained depletion of the CD3+/161+/56+/16+ phenotype in both viremic (median, 0.46% [IQR, 0.73%]) and suppressed children (median, 0.71% [IQR, 0.88%]), compared with levels in uninfected children (median, 1.22% [IQR, 1.28%]). Interestingly, aggregation of viremic children into 2 groups according to history of a decrease in CD4+ cell percentage did show significant differences in the distribution of NK subset changes, as shown in figure 2A. The NK cell CD56- subsets CD161+/56-/16-, CD161+/56+/16-, and CD161+ /56+/16+ (figure 2A, bottom 3 panels) were depleted in children who had a history of declining CD4+ cell count, compared with levels in uninfected control children (median, 0.2% [IQR, 0.1%]). It is of interest to note that NK cell subsets CD161+ /56-/16- and CD161+ /56+/16+ in viremic children with a history of stable CD4+ cell percentages were similar to those of uninfected control children.

The relative proportion of NK subsets within the general NK population is summarized as the mean percentage of total NK cells in figure 2B. Analysis of changes within NK cell subsets showed a reduced proportion of mature CD161+/56+/16+ NK

![Figure 1](https://academic.oup.com/jid/article-abstract/191/9/1451/861482)

Figure 1. Distribution of HIV RNA load in relation to CD4+ cell percentage and clinical history. A. Correlation between CD4+ cell percentage (clinical measurement) and serum HIV RNA load (copies/mL). Triangles, patients; dotted line, exponential growth trend. B. Serum HIV RNA load in viremic children with stable or decreasing CD4+ cell percentage, as defined in Subjects, Materials, and Methods. Boxes, median, 10th, 25th, 75th, and 90th percentile and mean (dashed line) of the HIV RNA distribution of each subset.

Defective IFN-γ production in HIV-positive suppressed children with retained plasmacytoid DC percentages. To assess the effect of HIV infection on plasmacytoid DC percentages, we evaluated the Lin-1+/HLA-DR+/CD123+ cell population. The plasmacytoid DC percentage was significantly lower (P < .05) in viremic children (median, 0.05% [IQR, 0.04%]) than that in uninfected children (median, 0.09% [IQR, 0.05%]) or that in HIV-positive suppressed children (median, 0.12% [IQR, 0.15%]). Plasmacytoid DC percentage was directly correlated with CD4+ cell percentage (figure 3A, r = 0.35; P < .05) and was inversely correlated to serum HIV RNA load (figure 3B, r = −0.48; P < .05), which confirms that the subset is depleted in viremic children and that disease progression (as indicated by a de-
Figure 2. Clinical history and percentage of NK phenotypes in HIV-infected children. A, Whole-blood NK phenotypes are analyzed as described in Patients, Materials, and Methods: values represent the percentage of total events (cells), electronically gated on the basis of forward- and side-scatter parameters. Analysis groups shown in every panel are: controls (C): HIV-negative control subjects; suppressed (S): serum HIV RNA load <400 copies/mL; viremic/stable CD4 (St): HIV RNA load >400 copies/mL with a history of stable CD4+ cell percentage; viremic/declining CD4 (D): HIV RNA load >400 copies/mL with a history of declining CD4+ cell percentage. Boxes, median, 10th, 25th, 75th, and 90th percentile and mean (dashed line) of the percentage distribution (percentage of total peripheral blood mononuclear cells) of each subset. Continuous line brackets indicate differences with a (Mann-Whitney U test). Dashed line bracket, differences of . CD3+/H11002/56+ and CD3+/H11002/161+/56+/16+ had (Kruskal-Wallis analysis of variance) for the entire distribution. B, Pie chart of the relative proportion of the indicated NK subsets, studied as described above. Values represent the percentage of total NK cells, defined as cells expressing any combination of CD161, CD56, or CD16 in the absence of CD3 (CD3+/H11002/161+/56+/16+ cells were excluded because they consist mainly of monocytes).
increasing CD4+ cell percentage) is associated with plasmacytoid DC depletion. Surprisingly, no difference in plasmacytoid DC percentage was observed between HIV-positive suppressed children and their HIV-negative counterparts, which suggests that, unlike adults [9], HIV-infected children may recover the plasmacytoid DC subset after viral suppression. When the history of CD4+ cell percentage retention was taken into consideration (figure 3C), a significantly lower (P < .05) plasmacytoid DC percentage was present in viremic children with declining CD4+ cell counts, compared with levels in children who had stable CD4+ cell percentages, who showed a plasmacytoid DC percentage similar to that of HIV-positive suppressed children (median, 0.13% [IQR, 0.13%]). However, plasmacytoid DC percentage for both the decreasing (median, 0.03% [IQR, 0.003%]) and the stable CD4+ cell percentage (median, 0.06% [IQR, 0.02%]) groups were significantly lower than that of HIV-negative children (median, 0.09% [IQR, 0.05%]; P < .05 for both comparisons). Further analysis of CD86 mean fluorescence intensity (MFI) on plasmacytoid DCs as a molecule highly expressed on terminally differentiated DCs or of CD95 showed no significant difference in expression among HIV-negative, HIV-positive suppressed, and viremic children, irrespective of the decline in CD4+ cell percentage (figure 3D).

To monitor plasmacytoid DC function, the secretion of IFN-α was assessed on stimulation of isolated PBMCs with 5HA influenza-PR8 or the Toll-like receptor (TLR)-9 ligand CpG-2216. Both stimuli are considered to be relatively plasmacytoid DC specific, given that only plasmacytoid DCs and B lymphocytes express relevant amounts of TLR-9 [38] and that type A CpGs (such as 2216) are known to preferentially activate plasmacytoid DCs [39, 40]. With both stimuli, we observed a significantly lower (P < .05) level of IFN-α production in all HIV-

Figure 3. Plasmacytoid dendritic cell (DC) percentage in HIV-infected children. A, Correlation between plasmacytoid DC and CD4+ cell percentages (r = 0.35; P < .05, Spearman test). B, Correlation between plasmacytoid DC percentage and serum HIV RNA load (r = −0.48; P < .05, Spearman test). C, Percentage of Lin-1/HLA-DR+/CD123+ plasmacytoid DCs, studied on whole blood by 4-color immunofluorescence in control subjects and HIV-infected children, as described in figure 2. Group assignment within each panel is according to CD4+ cell percentage history, as described in figure 2 (P < .05, Kruskal-Wallis analysis of variance). D, Mean fluorescence intensity (MFI) of CD86 or CD95 was assessed in separate staining on gated Lin-1/HLA-DR+/CD123+ cells, as described in figure 2.
positive children, with median values of 177 pg/mL (IQR, 187 pg/mL) and 235 pg/mL (IQR, 229 pg/mL) for 5HA influenza-PR8–induced IFN-α and of 69 pg/mL (IQR, 332 pg/mL) and 135 pg/mL (IQR, 190 pg/mL) for CpG-2216, compared with levels in control children (influenza, 456 pg/mL [IQR, 186 pg/mL]; CpG-2216, 298 pg/mL [IQR, 162 pg/mL]). No significant differences were observed between the HIV-infected viremic and suppressed subgroups when we analyzed secretion levels. In keeping with this observation, no correlation was observed between IFN-α production and CD4+ cell percentage, serum HIV RNA load, or a history of declining/stable CD4+ cell count in viremic children (figure 4), which indicates a sustained functional impairment, despite changes in the percentage of circulating plasmacytoid DCs.

To assess whether the observed impairment of IFN-α production was due exclusively to the loss of plasmacytoid DCs in PBMCs, we estimated the amount of IFN-α produced by each plasmacytoid DC on stimulation with the TLR-9–specific CpG-2216, as described in Subjects, Materials, and Methods. Whereas, in control children, each plasmacytoid DC produced a median 0.27 pg of IFN-α (IQR, 0.13 pg), this amount was significantly lower in HIV-infected children (median, 0.06 pg [IQR, 0.37 pg]; 1-sided P < .05). No significant differences were noted between HIV-infected viremic and suppressed children. A similar trend was also observed when PR-8 stimulation was used, which supports the hypothesis of a functional cellular impairment in plasmacytoid DC secretion.

**Depletion of myeloid DCs in children with advanced HIV infection.** We analyzed the percentage of Lin−1−/HLA-DR+/CD11c+ myeloid DCs, to determine how HIV infection affects this DC subset in HIV-infected viremic and suppressed children. Contrary to previous observations by our group and others in adults [7, 9, 10, 41], no significant difference was observed among uninfected age-matched control (median 0.20% [IQR, 0.14%]), suppressed (median, 0.17% [IQR, 0.16%]), and viremic (median, 0.21% [IQR, 0.21%]) children, which suggests that myeloid DCs in children might be less affected by viral replication than those in adults. However, the percentage of myeloid DCs was significantly lower (P < .05) in viremic children who had decreasing CD4+ cell percentages (median, 0.05% [IQR, 0.10%]) than in both control (median, 0.19% [IQR, 0.09%]) and HIV-positive suppressed (median, 0.21% [IQR, 0.17%]) children (figure 5A) children, which supports the hypothesis that disease progression is associated with loss of myeloid DCs in children. As was observed in plasmacytoid DC subsets, no significant difference in the expression (according to MFI) of CD86 and CD95 (figure 5B) was observed between groups.

**DISCUSSION**

We assessed innate immunity correlates of viral control and sustained CD4+ cell percentage in HIV-infected, ART-treat-
reported in viremic HIV-infected children [51]. Although, globally, the NK subsets expressing CD56 are similar to those in HIV-infected suppressed children, the persistent decrease in the major mature subset of CD161+CD56+CD16+ promotes the hypothesis that immune reconstitution in infected children might be impared with regard to NK function. Functional longitudinal studies will be necessary to establish whether ART-mediated viral suppression results in full reconstitution of the functions of the NK cell subset (e.g., IFN-γ production on IL-2/IL-12 stimulation).

In contrast to that in adult subjects [9, 10, 12], the plasmacytoid DC percentage was similar in control and HIV-infected suppressed children. However, similar to findings in infected adults [9, 10, 12], a significantly lower percentage of plasmacytoid DGs was observed in viremic children, compared with that in HIV-infected suppressed children, and a sustained impairment in IFN-α secretion was present irrespective of viral suppression. Potential factors that may influence the increase of a functionally impaired plasmacytoid DC subset on viral suppression in children include an increased plasticity of the innate immune system and the anticipated longer time on therapy in our cohort—most children had been treated since infancy. Altogether, even though the reduction of PDC percentage in viremic children might contribute to the impairment of IFN-α production, the observation of a reduced “per cell” production of IFN-α by plasmacytoid DCs on stimulation with TLR-9–specific CpG-2216 in all HIV-infected children, independent of viremia, supports a persistent functional impairment at the cellular level irrespective of viral suppression.

It is important to consider that results from our ongoing and published longitudinal studies in infected adults starting suppressive ART [52] indicate that, although normal (uninfected) levels of IFN-α secretion and plasmacytoid DC percentages are not recovered, an increase from pretreatment levels is observed after viral suppression. Because adults and children have differences in the plasticity of their immune systems, longitudinal studies in pediatric cohorts will be required to determine whether similar changes are observed in newly treated children.

In agreement with studies conducted in adults [7–10], myeloid DC percentage was reduced in HIV-infected children with advanced disease (i.e., a history of declining CD4+ cell percentage). However, similar percentages of Lin1-/HLA-DR+/CD11c+ cells were observed in HIV-negative control children and in HIV-infected children with stable CD4+ cell counts, independent of viremia, which indicates that either complete control of viral load resulted in myeloid DC recovery over time in this subgroup or that therapy was started at a time when this subset was not depleted.

It is important to note that children in our cohort were treated early in life and are generally older (mean age, 11.1 years) and more heavily ART-experienced than those in previous studies. Data on the exact duration of ART therapy (and possible interruptions) were not available for many of our subjects, making it difficult to interpret whether the amount of confirmed time on ART was a direct correlate to the levels of innate immunity effectors and/or history of CD4+ cell percentages. Furthermore, because most children in the cohort started ART shortly after birth, we interpret that their age is an indicator of the time receiving ART. However, our data do not rule out that the observed outcomes may depend, at least in part, on the clinical efficacy of ART in suppressing viral replication, relative to changes in CD4+ cell percentage during the course of child development. Interestingly, stratification of our HIV-infected children on the basis of US Centers for Disease Control and Prevention class did not segregate children with decreasing or stable CD4+ cell percentages and did not correspond to differential frequency or functionality of NK cells or DCs (data not shown), which suggests that prior “worse ever” status is not predictive of the innate immunity-related outcomes that we studied or of the patient’s ability to sustain their CD4+ cell
percentage. Finally, the presence of children with low viral loads (subjects 3 and 10) in the viremic cohort did not bias our results—testing repeated after excluding these samples resulted in the same trends as in the whole cohort (data not shown).

Although the association of a history of CD4+ cell retention in viremic children with increased retention of innate subsets of plasmacytoid and myeloid DCs and NK subsets suggests a role for immune function or “innate fitness” as a factor associated with a stable CD4+ cell percentage, the cross-sectional nature of the study does not allow us to determine whether the decrease in innate function is a cause or a consequence of the decrease in CD4+ count. This remains to be established in future longitudinal studies.

In conclusion, our results constitute, to our knowledge, the first comprehensive assessment of innate immunity cellular effectors in children, and they indicate that (1) selected innate immune cells and function are depleted in HIV-infected suppressed children; (2) differences may exist between infected adults and children in innate immune reconstitution after ART; and (3) innate immune effector cell (NK cells and plasmacytoid and myeloid DCs) percentage and function are impaired in viremic children, irrespective of the presence of partially suppressive ART, particularly in those with a history of decreasing CD4+ percentages.

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