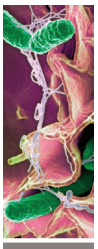

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RESEARCH ARTICLE

Defective dendritic cell response to Toll-like receptor 7/8 agonists in perinatally HIV-infected children

Anbalagan Selvaraj[1], Sudheesh Pilakka-Kanthikeel[2], Perumal Kannabiran Bhavani[1], Luke E. Hanna[1], Savita Pahwa[2] & Soumya Swaminathan[1]

1 Department of Clinical Research, National Institute for Research in Tuberculosis, Indian Council of Medical Research, Chennai, India
2 Department of Microbiology and Immunology, Centre for AIDS Research, University of Miami, Miller School of Medicine, Miami, FL, USA

Highly Active Antiretroviral Therapy (HAART) has forever changed the outcome of HIV infection, transforming a terminal syndrome that ravaged the immune system into a manageable chronic viral infection. However, the impact of HIV and HAART on pediatric populations has been studied less in detail than in adults. In this article, Selvaraj *et al.* report their observation on the impact of HIV infection and therapy on a cohort of children, focusing on innate immunity. Their data shows that in this population, HIV cripples dendritic cells, and that HAART regimen does not seem to fully reconstitute that damage. The data will be of interest to both immunologists and clinicians.

Keywords
dendritic cells; innate immunity; pediatric HIV.

Correspondence
Soumya Swaminathan, National Institute for Research in Tuberculosis, #1 Mayor Sathiyamoorthy Road, Chetpet, Chennai – 600 031, India.
Tel.: +91 (044) 2836 9700
fax: +91 (044) 2836 2525/2528
e-mail: doctorsoumya@yahoo.com

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Abstract

Understanding the defects in innate immunity associated with perinatal HIV infection is a prerequisite for effective antiretroviral treatment. We therefore compared the innate immune response [dendritic cell (DC) phenotype and function] in peripheral blood by flow cytometry at baseline and 12 months in HIV-infected children to determine the defect associated with perinatal HIV infection. As compared with controls, patients had decreased numbers of total DCs including plasmacytoid (p)DCs and myeloid (m)DCs and impaired function based on induction of maturation markers (CD83, CD80, CCR7) and cytokines tumor necrosis factor-α and interferon-α (exclusive to pDC) upon stimulation with the TLR7/8 agonist Resiquimod. These abnormalities were evident in all three CD4 immune categories and persisted over 12 months; pDC function worsened in HIV+ children without treatment and improved slightly in those on highly active antiretroviral therapy (HAART). In conclusion, a majority of perinatally HIV-infected older children without HAART remain clinically stable in the short term, but have demonstrable immunologic abnormalities indicative of defects in the innate immune system. Children initiated on HAART showed improvement in CD4 counts but did not show improvement in DC function over the short term.

Introduction

HIV infection is associated with activation of immune responses, as well as a gradual loss of immune function and increased susceptibility to opportunistic infections. Patients with HIV infection at both early and late stages show immunoregulatory defects that precede CD4 T-cell depletion (Fauci, 1993; Levy, 1993). While the loss of adaptive HIV-specific immune responses is an area of active investigation in HIV research, the potential role of the innate immune response in children is largely unknown. A recent review by Borrow *et al.* (2010) has emphasized the importance of innate immunity and the role of dendritic cells (DCs) in the control of HIV/AIDS. The course of perinatal HIV infection is rapid, with 50% of infected infants dying before the age of 2 years (Devi *et al.*, 2009). Hence, it is of interest to investigate phenotypic and functional changes in DC subsets associated with disease progression and immune reconstitution in children receiving suppressive highly active antiretroviral therapy (HAART).

DCs constitute a heterogeneous population of antigen presenting cells that are critical for bridging the innate and the adaptive immune responses. DCs are found virtually in every tissue and organ. In the peripheral blood, two major DC populations can be identified: CD11C+ myeloid DCs (mDCs) and CD123+ plasmacytoid DCs (pDCs) (Siegal *et al.*, 1999; Banchereau *et al.*, 2000; Liu, 2005; Zhang & Wang, 2005; Cao & Liu, 2007). The chief mechanism of pathogen recognition by DCs is via an evolutionarily conserved system of Toll-like receptors (TLRs), all of which

share an interleukin-1 (IL-1) receptor-like structural motif. mDCs and pDCs have different patterns of TLR expression. mDCs express TLR1–TLR6, TLR8, TLR10 and, possibly, TLR7, while pDCs express TLR1, TLR6, TLR10, and very high levels of TLR7 and TLR9 (Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001; Krug *et al.*, 2001; Ito *et al.*, 2002). In response to viral infections, both DC populations are involved in the generation of innate and acquired immune responses through secretion of type I interferons (IFN), tumor necrosis factor-α (TNF-α) and IL-12. Mimicking viral infections, resiquimod (RSQ) induces the production of IL-6, TNF-α and IFN-α from DCs. In addition, RSQ directly activates innate immune responses through an MyD88/TLR7-dependent pathway (Hemmi *et al.*, 2002).

Several studies have shown that, during HIV infection, both DC subsets are substantially reduced in patients' blood (Macatonia *et al.*, 1990; Grassi *et al.*, 1999; Feldman *et al.*, 2001; Soumelis *et al.*, 2001; Chehimi *et al.*, 2002; Barron *et al.*, 2003; Donaghy *et al.*, 2003). In some, this decrease correlated with plasma viral load and was partially restored following HAART (Feldman *et al.*, 2001; Soumelis *et al.*, 2001; Barron *et al.*, 2003). However, it remains unclear whether DC functions recover following suppressive HAART in HIV-infected children.

The main goal of the present study was to evaluate phenotypic and functional properties of the two major subsets of DCs in relation to immunological status of children with perinatally acquired HIV infection, and to evaluate immunologic changes over time in both untreated children and those initiated on HAART.

Materials and methods

Study subjects

Sixty-two HIV-1-infected treatment-naïve children attending government ART clinics in Chennai were enrolled in the study. Informed consent was obtained from their parents or legal guardians. They were followed up at 3-month intervals with clinical and laboratory evaluation up to 1 year. Nine patients were started on HAART (Lamivudine + stavudine with Nevirapine or Efavirenz) after baseline blood collection and continued therapy without modifying the regimen over a1-year follow-up period. Twenty age-matched healthy children served as controls. Venous blood was collected in heparinized vacutainers and processed for DC assays.

Viral load

Viral load measurements were performed on plasma samples from HIV-infected children at baseline, 6 months and 1 year using the standard Cobas Amplicor HIV-1 Monitor Test, v1.5 (Roche Diagnostic Systems) following the manufacturer's protocol.

Whole blood – DC assay

Staining was performed on heparinized blood as described (Ida *et al.*, 2006). Briefly, 180 μL whole blood was added to 20 μL R5 medium (5% pooled human AB serum, 1% HEPES buffer, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in RPMI 1640) containing 10 μM RSQ (stimulated cultures) or only R5 medium (unstimulated cultures). Cultures were set up in ventilation-capped 5-mL polystyrene round-bottomed plastic tubes. Tubes were incubated at 37 °C in a humid, 5% CO_2 atmosphere at a 5° slant.

Intracellular cytokine assay

After 1 h of incubation, Brefeldin A was added at a final concentration of 10 μg mL^{-1} and incubation was continued for an additional 2 h. Following incubation, surface staining was performed by adding the following antibody cocktails: Lineage-1 fluorescein isothicyanate (FITC), CD123/CD11c phycoerythrin (PE) and Human leucocyte antigen-DR (HLA-DR) peridinin chlorophyll protein (PerCP). Tubes were vortexed and incubated at room temperature for 30 min in the dark. After incubation, red cells were lysed using BD FACS lysing solution followed by washing with 2 mL wash buffer. Cells were permeabilized in 200 μL cytofix/cytoperm buffer for 20 min at 4 °C. Subsequently, samples were washed twice with 1 mL permeablization buffer. Anti-human TNF-α allophycocyanin (APC) (1 : 200) and anti-human IFN-α Alexa fluor-647 (1 : 400) antibodies diluted in permeablization buffer were added at 30 μL per tubes and samples were incubated for 30 min in the dark at 4 °C. Samples were washed twice with 1 mL permeablization buffer. Cell pellets were then resuspended in 200 μL 1% paraformaldehyde and stored in the dark at 4 °C prior to flow cytometric analysis.

Surface staining

After 5 h incubation, RSQ-stimulated and unstimulated whole blood cultures were subjected to surface staining by adding the following antibody cocktails: Lineage-1 FITC, CD11c/CD123 APC, HLA-DR PerCP and maturation/activation markers (CD80/CD83/CCR7) PE. Tubes were vortexed and incubated at room temperature for 30 min. After incubation, red cells were lysed using a BD lysing solution followed by washing with 2 mL wash buffer. Cell pellets were resuspended in 200 μL 1% paraformaldehye and stored in the dark at 4 °C prior to flow cytometric analysis.

Acquisition was performed on a FACS-Calibur machine using CellQuestPro software and analysed using FlowJo software version 7.1.3. Figure 1 shows the gating strategy used to detect cytokine production and expression of maturation factors by DC subsets in whole blood.

Statistical analysis

Statistical analysis was performed using ANOVA and a general linear model with planned contrasts was used to compare CD4 and viral load changes over time. P values < 0.05 were considered significant. SAS version 9.1 was

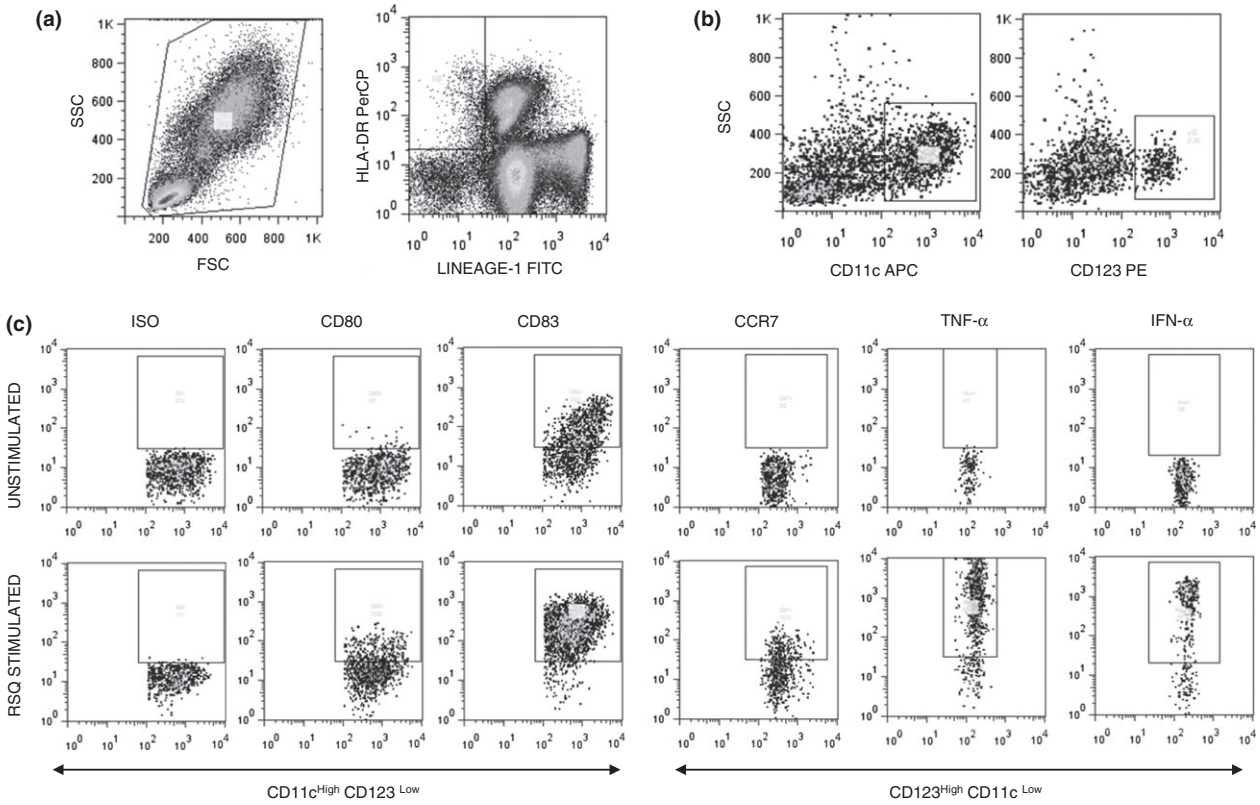

Fig. 1 Flow cytometry gating strategy to detect cytokine production and expression of maturation factors by DCs in whole blood. (a) Among viable cells, DCs are identified as negative for the lineage markers (Lin-1) and positive for HLA-DR. (b) HLA-DR^{pos} and Lineage-1^{neg} DCs are then phenotyped as myeloid (CD123^{low}, CD11c^{high}) or plasmacytoid (CD11c^{low}, CD123^{high}) DCs. (c) Expression of maturation factors (CD83, CD80 and CCR7) and cytokines (TNF-α and IFN-α) on DC subsets after TLR7/8 stimulation including isotype and unstimulated controls.

used for analyses; Sigma plot (version 11.0) was used to plot graphs.

Results

Characteristics of the patient population

Sixty-two HAART naïve children (28 males/34 females) with ages ranging from 9 months to 13 years, and median body mass index of 14.4 (6.4–24.1) were included in the study. At entry, CD4 counts in the study subjects ranged between 5 and 44% (median 23%) and plasma HIV-RNA between 4490 and > 750 000 (median 89 200) copies per mL. Patients were classified into age-specific immune categories based on their CD4 count at the time of entry into the study: immune category 1 (IC-1) [CD4% > 25, $n = 20$, (32.25%)], IC-2 [CD4% 15–25, $n = 33$ (53.23%)] and IC-3 [CD4% < 15, $n = 9$ (14.52%)] (CDC, 1994). Nine patients (five with CD4 < 15%, four with CD4 ≥ 15%) were started on HAART (Lamivudine + stavudine with Nevirapine or Efavirenz) during the study period and treatment was given as per National AIDS Control Organisation guidelines (NACO Report, 2006). Children who did not initiate HAART were also monitored every 3 months up to 1 year. The immunological and virological characteristics of different groups at baseline are described in Table 1 and during follow-up are described in Table 2. We also studied 20 (nine males/11 females) healthy age-matched HIV uninfected children as controls at one time point only.

Resiquimod induced cytokine expression on DC subsets

We evaluated the effect of RSQ, a chemical agonist of TLR7 and TLR8, on the DC subsets of HIV+ children. As shown in Table 2 the absolute number of mDCs was greater than that of pDCs in both HIV+ and age-matched healthy control subjects. At baseline, RSQ-induced cytokine expression in both DC subsets (mDC and pDC) showed considerable inter-individual variation in both HIV+ children and age-matched healthy controls. Compared with controls, HIV+ children had significantly lower percentages of RSQ-induced TNF-α-producing cells in both DC subsets. In contrast, IFN-α was expressed exclusively by pDCs and children with HIV had a significantly lower proportion of RSQ-induced IFN-α-secreting pDCs than healthy children (Fig. 2).

Table 1 Baseline immunological and virological characteristics of the study population

Category	Total no. of HIV subjects	IC-1	IC-2	IC-3	Control
No. of samples*	62	20	33 (4)	9 (5)	20
Age†	7 (0.9–13)	7 (0.9–13)	6 (1.5–12)	6 (3–13)	7 (1–12)
VL (log 10) (copies per mL)†	4.96 (3.65–5.87)	4.7 (3.65–5.88)	4.97 (3.75–5.88)	5.32 (4.73–5.69)	–
Hemoglobin (g dL^{-1})	10.96 ± 1.4‡	10.73 ± 1.45	10.96 ± 1.51	11.19 ± 1.23	10.51 ± 1.30
CD3					
%	79 ± 10	79 ± 8	78 ± 7	81 ± 14	66 ± 10
Cells per mm^3	2878 ± 1439	2954 ± 1079	2899 ± 1374	2780 ± 1864	4131 ± 2791
CD4					
%	21 ± 4	32 ± 5	21 ± 3	9 ± 3	33 ± 8
Cells per mm^3	766 ± 374	1213 ± 530	809 ± 459	275 ± 132	2147 ± 1452
CD8					
%	53 ± 10	42 ± 8	51 ± 8	66 ± 14	29 ± 10
Cells per mm^3	1909 ± 1041	1557 ± 578	1885 ± 883	2286 ± 1661	1764 ±1 446
CD19					
%	10 ± 6	12 ± 7	11 ± 5	8 ± 5	23 ± 8
Cells per mm^3	388 ± 335	459 ± 375	465 ± 450	240 ± 179	1338 ± 939
CD16+56					
%	17 ± 4	5 ± 3	7 ± 6	5 ± 4	9 ± 5
Cells per mm^3	220 ± 197	193 ± 130	306 ± 313	161 ± 149	485 ± 389
DC					
%	0.36 ± 0.03	0.31 ± 0.13	0.38 ± 0.28	0.40 ± 0.14	0.67 ± 0.32
Cells per mm^3	31 ± 2	27 ± 14	31 ± 17	33 ± 18	86 ± 66
mDC					
%	0.26 ± 0.02	0.24 ± 0.09	0.26 ± 0.20	0.28 ± 0.09	0.44 ± 0.20
Cells per mm^3	22 ± 2	21 ± 11	22 ± 13	23 ± 12	57 ± 43
pDC					
%	0.1 ± 0.01	0.07 ± 0.05	0.12 ± 0.10	0.12 ± 0.07	0.23 ± 0.14
Cells per mm^3	9 ± 1	6 ± 4	10 ± 6	10 ± 7	29 ± 24

*Values in parentheses are the number of children initiated on HAART.
†Data are median (range).
‡Data are mean ± SD.

Reduced RSQ-induced cytokine expression persisted over follow-up with a further decline in IFN-α induction in pDCs at 12 months (Fig. 3). By contrast, TNF-α expression on both DC subsets was stable between 0 and 12 months in all HIV+ children but significantly lower than in controls. When cytokine expression was compared in patients across different immune stages (IC-1, IC-2 or IC-3), RSQ-induced cytokine expression did not correlate significantly with disease stage. In comparison with untreated subjects, children initiating HAART exhibited stable expression of TNF-α in mDCs. By contrast, a profound reduction of TNF-α and IFN-α expression in pDCs was seen in HIV+ children even after treatment.

Resiquimod induced DC maturation

To examine the effect of reduced cytokine expression on DC maturation in HIV+ children, we analysed the expression of CD83, CD80 and CCR7, all of which are associated with the maturation status of DCs. As shown in Fig. 4, RSQ-induced CD80 and CD83 expression was significantly lower in mDCs of HIV+ children at baseline than controls. In contrast, RSQ-induced CD80 expression was non-significant in pDCs. Also, in comparison with age-matched healthy controls, RSQ-induced CD83 and CCR7 expression was significantly lower in pDCs of HIV+ children.

The defect in DC maturation persisted over follow-up with a further decline in the expression of maturation markers especially CD80 on pDCs at 12 months. CD83, CD80 and CCR7 expression on pDCs varied greatly among different IC groups and the group of HIV+ children as a whole during follow-up. As shown in Fig. 5, RSQ-induced pDC maturation did not correlate significantly with changes in CD4 T-cell counts. In comparison with the untreated children, the HIV+ children initiating HAART showed increased expression of CD83 and CCR7 on pDCs. By contrast, a further decline in CD80 expression was seen on pDCs in HIV+ children even after treatment.

Table 2 Immunological and virological changes in different IC groups during follow-up

	Baseline	3rd Month	6th Month	9th Month	12th Month
Total					
*n**	52 (62)	52 (58)	52 (58)	52 (53)	52 (52)
DC					
%	0.36 ± 0.03	0.29 ± 0.02	0.39 ± 0.03	0.41 ± 0.02	0.49 ± 0.03
Cells per mm^3	31 ± 2	25 ± 2	34 ± 3	34 ± 2	40 ± 3
mDC					
%	0.26 ± 0.02	0.22 ± 0.02	0.29 ± 0.02	0.3 ± 0.02	0.38 ± 0.03
Cells per mm^3	22 ± 2	18 ± 1	25 ± 2	25 ± 1.41	31 ± 2
pDC					
%	0.1 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Cells per mm^3	9 ± 1	6 ± 1	9 ± 1	9 ± 1	9 ± 1
CD3					
%	79 ± 10	81 ± 2	81 ± 1	82 ± 1	79 ± 2
Cells per mm^3	2878 ± 1439	3100 ± 198	2963 ± 195	3093 ± 154	2901 ± 172
CD4					
%	21 ± 4	25 ± 2	26 ± 1	28 ± 2	26 ± 1
Cells per mm^3	766 ± 374	947 ± 63	958 ± 80	1048 ± 77	867 ± 66
CD8					
%	53 ± 10	51 ± 2	50 ± 1.6	50 ± 1.92	49 ± 2
Cells per mm^3	1909 ± 1041	1953 ± 143	1820 ± 133	1880 ± 111	1694 ± 125
VL (log10) (copies per mL)	4.94	–	4.42	–	4.31
IC-1					
*n**	18 (20)	18 (20)	18 (20)	18 (18)	18 (18)
DC					
%	0.31 ± 0.13	0.30 ± 0.15	0.36 ± 0.14	0.43 ±0.23	0.53 ± 0.26
Cells per mm^3	27 ± 14	25 ± 15	32 ± 19	35 ± 15	42 ± 22
mDC					
%	0.24 ± 0.09	0.23 ± 0.14	0.28 ± 0.12	0.34 ± 0.2	0.43 ± 0.23
Cells per mm^3	21 ± 11	19 ± 13	24 ± 12	27 ± 12	34 ± 18
pDC					
%	0.07 ± 0.05	0.07 ± 0.03	0.08 ± 0.05	0.09 ± 0.04	0.10 ± 0.06
Cells per mm^3	6 ± 4	6 ± 3	8 ± 7	7 ± 4	8 ± 5
CD3					
%	79 ± 8	83 ± 7	82 ± 6	81 ± 6	78 ± 9
Cells per mm^3	2954 ± 1079	2736 ± 870	2789 ± 1410	2743 ± 1073	2365 ± 774
CD4					
%	32 ± 5	33 ± 7	31 ± 5	34 ± 7	34 ± 8
Cells per mm^3	1213 ± 530	1086 ± 395	1068 ± 619	1150 ± 549	1033 ± 448
CD8					
%	42 ± 8	46 ± 9	46 ± 9	43 ± 11	40 ± 11
Cells per mm^3	1557 ± 578	1517 ± 548	1560 ± 802	1461 ± 703	1197 ± 437
VL (log10) (copies per mL)	4.7	–	4.73	–	4.53
IC-2					
*n**	22 (33)	22 (25)	22 (25)	22 (22)	22 (22)
DC					
%	0.38 ± 0.28	0.27 ± 0.15	0.43 ± 0.24	0.38 ± 0.10	0.49 ± 0.25
Cells per mm^3	31 ± 17	22 ± 12	30 ± 12	35 ± 11	40 ± 22
mDC					
%	0.26 ± 0.20	0.2 ± 0.13	0.32 ± 0.21	0.26 ± 0.07	0.39 ± 0.21
Cells per mm^3	22 ± 13	17 ± 9	22 ± 8	24 ± 9	31 ± 13
pDC					
%	0.12 ± 0.10	0.07 ± 0.05	0.10 ± 0.05	0.12 ± 0.06	0.10 ± 0.07
Cells per mm^3	10 ± 6	6 ± 4	8 ± 5	11 ± 4	9 ± 6
CD3					
%	78 ± 7	80 ± 9	79 ± 9	82 ± 9	79 ± 12
Cells per mm^3	2899 ± 1374	3546 ± 2039	2639 ± 1243	3608 ± 1237	2830 ± 1316

Table 2 (continued)

	Baseline	3rd Month	6th Month	9th Month	12th Month
CD4					
%	21 ± 3	21 ± 2	21 ± 3	20 ± 3	19 ± 3
Cells per mm^3	809 ± 459	933 ± 510	692 ± 326	909 ± 451	684 ± 286
CD8					
%	51 ± 8	53 ± 10	53 ± 9	58 ± 11	55 ± 11
Cells per mm^3	1885 ± 883	2341 ± 1411	1757 ± 853	2488 ± 738	2008 ± 1048
VL (log10) (copies per mL)	5.0	–	4.63	–	4.6
IC-3					
*n**	4 (9)	4 (4)	4 (4)	4 (4)	4 (4)
DC					
%	0.40 ± 0.14	0.37 ± 0.06	0.42 ± 0.39	0.47 ± 0.12	0.57 ± 0.10
Cells per mm^3	33 ± 18	26 ± 12	51 ± 35	36 ± 10	51 ± 22
mDC					
%	0.28 ± 0.09	0.26 ± 0.06	0.28 ± 0.26	0.33 ± 0.13	0.40 ± 0.08
Cells per mm^3	23 ± 12	18 ± 9	31 ± 27	26 ± 10	36 ± 15
pDC					
%	0.12 ± 0.07	0.11 ± 0.04	0.14 ± 0.10	0.14 ± 0.03	0.16 ± 0.03
Cells per mm^3	10 ± 7	8 ± 5	19.89 ± 8.13	11 ± 3	15 ± 7
CD3					
%	81 ± 14	77 ± 20	87 ± 6	84 ± 6	79 ± 10
Cells per mm^3	2780 ± 1864	2424 ± 1413	4691 ± 1652	2492 ± 435	2329 ± 1022
CD4					
%	9 ± 3	12 ± 2	13 ± 2	12 ± 2	11 ± 3
Cells per mm^3	275 ± 132	357 ± 177	670 ± 158	376 ± 118	348 ± 185
CD8					
%	66 ± 14	60 ± 18	68 ± 8	67 ± 8	63 ± 12
Cells per mm^3	2286 ± 1661	1923 ± 1190	3766 ± 1663	1988 ± 341	1846 ± 805
VL (log10) (copies per mL)	5.24	–	5.48	–	5.23
HAART					
*n**	8† (9)	8 (9)	8 (9)	8 (9)	8 (8)
DC					
%	0.42 ± 0.25	0.28 ± 0.15	0.44 ± 0.17	0.40 ± 0.08	0.34 ± 0.04
Cells per mm^3	33 ± 28	27 ± 18	36 ± 22	32 ± 3	27 ± 6
mDC					
%	0.31 ± 0.17	0.21 ± 0.08	0.31 ± 0.13	0.31 ± 0.02	0.24 ± 0.03
Cells per mm^3	25 ± 19	20 ± 11	27 ± 19	26 ± 7	19 ± 4
pDC					
%	0.11 ± 0.08	0.07 ± 0.08	0.13 ± 0.05	0.09 ± 0.07	0.1 ± 0.05
Cells per mm^3	9 ± 9	7 ± 7	9 ± 4	6 ± 4	8 ± 3
CD3					
%	75 ± 14	83 ± 4	81 ± 8	83 ± 4	80 ± 3
Cells per mm^3	2969 ± 1808	3103 ± 994	3190 ± 1185	3122 ± 1160	2686 ± 545
CD4					
%	13 ± 2	25 ± 2	24 ± 6	34 ± 7	30 ± 3
Cells per mm^3	477 ± 156	906 ± 185	1059 ± 351	1303 ± 489	942 ± 99
CD8					
%	55 ± 16	53 ± 7	53 ± 8	45 ± 10	44 ± 3
Cells per mm^3	2243 ± 1596	1991 ± 740	1963 ± 888	1681 ± 978	1564 ± 408
VL‡ (log10) (copies per mL)	5.5	–	3.25	–	3.01

*Number of samples taken for analysis (total number of samples collected).
†Before initiation of HAART (data are mean ± SD).
‡VL, viral load (data are median values).

We observed significantly lower expression of CD83 and CD80 on mDCs in the group of HIV+ children as well as different IC groups and during follow-up. As shown in Fig. 6, expression of CD83 and CD80 on mDCs varied greatly in HIV+ children but was significantly lower than in controls. In comparison with the untreated children, HIV+ children

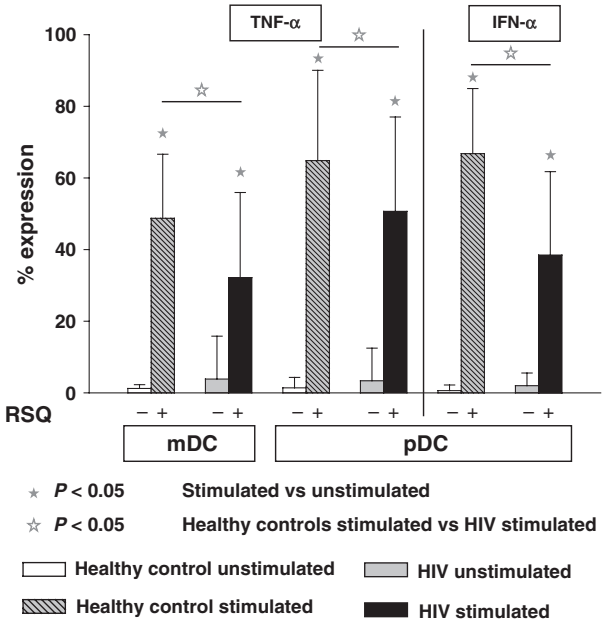

Fig. 2 Percentage of DC subsets (mDC, pDC) expressing cytokines TNF-α and IFN-α after TLR7/8 stimulation in whole blood taken from HIV+ children (n = 62) and age-matched controls (n = 20). Whole blood was incubated without any stimulus or with resiquimod (10 μM) for 3 h. Cells were then processed and acquired by flow cytometry as described in the Methods. The data are shown as the mean and SD.

initiating HAART showed a non-significantly higher expression of CD83 and CD80 on mDCs.

Discussion

We studied DC-specific markers of phenotype and function in children with perinatal HIV infection and evaluated their change over a period of 1 year. We also compared these markers across different CD4 strata and studied the impact of HAART on DC functions and phenotype.

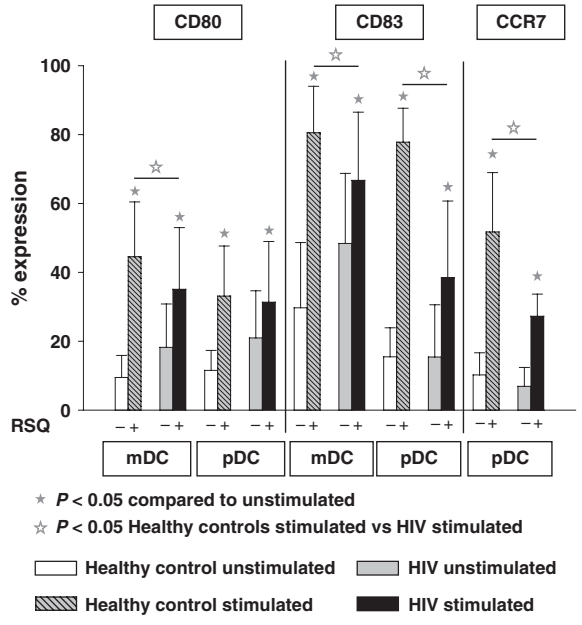

Fig. 4 Expression of CD80 and CD83 on DC subsets (mDC, pDC) and expression of CCR7 on pDCs only after TLR7/8 stimulation in whole blood taken from HIV+ children (n = 62) and age-matched controls (n = 20). Whole blood was incubated without any stimulus or with resiquimod (10 μM) for 5 h. Cells were then processed and acquired by flow cytometry as described in the Methods. The data are shown as the mean and SD.

Although previous research has shown that HIV-1-infected individuals have reduced mDC and pDC levels, information on the functional capability of blood DC subsets and their role during immune reconstitution in HAART-treated pediatric HIV patients is not well described (Zhang *et al.*, 2006; Usuga *et al.*, 2008). We found that circulating mDCs and pDCs were deficient qualitatively and quantitatively at study entry in HIV-infected children when compared with age-matched controls. The persistence of

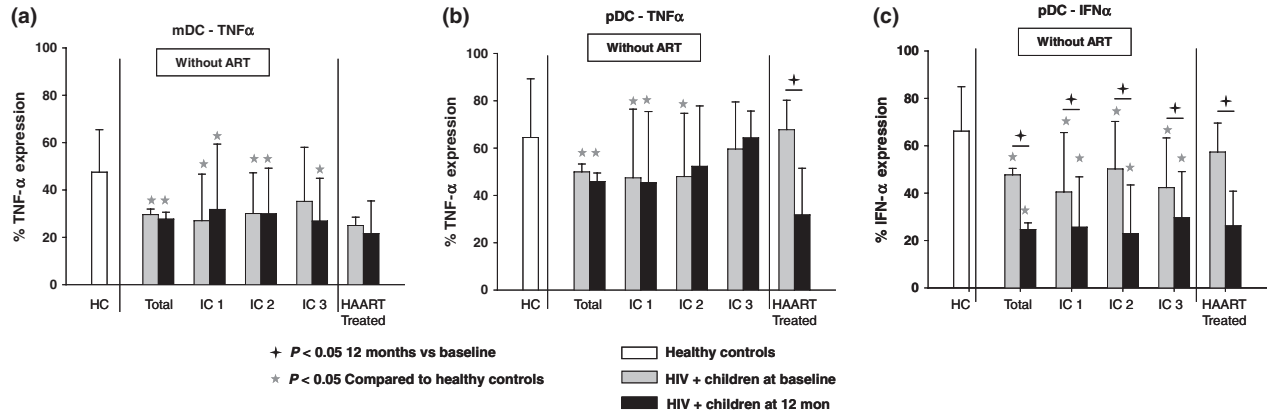

Fig. 3 Percentage of DC subsets (mDC, pDC) expressing cytokines. (a) mDC-TNF-α (b) pDC-TNF-α and (c) pDC-IFN-α after TLR7/8 stimulation in whole blood taken from age-matched controls, HIV+ children at baseline, HIV+ children at 12 months follow-up without treatment (categorized as IC-1, IC-2 and IC-3) and HIV+ children at 12 months after HAART. Whole blood was incubated without any stimulus or with resiquimod (10 μM) for 3 h. Cells were then processed and acquired by flow cytometry as described in the Methods. The data are shown as the mean and SD.

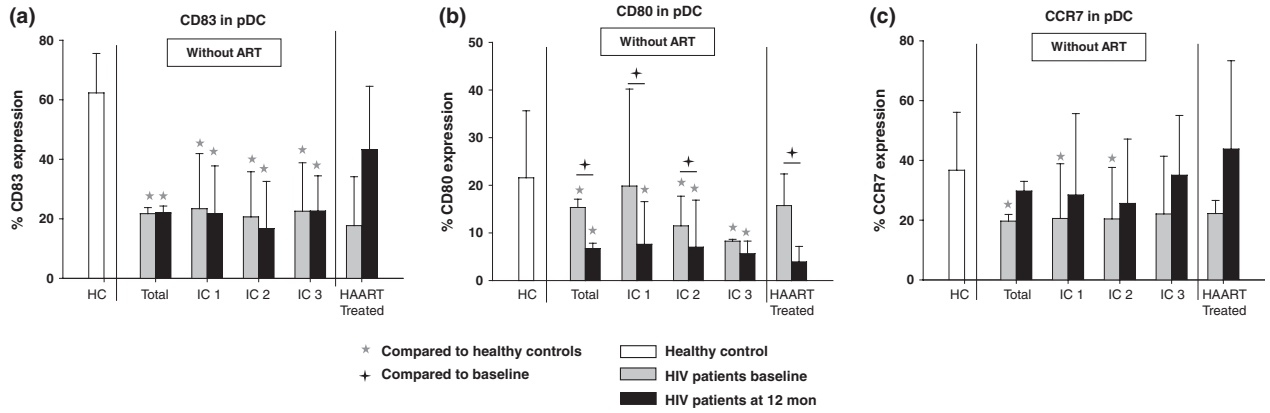

Fig. 5 Expression of (a) CD83, (b) CD80 and (c) CCR7 on pDC subsets after TLR7/8 stimulation in whole blood taken from age-matched controls, HIV+ children at baseline, HIV+ children at 12 months follow-up without treatment (categorized as IC-1, IC-2 and IC-3) and HIV+ children at 12 months after HAART. Whole blood was incubated without any stimulus or with resiquimod (10 μM) for 5 h. Cells were then processed and acquired by flow cytometry as described in the Methods. The data are shown as the mean and SD.

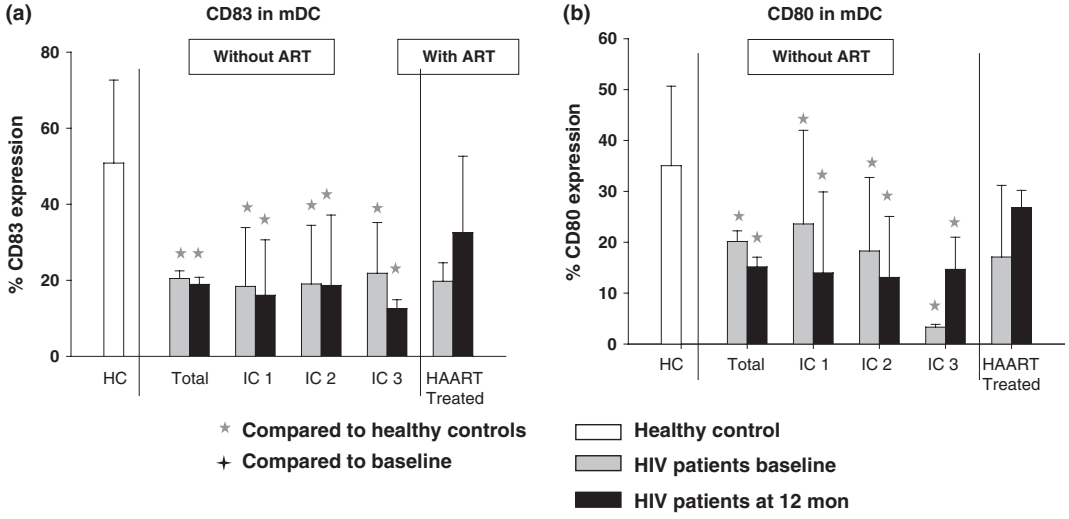

Fig. 6 Expression of (a) CD83 and (b) CD80 on mDC subsets after TLR7/8 stimulation in whole blood taken from age-matched controls, HIV+ children at baseline, HIV+ children at 12 months follow-up without treatment (categorized as IC-1, IC-2 and IC-3) and HIV+ children at 12 months after HAART. Whole blood was incubated without any stimulus or with resiquimod (10 μM) for 5 h. Cells were then processed and acquired by flow cytometry as described in the Methods. The data are shown as the mean and SD.

these defects over 1 year, with a further decline in some of their characteristics, suggests that these functional properties may play an important role in mounting or maintaining a successful immune response against perinatal HIV infection. We also found that HAART treatment partially restored mDCs, but that recovery of pDCs was incomplete. Our data showing a further decline in CD80 and IFN-α induction in pDCs over follow-up, despite HAART, supports the idea that pDC function is profoundly impaired in pediatric patients with HIV-1 infection and that delayed HAART initiation does not restore this immunological defect. Collectively, these defects were evident in analysis of all patients, and were variably expressed in patients in immune categories IC-1, IC-2 and IC-3.

In agreement with studies conducted in adult populations (Macatonia *et al.*, 1990; Grassi *et al.*, 1999; Feldman *et al.*, 2001; Chehimi *et al.*, 2002; Hemmi *et al.*, 2002; Barron *et al.*, 2003; Donaghy *et al.*, 2003), both mDC percentages and their ability for cytokine (TNF-α) production were reduced in therapy naïve HIV-infected children, indicating that this DC subset is also depleted in pediatric HIV-1 infection. The functional impairment of mDCs partially recovered in children on HAART. The differential restoration of mDCs and pDCs in HIV-1-infected children after HAART might be explained in part by differences in HIV susceptibility, variable sensitivities to HAART treatment, and selective interaction of HIV-1 with mDCs and pDCs.

Based on our examination of circulating DC subsets throughout the period of our study, we draw the following conclusions. First, HIV-1 infection leads to a decrease in numbers of circulating DC subsets along with a functional impairment in these cells. Impaired functions include the

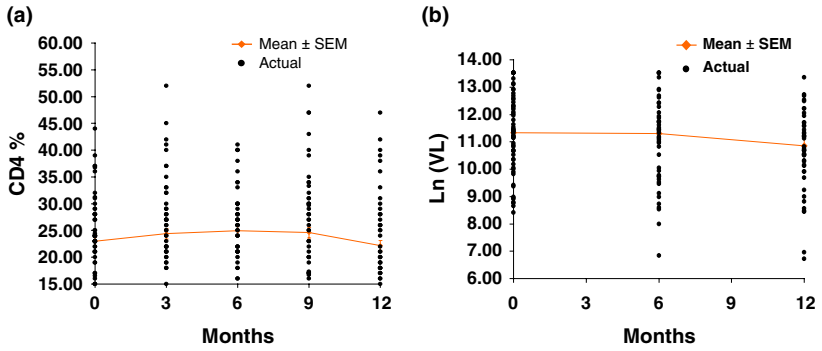

Fig. 7 CD4 (a) and viral load (b) over the 12-month follow-up period were relatively stable in untreated HIV+ children.

decreased expression of maturation markers (CD80, CD83 and CCR7) and decreased cytokine-releasing capacities of mDCs (TNF-α) and pDCs (TNF α and IFN-α). Secondly, while HAART can successfully control HIV-1 replication, there is only a partial recovery in DCs; pDC frequency and function recovery is less than mDC recovery. Time of initiation of HAART may influence the degree of recovery of functionally impaired pDCs, as most children in this cohort started treatment quite late. Thus, cellular immune responses that involve CD4 T cells may be induced, coordinated and regulated by DCs.

The cohort studied by us were children who had survived infancy and thus had a less aggressive course of the disease (Fig. 7). Furthermore, only a small number were initiated on HAART during the 12-month period of follow-up, limiting our observation on the impact of HAART on recovery of innate immune functions. However, future studies are planned to examine this phenomenon in infants initiated on HAART early in life, and examine the long-term effect of HAART on the immune system.

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Authors' Contribution

S.S. and S.P. contributed equally to the work.

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