Baseline Viral Load and Immune Activation Determine the Extent of Reconstitution of Innate Immune Effectors in HIV-1-Infected Subjects Undergoing Antiretroviral Treatment

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Baseline Viral Load and Immune Activation Determine the Extent of Reconstitution of Innate Immune Effectors in HIV-1-Infected Subjects Undergoing Antiretroviral Treatment

Jihed Chehimi,† Livio Azzoni,‖ Matthew Farabaugh,*, Shenoa A. Creer,*, Costin Tomescu,*, Aidan Hancock,*, Agnes Mackiewicz,*, Lara D’Alessandro,*, Smita Ghanekar,† Andrea S. Foulkes,‡ Karam Mounzer,§ Jay Kostman,¶ and Luis J. Montaner4*

We analyzed dendritic cell (DC) and NK cell compartments in relation to CD4 recovery in 21 HIV-infected subjects followed to <50 copies/ml once starting antiretroviral therapy (ART) and observed for 52 wk of sustained suppression. Although CD4 counts increased in all subjects in response to ART, we observed a restoration of functional plasmacytoid DC (PDC) after 52 wk of sustained suppression under ART (from 1850 cells/ml to 4550 cells/ml) to levels comparable to controls (5120 cells/ml) only in subjects with a low baseline viral load, which also rapidly suppressed to <50 copies/ml upon 60 days from ART initiation. Recovery of PDC at week 52 correlates with level of CD95 expression on CD8 T cells and PDC frequency following first ART suppression. NK cytotoxic activity increased rapidly upon viral suppression (VS) and correlated with PDC function at week 52. However, restoration of total NK cells was incomplete even after 52 wk on ART (73 cells/μl vs 122 cells/μl in controls). Direct reconstitution experiments indicate that NK cytotoxic activity against virally infected target cells requires DC/NK cooperation, and can be recovered upon sustained VS and recovery of functional PDC (but not myeloid DC) from ART-suppressed subjects. Our data indicate that viremic HIV-infected subjects may have different levels of reconstitution of DC and NK-mediated function following ART, with subjects with lower initial viremia and the greatest reduction of baseline immune activation at VS achieving the greatest level of innate effector cell reconstitution. The Journal of Immunology, 2007, 179: 2642–2650.

Human immunodeficiency virus infection is associated with chronic immune activation, progressive immune suppression, and deletion of memory adaptive responses, resulting in increased susceptibility to opportunistic infections (1, 2). The loss of adaptive memory responses and their functional recovery following viral suppression (VS) have been areas of active investigation; however, even though NK cells and dendritic cells (DC) contribute to early host defenses and shape subsequent adaptive immune responses via a complex cross-talk regulating the early phase of the immune response (3–10), their specific role in AIDS pathogenesis remain uncertain (11–13).

NK cells, characterized by their ability to spontaneously kill tumor and virus-infected cells, play an important role in early immune defense against pathogens (3). NK effector function is regulated by a dynamic balance between positive and negative signals derived from membrane receptors, maturation, and accessory cell help (14).

DCs are critical in initiating and regulating both innate (NK and macrophages) and adaptive responses (T and B cells). Based on phenotypic markers, at least two distinct DC subsets have been characterized in human peripheral blood: myeloid (MDC) and plasmacytoid (PDC). Although PDC express preferentially the endosomal TLR7 and TLR9, MDC express TLR2, TLR3, TLR4, and TLR8 (15, 16). Both DC subsets effectively contribute to the activation of NK cells: MDC secrete IL-12, IL-15, IL-18 following pathogen stimulation, inducing NK cell-mediated IFN-γ secretion; PDC secrete IFN-α following viral stimulation, which results in increased NK cell function (8–10).

Significant depletion and functional impairment of both NK and DC compartments have been documented in HIV infection (17–27) but the mechanisms underlying innate immune dysfunction remain to be elucidated. A loss in circulating CD123+ PDC was described in HIV-infected subjects with high viral loads (VL) and/or opportunistic infections or cancer, indicating that lower levels of PDC are associated with disease progression (17, 21). HIV-1 infection is also associated with NK cell dysfunction (13), as indicated by: 1) loss of the major NK cell subset (CD56+CD16+) in conjunction with an overall reduction of cytotoxic function; 2) expansion of the minor NK cell subset CD56–CD16+; 3) imbalance in inhibitory NK receptors/natural cytotoxic receptors; 4) reduction in secretion of cytokines/chemokines with anti-HIV properties; 5) alterations of gene expression; and, possibly, 6) direct infection of NK
cells by HIV. Overall, the concept that innate function may have a direct relationship with HIV replication and disease progression is supported by studies showing a positive association between PDC or NK function and a lack of disease progression (17, 20, 21) as well as genetic studies demonstrating that expression of NK-associated genes (e.g., the activating allele KIR3DS1 in combination with HLA-B Bw4–80Ile) are associated with delayed disease progression (29, 30).

Introduction of combination antiretroviral therapy (ART) has resulted in a decline in the morbidity and mortality of HIV infection; partial restoration of CD4 T cells and a degree of regeneration within the immune system. The recovery of the innate immune compartment has remained relatively unexplored, particularly with regard to longitudinal clinical studies focusing on the recovery of the DC and NK compartments in the context of the recovery of CD4 T cells following ART-mediated VS.

In prior work, we and others (22, 23) reported cross-sectional studies showing normal MDC frequency and function in HIV-1-infected, ART-suppressed subjects; however, the detection of decreased levels in PDC numbers and function in both viremic and suppressed subjects as compared with uninfected controls raised the hypothesis of a persistence of low levels of circulating PDC even following ART (22). Although cross-sectional studies have shown a degree of ART-mediated restoration of the NK compartment in adults and children (13, 24), few studies have addressed the time-related effects of suppressive ART on the functional and phenotypic recovery of NK in relation to CD4 and DC changes.

In this study, we describe longitudinal changes observed in NK and DC subsets (phenotype and function) following suppressive ART in a group of chronically infected adult subjects over a period of 52 wk after achieving <50 copies/ml VS on ART.

Materials and Methods

Study subjects

HIV-1-seropositive subjects (n = 21) naive to ART were recruited and completed follow-up at The Jonathan Lax Immune Disorders Clinic (Philadelphia Field Initiation Group for HIV Trials [FIGHT]). The study population was composed of 6 females and 15 males with a median age of 36 years (range 22–50), with CD4 and VL ranging from 85 to 435 cells/ml and 1,170 to 339,956 HIV RNA copies/ml. ART was initiated based on clinical evaluation by the patient’s healthcare provider. Blood was drawn at baseline (BL) before ART initiation, and every 4 wk thereafter for up to 24 wk, or until achievement of VS (serum HIV RNA <50 copies/ml). Follow-up visits were at weeks 4, 8, 16, 32 and 52 following VS. All visits included testing of CD4 and CD8 T cell counts, WBC differential, leukocyte count, and VL determinations. Subjects that did not achieve VS after 24 wk on ART and subjects with poor adherence to individual treatment regimen were excluded from follow-up. Results at BL, VS (first VL <30 copies/ml), and 52-wk follow-up were analyzed for this study. Healthy HIV-1- seronegative donors age- and gender-matched from The Wistar Institute Blood Donor Program were included as controls. The clinical characteristics of the study subjects are shown in Table I. Informed consent was obtained from all study participants at enrollment. The study was approved and monitored by Institutional Review Boards at the Philadelphia FIGHT and The Wistar Institute.

PBMC preparation and whole blood phenotypic analysis of T cells, PDC, MDC, and NK subsets

Blood was processed within 6 h of drawing. PBMC were separated on Ficoll-Paque (Amersham Pharmacia Biotech) density gradient and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, l-glutamine, and antibiotics. Multicolor FACS analysis was performed on whole blood (200 µl) as described (22). All MAbs used were obtained from BD Biosciences, DC, NK cells, and activated T cells were obtained from The Wistar Institute Blood Donor Program were included as controls. The clinical characteristics of the study subjects are shown in Table I. Informed consent was obtained from all study participants at enrollment. The study was approved and monitored by Institutional Review Boards at the Philadelphia FIGHT and The Wistar Institute.

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IFN-α production

PBMC (2.5 × 10⁶/well) were cultured in 24-well plates for 18 h with heat-inactivated influenza virus (strain PR8, 8-10 HA) interacting through TLR7, or CpG oligodeoxynucleotide class A (CpG-2216, 10 µg/ml from
Integrated DNA Technologies) interacting with TLR9. All reagents used were selected for their low levels of endotoxin contamination. Allantoin fluid and CpG 1040 used as controls did not induce any detectable amount of IFN-α (data not shown). In selected experiments, PDC were enriched using BDCA2/4 microbeads (Miltenyi Biotec), stimulated with CpG-2216 for 18 h and tested for IFN-α by ELISA (Pierce Endogen). Sensitivity of the assay was ~8–12 pg/ml. For intracellular detection of IFN-α in PDC, PBMC (106 cells/ml) were stimulated for 18 h with medium or CpG-2216 (10 μM) and treated with brefeldin A (5 μg/ml; Sigma-Aldrich) added 2 h after the start of the stimulation. Cells were stained for PDC for 15 min, washed, fixed, and permeabilized using Cytofix/Cytoperm (BD Biosciences) and stained with PE-conjugated anti-IFN-α (clone 7N4-1; BD Biosciences).

**NK assays**

PBMC (5 × 10^6/well) from HIV+ and control subjects were cultured in 24-well plates for 18 h in the presence of medium (unstimulated), CpG-2216 (10 μM), IL-12/IL-15 (10 ng/ml each from R&D Systems), IFN-γ (data not shown). In selected experiments, PDC were enriched using HLA-DR-depleted PBMC at a ratio of 1:1 and 1:10, respectively. PBMC were depleted of HLA-DR+ cells was performed using HLA-DR mAbs. The average yield from 106 viable cells) were labeled with Na2CrO4 (100 μg/ml) with brefeldin A (5 μg/ml; Sigma-Aldrich) added 2 h after the incubation of 0.1. When ~90% of cells exhibited cytotoxic changes (24–36 h), viable cells were trypsinized, washed, and cryopreserved at −80°C. Target cells (2 × 10^5 viable cells) were labeled with Na2CrO4 (−100 μCi) for 1 h (K562) or 2 h (HIV-SUP-T1 and HSV-FS4) at 37°C washed and suspended in culture medium (5 × 10^5 cells/ml). Effector cells (5 × 10^6/ml) and 51Cr-labeled targets were incubated at the desired E:T ratios (50:1, 25:1, 12.5:1, and 6.25:1) in 0.2 ml of culture in round-bottom 96-well plates and incubated for 4 h for K562, 8 h for HIV-SUP-T1, or 18 h for HSV-FS4. Cell-free supernatant was collected and 51Cr release was measured on cell-free supernatants. Percent-specific lysis was determined as described (18). To standardize cytolysis activity for quantitative analysis, the area under the curve (AUC) was calculated using percent-specific lysis of all four E:T ratios modified from the method of Stroehlein et al. (32).

**Enrichment of DC subsets**

Enrichment of circulating blood DC subsets from 10 HIV-infected subjects and 4 controls was performed using BDCA1 (MDC), and BDCA2/4 (PDC) isolation kits (Miltenyi Biotec). The isolation procedure was repeated twice to increase purity. Purity was determined using flow cytometry and directly cultured BDCA1 and BDCA2/4 (5 × 10^5 cells/ml). Effector cells (5 × 10^6/ml) and 51Cr-labeled targets were incubated at the desired E:T ratios (50:1, 25:1, 12.5:1, and 6.25:1) in 0.2 ml of culture in round-bottom 96-well plates and incubated for 4 h for K562, 8 h for HIV-SUP-T1, or 18 h for HSV-FS4. Cell-free supernatant was collected and 51Cr release was measured on cell-free supernatants. Percent-specific lysis was determined as described (18).

**Depletion and reconstitution experiments**

PBMC were depleted of HLA-DR+ cells using anti-HLA-DR mAb (B53.1; The Wistar Institute) and rabbit complement (1:2) as described (33). PDC and MDC were depleted using BDCA4 or BDCA1 microbeads and NK cells depleted using NK column (Stem Cell Technologies). Cells were retested by flow cytometry to confirm depletion outcomes. For reconstitution experiments, HLA-DR+ cells, purified PDC and MDC were added to HLA-DR-depleted PBMC at a ratio of 1:1 and 1:10, respectively.

**Statistical analysis**

Within groups, time-related changes (overall time trend) were first tested by categorical tests were performed the using Fisher’s exact test. All tests were two-tailed, α = 0.05. Analysis was performed with JMP (SAS) or Prism (Graphpad Software).

**Results**

**Clinical characteristics of the study population and response to ART**

Table I lists the clinical characteristics of the study subjects. To determine the extent to which the kinetics of ART-mediated VS (<50 HIV RNA copies/ml) affect immune reconstitution, we divided our HIV+ subjects into two groups: group 1, subjects achieving rapid VS (VL <50 copies/ml by week 12 of treatment; median VL at BL = 4134 copies/ml; median days to suppression = 45), and group 2, subjects with delayed VS (VL >50 copies/ml after week 12 of ART; median BL VL = 29254 copies/ml; median days to suppression = 92). CD4+ T cell number was similar in both groups (median 231 vs 261/μl, respectively) despite a significant difference in median VL at BL (p = 0.0054, between groups 1 and 2). In accordance with prior reports and our recently published study (26, 34–36), we detected a significant increase in CD4 T cells by week 52 in the whole population (p = 0.002, repeated measures ANOVA). Both groups had comparable CD4+ T cells recovery by week 52, with a median gain of 141 cells/μl in group 1 (p = 0.0039 compared with BL) and 174 cells/μl (p = 0.0216 compared with BL) in group 2 (Fig. 1A). As described by others (37), we observed a significant association between therapy regimen and time to VS, with subjects receiving NNRTI on a NRTI backbone being mostly in group 1 (rapid suppressors) and subjects PI on NRTI backbone in group 2 (Fisher, p = 0.02). No difference in CD4 T cell reconstitution was noted between subjects with high or low VL starting ART, nor by the VS rate.

Association of ART-mediated PDC recovery with pre-ART VL and CD8+ T cell activation

In agreement with previous findings (12), PDC frequency (%) and absolute numbers were significantly lower in viremic pre-ART patients (BL) as compared with control individuals (median 0.17 vs 0.72%, p = 0.0017, and 1800 cells/ml vs 5120 cells/ml, p = 0.0017, Fig. 1B). Following ART initiation, we observed an increase in PDC numbers to a median 2190 cells/ml at VS and 4465 cells/ml after week 52 (Fig. 1B). Upon segregation of rapid and delayed recovery subjects, BL PDC numbers were similar in both groups (1850 and 1750 cells/ml, Fig. 1C); however, only subjects in group 1 showed a significant rise in PDC numbers after week 52 (median 1850 cells/ml at BL and 4550 cells/ml at week 52, p = 0.04, Fig. 1C). This observation was also reflected in a significant difference in median PDC gain (between BL and week 52) between groups, with 3070 PDC/ml for group 1 and 490 PDC/ml for group 2; the analysis of the relationship between BL VL and week 52 PDC recovery indicated a correlation trend (r = −0.47, p = 0.08) further supporting differences observed between subjects with high or low pre-ART VL. Repeated measures ANOVA did not indicate a significant effect of time in the whole population (Fig. 1A), likely due to the lack of change in group 2. Even though time to suppression was found to be associated with NNRTI or PI-based ART regimen, it was not directly associated with PDC recovery at week 52.

Together with increased CD4 count, decreased immune activation is an early outcome of suppressive ART; thus, we evaluated the relationship between suppression of T cell activation and long-term PDC recovery. At the time of first VS, both PDC levels (r = 0.65, p = 0.01) and the expression of CD95 on CD8+ T cells (r = −0.55, p = 0.04) were associated with week 52 PDC frequency (Fig. 2A and B), supporting the interpretation that higher BL VLs and levels of CD8 activation after therapy impact PDC recovery. This was sustained by the observation of a significant relationship between PDC and CD28 expression on CD8+ T cells (CD28 serving as a marker of CD8 differentiation of effector subsets, inversely associated with CD95 expression at all time points, data not shown); both CD28 expression on CD8+ T cells at BL and the change in CD28 frequency at VS were associated with 52-wk PDC frequency (r = 0.69, p = 0.008; r = −0.54, p = 0.052, respectively). Association between PDC frequency and CD28 (r = 0.60,
To assess the functional impact of the observed PDC depletion and ART-mediated reconstitution, we evaluated the production of IFN-γ. First, we tested the secretion of IFN-γ upon 18-h engagement of TLR7 (influenza virus PR8) or TLR9 (CpG-2216). IFN-γ production at BL was significantly lower than controls (PR8: 201 vs 1634 pg/ml in controls, *p* < 0.0001, Fig. 3A; CpG-2216: 68.30 pg/ml vs 1011 pg/ml in controls, *p* < 0.0001, Fig. 3B). We also measured IFN-γ plasma levels in pre-ART, viremic HIV-infected subjects and controls (median 20.24 and 12.2 pg/ml, respectively), and found no significant difference between groups, and no correlation with in vitro-induced IFN-α levels.

Repeated measures ANOVA indicated a significant difference in IFN-γ production between BL and week 52 (*p* = 0.010 for PR8 and *p* < 0.0001 for CpG), indicative of a recovery of function following sustained VS. However, IFN-γ production remained significantly lower than controls throughout the follow-up (Fig. 3, A and B), while recovery was greater at 52 wk rather than at first VS. We confirmed these results using intracellular IFN-γ detection on PBMC stimulated with CpG-2216. Fig. 3C illustrates expression of IFN-γ in PDC from the same representative HIV-infected subject at BL (left panel), and at week 52 (<50 RNA copies/ml, central panel), demonstrating a rise in IFN-γ production by PDC.
FIGURE 3. Partial restoration of IFN-α production upon engagement of TLR7 and TLR9. PBMC (2.5 × 10⁶ cells) of HIV⁺ and controls were stimulated for 18 h with heat-killed PR8 (A) or CpG2216 (B) for IFN-α production (ELISA). Horizontal bars represent the median values. Circles (●) represent HIV⁺ subjects at BL, VS, and S2. Controls are HIV⁻ healthy individuals (○). C, Expression of intracellular IFN-α on PDC from a representative HIV⁺ VS, and 52. Controls are HIV⁻ (Table II), comparing the levels of IFN-α production in virally suppressed vs 0.05, Table II), supporting a partial recovery of PDC function following VS, irrespective of cell number. Taken together, our data indicate a median of 0.59 pg/cell in virally suppressed vs 0.14 pg/cell in viremic subjects (p = 0.008); both values were significantly lower than controls (1.74 pg/PDC in controls, both p < 0.015, Table II), supporting a partial recovery of PDC function following VS, irrespective of cell number. Taken together, our data indicate that 1) impaired IFN-α production in viremic HIV-infected individuals reflects both PDC depletion and functional impairment of remaining PDCs; 2) a partial functional PDC recovery is achieved with long-term suppressive ART (52 wk and over), and 3) BL VL and rapid VS in response to therapy may predict the degree of PDC recovery after 52 wk of ART, as indicated by greater recovery with lower pre-ART VL and time to suppression on ART, while also showing a negative correlation with CD8 T cell activation.

Delayed recovery of mature NK cells despite rapid cytotoxic function restoration

Compromised NK cell numbers and function are observed as a result of chronic HIV infection (13). To assess the corrective impact of suppressive ART on the NK cell compartment, we assessed the frequency and phenotype of NK cell subsets and NK cell-mediated cytotoxicity in HIV-infected subjects before and during suppressive ART. As expected, total NK cells (a combination of all three phenotypes analyzed, CD3⁻ CD161⁺/⁻ CD56⁻ CD16⁺, CD3⁻ CD161⁺/⁻ CD56⁺ CD16⁻, and CD3⁻ CD161⁺ CD56⁻ CD16⁻) were significantly lower than in control donor, right panel). We further confirmed this observation in a cross-sectional group of 10 HIV-infected and 4 control subjects (Table II), comparing the levels of IFN-α produced upon TLR9 engagement using enriched circulating PDC (>85% purity). An estimation of the IFN-α secretion per PDC indicates a median of 0.59 pg/cell in virally suppressed vs 0.14 pg/cell in viremic subjects (p = 0.008); both values were significantly lower than controls (1.74 pg/PDC in controls, both p < 0.05, Table II), supporting a partial recovery of PDC function following VS, irrespective of cell number. Taken together, our data indicate that 1) impaired IFN-α production in viremic HIV-infected individuals reflects both PDC depletion and functional impairment of remaining PDCs; 2) a partial functional PDC recovery is achieved with long-term suppressive ART (52 wk and over), and 3) BL VL and rapid VS in response to therapy may predict the degree of PDC recovery after 52 wk of ART, as indicated by greater recovery with lower pre-ART VL and time to suppression on ART, while also showing a negative correlation with CD8 T cell activation.

**Table II. Intrinsic defect of PDC from viremic patients to produce IFN-α upon TLR9 engagement***

<table>
<thead>
<tr>
<th>Donors</th>
<th>HIV RNA</th>
<th>PDC</th>
<th>pg IFN-α/PDC</th>
<th>Median pg IFN-α/PDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;100,000</td>
<td>1,800</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&gt;100,000</td>
<td>1,558</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&gt;100,000</td>
<td>1,750</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39,800</td>
<td>3,600</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>&gt;100,000</td>
<td>2,800</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>&lt;50</td>
<td>4,520</td>
<td>0.43</td>
<td>0.14 (p = 0.016)***</td>
</tr>
<tr>
<td>7</td>
<td>&lt;50</td>
<td>4,910</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>&lt;50</td>
<td>6,840</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&lt;50</td>
<td>3,824</td>
<td>0.35</td>
<td></td>
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<td>10</td>
<td>&lt;50</td>
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<td>0.59</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Control</td>
<td>5,625</td>
<td>1.06</td>
<td></td>
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<td>12</td>
<td>Control</td>
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<td>1.20</td>
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<tr>
<td>13</td>
<td>Control</td>
<td>5,990</td>
<td>2.51</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Control</td>
<td>14,000</td>
<td>2.28</td>
<td></td>
</tr>
</tbody>
</table>

* PDC from HIV⁺ (different from subjects described in Table I) and control subjects were enriched using two consecutive passages through a BDCA-4 isolation kit and incubated with CpG-2216 (10 mg/ml) for 18 h. Supernatants were harvested and tested for IFN-α by ELISA.

** PDC absolute numbers per milliliter were determined in whole blood before column enrichment.

* A total of 1 × 10⁶ enriched PDC were stimulated for 18 h by CpG-2216; pg IFN-α/PDC = picograms of IFN-α per PDC corrected according to the percent enrichment.

*** Denotes statistical analysis between viremic subjects (median 0.14 pg/PDC) and controls (median 1.74 pg/PDC) and suppressed subjects (0.59 pg/PDC) and controls (1.74 pg/PDC by Mann-Whitney).

**** Denotes statistical difference between viremic (0.14 pg/PDC) and controls (1.74 pg/PDC) and suppressed (0.59 pg/PDC) subjects (Mann-Whitney).
and the major NK subset (CD3<sup>+</sup>CD16<sup>+</sup>/CD56<sup>−</sup>CD16<sup>+</sup>) were significantly reduced in viremic, pre-ART subjects as compared with controls (median 50 vs 122 cells/µL, p = 0.0038, Fig. 4A, median 29 vs 87 cells/µL, p = 0.0287, Fig. 4B). Following ART, repeated measures ANOVA, indicated a trend for a positive change for the major NK subset (p = 0.09), which was reflected by total NK and CD3<sup>+</sup>CD16<sup>+</sup>/CD56<sup>−</sup>CD16<sup>+</sup> numbers increases by week 52 (from a median 50 cells/µL to 73 cells/µL for total NK and from 29 to 64 cells/µL for the major subset). No correlation was found between NK subsets at week 52 and VL at BL, nor with CD28 or CD95 expression on CD8 T cells. In contrast to previous reports (38–41), no statistical differences between HIV-infected and control subjects were detected for the CD3<sup>+</sup>CD16<sup>+</sup>/CD56<sup>−</sup>CD16<sup>−</sup> and CD3<sup>+</sup>CD16<sup>+</sup>/CD56<sup>−</sup>CD16<sup>−</sup> subsets at any time point tested (Fig. 4, C and D); however, repeated measures ANOVA indicates a decrease in the minor subset CD3<sup>+</sup>CD16<sup>+</sup>/CD56<sup>−</sup>CD16<sup>−</sup> (p = 0.047).

NK cell cytotoxicity was assessed against HIV-1-infected target cells at all time points. At BL, both percent-specific lysis and AUC were significantly lower in viremic subjects (median-specific lysis = 17.89% at E:T ratio of 1:50; AUC = 745.3) compared with controls (median-specific lysis = 42.5%, and AUC = 2594, p < 0.0001 for specific lysis and AUC, Fig. 5A, Table III). Similar to PDC function (i.e., IFN-α production) repeated measures ANOVA indicated a significant overall increase of NK cytotoxic activity against HIV-infected targets (p < 0.0001). Surprisingly, NK-mediated killing was rapidly increased at a level comparable to controls at both VS and week 52 (median-specific lysis = 46.32%, AUC = 2486 at week 52 vs median-specific lysis = 44.46% and AUC = 2594 in controls, Fig. 5A), despite the observed delayed recovery of the major NK subset (CD3<sup>+</sup>CD16<sup>+</sup>/CD56<sup>−</sup>CD16<sup>−</sup>). Indicative of the relationship between NK cytotoxicity and PDC function, we did observe a positive relationship between cytotoxic activity against virally infected targets following TLR stimulation and TLR stimulated IFN-α secretion at week 52 (r = 0.66, p = 0.003, Fig. 5B).

To test the responsiveness of NK cells from HIV<sup>+</sup> subjects to exogenous cytokines produced by MDC and PDC, we tested the ability of PBMC to lyse HIV-1-infected targets after treatment with IL-12/IL-15 or IFN-α (known to enhance NK cell-mediated cytotoxic activity (18, 19, 31)) for 18 h. As seen in Table III, the cytotoxic activity mediated by PBMC of viremic subjects was significantly increased by exogenous IL-12/IL-15 or IFN-α to values comparable to those observed with PBMC from controls, indicating NK responsiveness to killing-enhancing stimuli, irrespective of VL.

Taken together, our results suggest a dichotomy between functional and phenotypic recovery in NK subsets following ART, with

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**Table III.** NK cells from viremic subjects respond to exogenous stimuli with restoration of cytotoxic function against virally infected targets<sup>a</sup>

<table>
<thead>
<tr>
<th>Donors</th>
<th>Medium</th>
<th>IL-12/IL-15</th>
<th>IFN-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV&lt;sup&gt;+&lt;/sup&gt; (BL)</td>
<td>745 (466–1235)</td>
<td>2795 (2077–4613)</td>
<td>1794 (1021–2598)</td>
</tr>
<tr>
<td>HIV&lt;sup&gt;+&lt;/sup&gt; (VS)</td>
<td>1900 (1193–2954)</td>
<td>3675 (2805–5902)</td>
<td>4005 (2487–5082)</td>
</tr>
<tr>
<td>HIV&lt;sup&gt;+&lt;/sup&gt; (52)</td>
<td>2486 (1624–3230)</td>
<td>4978 (3190–5693)</td>
<td>4082 (2689–5259)</td>
</tr>
<tr>
<td>Controls</td>
<td>2594 (1947–3718)</td>
<td>4619 (4040–5154)</td>
<td>4163 (3670–4733)</td>
</tr>
</tbody>
</table>

<sup>a</sup> PBMC from chronically HIV<sup>+</sup> subjects and controls were stimulated for 18 h with IL-12/IL-15 (10 ng/ml) or IFN-α (1000 U/ml), and used as effector cells in a killing assay against HIV-infected targets. Values shown represent median (25th–75th percentile) of AUC.
Killing of target cells (Table IV, 4.85, 4.20, 6.90 vs 42.16% PBMC (from control subjects) completely abrogated NK-mediated toxicity. Depletion of NK cells, PDC or HLA-DR-depleted PBMC completely restored NK killing (4.20 vs 36.33% lysis 38.15 vs 42.16%). Addition of exogenous IFN-α further enhanced cytotoxic activity against virally infected targets (29.50 vs 35.90%), whereas addition of MDC had a lesser effect (13.98 vs 38.60%, Table IV). Using PDC, but not MDC, derived from HIV-infected, ART-suppressed subjects restored NK cell-mediated cytotoxicity (29.50 vs 42.16%), demonstrating an impairment of PDC-dependent TLR-signaling following VS, indicating that suppression of viral replication increases the intrinsic capacity of PDC to provide accessory function for NK-mediated lysis of virus-infected cells (as well as an increase of CD69 expression, data not shown). Together with long-term increases in PDC frequency upon sustained VS observed in selected individuals, these results support the potential for ART-mediated reconstitution of PDC/NK-mediated viral control mechanisms in chronic HIV-1 infection.

**Discussion**

In this study, we analyze for the first time the degree of functional ART-mediated reconstitution of the DC and NK innate compartments in a longitudinal cohort of patients achieving VS and immune monitoring for 52 wk of sustained suppression. Importantly, we provide the first evidence that the levels of BL viral replication or changes in CD8 T cell immune activation following suppressive therapy may predict the degree of reconstitution of innate effector and function on ART. Using enriched cell populations, we also provide the first data addressing the intrinsic function of PDC following VS, demonstrating an impairment of PDC-dependent TLR-mediated responses in viremic patients that is partly recovered from ART-suppressed subjects provide accessory help to NK cells to lyse virally infected targets

To directly determine whether the functional state of the HLA-DR+ accessory cells had an effect on NK function, we tested the ability of PDC and MDC from viremic or suppressed HIV-infected subjects to provide accessory function for NK cell-mediated cytotoxicity. Depletion of NK cells, PDC or HLA-DR+ cells from PBMC (from control subjects) completely abrogated NK-mediated killing of target cells (Table IV, 4.85, 4.20, 6.90 vs 42.16%, p = 0.001), whereas depletion of MDC had minimal effect on specific lysis (38.15 vs 42.16%). Addition of exogenous IFN-α to PDC-depleted PBMC completely restored NK killing (4.20 vs 36.33% compared with 42.16%) suggesting that the effects of PDC or HLA-DR+ cell depletion are mediated by the consequent lack of IFN-α production.

We then compared the ability of HLA-DR+ cells from HIV-infected subjects (viremic or ART-suppressed) to provide accessory help to NK cells. Enriched HLA-DR+ cells (including both DC subsets) from ART-suppressed and viremic subjects were mixed with HLA-DR-depleted PBMC from control donors and tested in an 18-h cytotoxicity assay against HSV-FS4. Addition of HLA-DR+ cells from HIV+ ART-suppressed, but not viremic, individuals restored NK mediated killing of HLA-DR-depleted PBMC in controls (38.60 vs 42.11%, for HIV suppressed, and 5.20 vs 42.11% for HIV viremic, p = 0.001). We next tested the role of highly enriched PDC and MDC subsets within the HLA-DR+ subset. Addition of PDC from controls to HLA-DR-depleted PBMC from ART-suppressed subjects restored NK cell-mediated cytotoxicity (29.50 vs 35.90%), whereas addition of MDC had a lesser effect (13.98 vs 38.60%, Table IV). We obtained similar results using PDC, but not MDC, derived from HIV-infected, ART-suppressed subjects (33.41 vs 42.16% for PDC and 9.47 vs 42.16% for MDC, Table IV). Further stressing a restriction for the role of PDC in enhancing cytotoxic activity against virally infected targets, depletion of PDC had no effect on the killing of K562 targets (PBMC = 48.75%; NK-depleted PBMC = 5.6%; PDC-depleted PBMC = 41.8%). Altogether, our reconstitution experiments indicate that suppression of viral replication increases the intrinsic capacity of PDC to provide accessory function for NK-mediated lysis of virus-infected cells (as well as an increase of CD69 expression, data not shown). Together with long-term increases in PDC frequency upon sustained VS observed in selected individuals, these results support the potential for ART-mediated reconstitution of PDC/NK-mediated viral control mechanisms in chronic HIV-1 infection.

**Table IV.** PDC from HIV-1 suppressed subjects restore NK cell-mediated killing of herpes-infected targets

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC (HIV+)</td>
<td>42.16 ± 7.88</td>
</tr>
<tr>
<td>PBMC (HIV+)-NK depleted</td>
<td>4.85 ± 1.0</td>
</tr>
<tr>
<td>PBMC (HIV+)-HLA-DR depleted</td>
<td>6.90 ± 2.9</td>
</tr>
<tr>
<td>PBMC (HIV+)-PDC depleted</td>
<td>4.20 ± 1.0</td>
</tr>
<tr>
<td>PBMC (HIV+)-PDC depleted + IFN-α</td>
<td>36.33 ± 9.8</td>
</tr>
<tr>
<td>PBMC (HIV+)-MDC depleted</td>
<td>38.15 ± 6.0</td>
</tr>
<tr>
<td>PBMC (HIV+)-HLA-DR depleted + HLA-DR+ (HIV+, RNA &lt;50)</td>
<td>38.60 ± 7.7</td>
</tr>
<tr>
<td>PBMC (HIV+)-HLA-DR depleted + HLA-DR+ (HIV+, RNA &gt;100)</td>
<td>5.20 ± 2.5</td>
</tr>
<tr>
<td>PBMC (HIV+, RNA &lt;50)</td>
<td>35.90 ± 4.97</td>
</tr>
<tr>
<td>PDC (HIV+)</td>
<td>4.23 ± 1.77</td>
</tr>
<tr>
<td>HLA-DR-depleted PBMC (HIV+)+</td>
<td>29.50 ± 7.94</td>
</tr>
<tr>
<td>PDC (HIV+)</td>
<td>13.98 ± 3.80</td>
</tr>
<tr>
<td>MDC (HIV+)</td>
<td>9.47 ± 6.35</td>
</tr>
<tr>
<td>HLA-DR-depleted PBMC (HIV+) + MDC (HIV+)</td>
<td>33.31 ± 5.02</td>
</tr>
<tr>
<td>PDC (HIV+)</td>
<td>7.88</td>
</tr>
</tbody>
</table>

a Results represent the summary of four sets of experiments using 15 controls, 8 HIV+ suppressed, and 4 HIV+ viremic subjects.

b Effector cells were prepared as described in Materials and Methods.

c Percent-specific killing at an E:T ratio of 50:1 ± SD are shown.

d Exogenous IFN-α (1000 U/ml) added.

e All HIV+ subjects used in these experiments are virally suppressed (<50 copies/ml).
after long-term suppression. These experiments indicate that in chronic HIV-1 infection, PDC depletion in circulation is compounded by an impairment of PDC activation mechanisms (such as TLR-mediated signaling or IFN-α synthesis and/or secretion). We also define a requirement for PDC to functionally support NK cytolytic function against virus-infected targets in HIV-infected, ART-suppressed subjects.

In our cohort, which shared a uniform rise in CD4 count and sustained suppression, the observed uneven recovery of innate immune effectors across all subsets analyzed may help explain the broad heterogeneity of innate cells frequencies described in prior cross-sectional studies which, unlike this study, did not control for pre-ART VL, or ART-related outcomes (e.g., CD4 count rise or levels of immune CD8+ T cell activation). Importantly, our data strongly suggest that additional parameters, independent of VS, may affect PDC reconstitution on ART, with pretreatment viremia and CD8+ T cell activation serving as predictor of PDC recovery. With regards to HIV-1 replication, a relationship between viral replication and PDC levels after long-term ART-mediated suppression has also been described in ART-interruption studies, where preinterruption PDC frequency was inversely correlated with viral rebound (42). Our data are consistent with an adverse effect of commonly interleaved factors of chronic HIV-1 infection (immune activation and viral replication) on PDC function, as also reflected by the restored PDC function observed in suppressed subjects after enrichment. However, the role of PDC in HIV pathogenesis remains controversial, with proposed pro- and antiapoptotic roles after enrichment. Nevertheless, the restored PDC function observed in suppressed ART-suppressed subjects.

With regards to HIV-1 replication, a relationship between viral replication and PDC levels after long-term ART-mediated suppression has also been described in ART-interruption studies, where preinterruption PDC frequency was inversely correlated with viral rebound (42). Our data are consistent with an adverse effect of commonly interleaved factors of chronic HIV-1 infection (immune activation and viral replication) on PDC function, as also reflected by the restored PDC function observed in suppressed subjects after enrichment. However, the role of PDC in HIV pathogenesis remains controversial, with proposed pro- and antiapoptotic roles for sustained PDC-derived IFN-α (43–47); PDC have also been postulated as a source of increased levels of plasma IFN-α in vivo following interactions with HIV-infected cells or TLR ligands originated from gut microbial translocation (48). Of interest, we were unable to document a difference in IFN-α plasma levels between pre-ART viremic HIV-infected subjects and controls (over 100 HIV+ subjects tested; J. Chehimi, L. Azzoni, L. J. Montaner, unpublished observation), consistent with data reported by Killian et al. (25), and supporting the decrease in PDC function reported in the present study. The reason for discrepancy between these observations and other reports suggesting increased IFN-α levels in viremic subjects remains undetermined.

We show that NK cytotoxic function is rapidly restored upon VS, despite a delay in the recovery of the major CD56+/CD16+ NK cell subset, thus confirming our previous studies on samples from the Multicenter AIDS Cohort Study and Women’s Interagency HIV Study cohorts, which established that NK subset recovery is delayed until after ≥270 days on ART (26). The unexpected observation of an early ART-mediated recovery of NK cytotoxic function, independent of subset reconstitution, strongly suggests a dichotomy between functional and phenotypic recovery, highlighting a potential for underestimation of NK functionality as inferred from subset frequency, at least early after ART initiation. Interestingly, NK cells in our cohort were able to respond to exogenous cytokines (IFN-α, IL-12/IL-15) both before and after VS. Together, these data support the interpretation that impairments of NK cytotoxic function depend on negative regulatory mechanisms directly associated with viral replication (e.g., gp120-mediated NK dysfunction (13, 49), which are partly reversed in the presence of: 1) counterregulation, as reflected by effects of in vitro (Table III) or in vivo cytokine treatment (PEG-IFN-α 2A-treated HIV/ HCV infected subjects, J. Chehimi, L. Azzoni, and L. J. Montaner, unpublished observation) or 2) VS in vivo. Based on the multiple changes associated with VS, additional experiments are needed to determine whether the functional recovery of NK cell cytotoxicity upon VS is also associated with acute changes in inhibitory NK receptor/natural cytotoxic receptor expression patterns as described by Mavilio et al. (39).

In contrast to earlier work (38–41), we did not detect any increase in the frequency of the CD3+ CD56- CD16+ NK cell subset at viremia at time of ART start. The reason for this discrepancy is not clear, but may be related to the specific methodologies used (whole blood-based assays in this manuscript vs PBMC or purified NK cells in other works). Finally, a direct relationship between NK cytotoxicity and PDC function was evidenced by our data also consistent with PDC’s role in activating NK responses following viral infection (8, 9).

Although the mechanism(s) responsible for the observed delay in recovery of circulating PDC or mature NK cell frequency remain undefined (e.g., potential for a greater retention in tissues following VS or a slower recovery of subset replenishment from bone marrow, in addition to direct infection), our data indicate that the overall recovery of the NK-DC functional compartments is adversely affected by higher pre-ART VL and immune activation. However, our data do not directly provide the potential for added factors such as BL CD4 count as an independent variable affecting innate recovery, as our typical pretreatment U.S. cohort did not include subjects with extremely low (<50) or high (>500) CD4 counts. Due to the overall contribution of innate immune cells to general immunological homeostasis (i.e., resistance against opportunistic infection, vaccine response, etc.), the potential for identifying differential levels of innate effector function and reconstitution following ART among otherwise equally suppressed subjects with similar rises in CD4 count may have important clinical implications with regard to frequency of comorbidities, vaccine responses, and treatment response against coinfection.

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Disclosures
The authors have no financial conflict of interest.

References