Inability of Plasmacytoid Dendritic Cells To Directly Lyse HIV-Infected Autologous CD4+ T Cells despite Induction of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Jihed Chehimi, Emmanouil Papasavvas, Costin Tomescu, Shaheed Abdulhaqq, Andrea Raymond, Aidan Hancock, Kavita Vinekar, Craig Carty, Griffin Reynolds, Maxwell Pistilli, Karam Mounzer, Jay Kostman, and Luis J. Montaner


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The function of plasmacytoid dendritic cells (PDC) in chronic human immunodeficiency virus type 1 (HIV-1) infection remains controversial with regard to its potential for sustained alpha interferon (IFN-α) production and induction of PDC-dependent tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated cytoxicity of HIV-infected cells. We address these areas by a study of chronically HIV-1-infected subjects followed through antiretroviral therapy (ART) interruption and by testing PDC cytolytic function against autologous HIV-infected CD4+ T cells. Rebound in viremia induced by therapy interruption showed a positive association between TRAIL and viral load or T-cell activation, but comparable levels of plasma IFN-α/β were found in viremic ART-treated and control subjects. While PDC from HIV-infected subjects expressed less interferon regulator factor 7 (IRF-7) and produced significantly less IFN-α both secreted and membrane bound receptor 7/9 (TLR7/9) engagement than controls, membrane TRAIL expression in PDC from HIV+ subjects was increased. Moreover, no significant increase in death receptor 5 (DR5) expression was seen in CD4+ T cells from viremic HIV+ subjects compared to controls or following in vitro infection/exposure to infectious and noninfectious virus or exogenous IFN-α, respectively. Although activated PDC killed the DR5-expressing HIV-infected Sup-T1 cell line, PDC did not lyse primary autologous HIV+ CD4+ T cells yet could provide accessory help for NK cells in killing HIV-infected autologous CD4+ T cells. Taken together, our data show a lack of sustained high levels of soluble IFN-α in chronic HIV-1 infection in vivo and document a lack of direct PDC cytolytic activity against autologous infected or uninfected CD4+ T cells.

Human immunodeficiency virus (HIV) infection is associated with chronic immune activation, progressive immune suppression, and deletion of memory adaptive responses, resulting in increased susceptibility to opportunistic infections (23, 51, 52). Loss of CD4+ T cells is the hallmark of HIV infection, with multiple mechanisms proposed as contributing to this loss (activation-induced cell death, direct cytopathic effect, immune cells, and death receptor-mediated apoptosis induction) (reviewed in references 33 and 34). One of the most puzzling observations in AIDS pathogenesis has been the progressive depletion of bystander T cells, especially in lymphoid tissues (25, 33, 34, 55). While antiretroviral therapy (ART) initiated in the early stages of HIV infection, when CD4+ T-cell counts are high (>500 cells/μl), may prevent the destruction of lymph node (LN) tissue and the massive depletion of CD4+ T lymphocytes by decreasing the rate of virally induced apoptosis (20), a persistent, albeit decreased, level of apoptosis of peripheral blood CD4+ and CD8+ T cells is seen in ART-treated HIV+ subjects despite long-term viral suppression (36).

A member of the tumor necrosis factor (TNF) family, TNF-related apoptosis-inducing ligand (TRAIL), has been shown to be involved in HIV-1-associated T-cell apoptosis (33, 34). TRAIL (soluble or membrane bound) induces apoptosis upon binding to death receptor 4 (DR4; also named TRAIL-R1) or DR5 (also named TRAIL-R2, TRICK2, or Killer/DR5).

On the basis of the in vitro observation that alpha interferon (IFN-α) and interferon regulator factor 7 (IRF-7) are involved in plasmacytoid dendritic cells (PDC) exposed to HIV-1 (40), the hypothesis that PDC activation by HIV-1 is responsible for an increased level of IFN-α throughout chronic disease has been proposed. It has also been proposed that the activation of the PDC compartment by HIV-1 participates in the initial immune activation following acute infection and contributes to CD4+ T-cell depletion by inducing, through IFN-α, the production of TRAIL, which mediates apoptosis of DR5-expressing CD4+ T cells following HIV-1 infection (37, 38, 40). However, several lines of evidence question the direct involvement of PDC in the loss of T cells during HIV infection, as PDC numbers are depleted during chronic HIV infection and PDC remaining in circulation are functionally impaired (10). Recent data show that circulating PDC in HIV-infected subjects, although unable to secrete IFN-α after Toll-like receptor (TLR)-mediated activation, constitutively express an increased level of IFN-α mRNA, indicating that during HIV infection PDC are activated yet impaired (71). Rodriguez et al. demonstrated the prevention of spontaneous apoptosis of CD4+ and CD8+ T cells by IFN-α (63), a major product of
PDC following HIV-1 stimulation (3, 28). In addition, Audige et al. (2) showed that blockade of IFN-α and IFN-γ receptor during in vitro HIV infection of CD4+ T cells isolated from human tonsils did not prevent apoptosis or TRAIL production, suggesting a lack of a central link between IFN-α production and apoptosis of tonsilar CD4+ T cells in HIV-1 infection. These data are also consistent with the observation that, in the human peripheral blood lymphocyte-transplanted SCID mouse (hu-PBL-SCID) model, IFN-α efficiently increases the survival of CD4+ T cells (49). Thus, controversy remains on the role of IFN-α as an indirect or direct inducer of apoptosis of CD4+ T cells through PDC/TRAIL induction, whereas the possibility that IFN-α acts as an antiviral agent by controlling HIV-1 replication and thus reducing virally mediated T-cell loss appears to be supported by several studies (reviewed in references 26, 47, and 58). In this regard, endogenous IFN-α produced by PDC has been shown to play an important role in controlling HIV infection in the human thymus (35), upregulating host antiviral factors such as APOBEC (1, 32, 44, 70) and stimulating NK cell-mediated cytotoxic activity against autologous HIV-infected targets (72).

In this report, we investigated the in vivo correlates of viremia in chronically infected subjects by studying the relationship between therapy interruption-associated viremia and plasma IFN-α and TRAIL levels. We also tested in vitro the functional outcome of HIV-1-activated PDC in terms of their ability to mediate lysis of primary autologous CD4+ T cells (infected or not with HIV-1), compared to indirect PDC-mediated lysis effects on the NK-dependent antiviral cytotoxic response.

**MATERIALS AND METHODS**

**Study subjects.** Two cohorts recruited at the Jonathan Lax Immune Disorders Clinic (Philadelphia FIGHT) were used in this study. The first cohort was composed of 21 suppressed HIV-1 infected subjects on ART (asymptomatic without opportunistic infections), who had CD4 counts of >400 cells/µl and plasma HIV-1 RNA levels of <500 copies/ml for >6 months and <50 copies/ml at recruitment; these subjects were participating in a parent study investigating the effect of treatment interruption (TI) on the viral set point (set point = average plasma HIV-1 RNA of the first three consecutive measurements within a <0.5 log difference) and immune reconstitution. The details and main findings of this cohort were published elsewhere (60). The second cohort (without opportunistic infection) was composed of 69 subjects with chronic HIV-1 infection (24 females and 45 males), whose CD4 numbers and viral loads ranged from 111 to 1,408 cells/(24 females and 45 males), whose CD4 numbers and viral loads ranged from 111 to 1,408 cells/(24 females and 45 males), whose CD4 numbers and viral loads ranged from 111 to 1,408 cells/ml to 50 copies/ml, respectively (Table 1).

Healthy HIV-1-seronegative donors from the Wistar Institute Blood Donor Clinic did not with HIV-1), compared to indirect PDC-mediated lysis effects on the NK-dependent antiviral cytotoxic response.

**Viral strains.** All HIV-1 strains (HIV-1, NL4-3, Bal, DOGE, and SHIP), used at 150 to 300 ng/ml in this study, were isolated, expanded, and titered at the University of Pennsylvania Center for AIDS Research Viral Core Facility (UPENN CFAR). In some experiments, aldrithiol-2 (AT2)-treated HIV strains (Bal and NL4-3, provided by UPENN CFAR) were used at 300 ng/ml p24 antigen (Ag) equivalent.

**HIV-1 infection.** PDC were stimulated for 2 days with 10 µg/ml phytohemagglutinin (PHA-P; Sigma Aldrich, St. Louis, MO) and 100 U/ml human interleukin-2 (hIL-2) (BD Pharmingen, San Jose, CA) prior to CD4+ primary T-cell isolation and infected as previously described (72). HIV-1-infected and uninfected CD4+ primary T cells were then grown in complete medium with 100 U/ml IL-2 for 4 days, and nonviable cells were removed by Ficoll density gradient separation. At day 4 postinfection, the percentages of infected CD4+ primary T cells varied between 25 and 70% according to intracellular p24 Ag content as measured by flow cytometry.

**Detection of mTRAIL, DR5 (CD262), and IRF-7.** Freshly isolated PBMC were used to detect constitutive membrane TRAIL (mTRAIL) expression on PDC and DR5 expression on CD4+ T cells. Cells (106 cells/100 µl) were incubated for 20 min at room temperature in FACS buffer (BD Bioscience) with PE-conjugated mouse IgG1 anti-human TRAIL monoclonal antibody (clone RIK-2, 0.5 µg/100 µl; eBioscience, San Diego, CA) and APC-conjugated anti-human BDC2/BDCA4 monoclonal antibodies (Miltenyi Biotech) for PDC, CD3-PE/CD4-APC (BD Bioscience), and CD56-PE (clone CD56-17A4, 0.5 µg/100 µl; eBioscience). Irrelevant PE-conjugated isotype-matched monoclonal antibodies for TRAIL- or DR5-IgG1 (clone MOPC-21/P3; eBioscience) were used. Sup-T1, K562, and WM793 were always used as positive controls for DR5 detection. Intracellular staining for IRF-7 detection was performed on freshly isolated PBMC as previously described by Dai et al. (15) using intracellular staining (IRF-7) combined with surface staining (PDC). Briefly, PDC were first stained with APC-conjugated BDC2/BDCA4 for 20 min at 4°C and then washed, fixed with paraformaldehyde, and permeabilized for 15 min at room temperature. Cells were then incubated with rabbit anti-human IRF-7 (500 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. Cells were washed and incubated with goat anti-rabbit IgG-FITC for 30 min. Normal rabbit IgG was used as a control in all IRF-7 stainings. Cells were washed twice, and fluorescence data on 150,000 to 200,000 events were acquired. Results were expressed as percentages of positive events or MFI. Data analysis was performed using FlowJo software (Tree Star, San Carlos, CA).

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Healthy HIV-1-seronegative donors from the Wistar Institute Blood Donor Program were included as controls. Informed consent was obtained from all study participants at enrollment. The study was approved and monitored by Institutional Review Boards at Philadelphia FIGHT and The Wistar Institute.

PBMC preparation and phenotypic analysis. Blood was processed within 6 h of being drawn. Peripheral blood mononuclear cells (PBMC) were separated on a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient and resuspended in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate).

Multicolor fluorescence-activated cell sorter (FACS) analysis was performed on isolated PBMC as described previously (10, 11). All monoclonal antibodies were purchased from BD Biosciences, except where indicated. PDC, NK, and activated T cells were identified using the following monoclonal antibody cocktail with paraformaldehyde, and permeabilized for 15 min at room temperature. PBMC as previously described by Dai et al. (15) using intracellular staining (IRF-7) combined with surface staining (PDC). Briefly, PDC were first stained with APC-conjugated BDC2/BDCA4 for 20 min at 4°C and then washed, fixed with paraformaldehyde, and permeabilized for 15 min at room temperature. Cells were then incubated with rabbit anti-human IRF-7 (500 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. Cells were washed and incubated with goat anti-rabbit IgG-FITC for 30 min. Normal rabbit IgG was used as a control in all IRF-7 stainings. Cells were washed twice, and fluorescence data on 150,000 to 200,000 events were acquired. Results were expressed as percentages of positive events or MFI. Data analysis was performed using FlowJo software (Tree Star, San Carlos, CA).
Killing assays. PBMC, NK cells, or purified PDC were activated for 18 h with CpG oligodeoxynucleotide (ODN) class A (CpG-2216, 10 μg/ml; InvivoGen, San Diego, CA), resiquimod (3M Pharmaceuticals, St. Paul, MN), or autologous HIV-infected targets and used as effector cells. These cells were used in a chromium release assay of K562 cells, Sup-T1 cells (infected or not with HIV-1), and autologous primary CD4+ T cells infected or not with HIV-1 as previously described (10, 72). Target cells (2 × 10^6 viable cells) were labeled with Na251CrO4 (∼100 μCi) for 2 to 3 h at 37°C, washed, and resuspended in complete medium (5 × 10^5 cells/ml). Effectors and 51Cr-labeled targets were incubated in duplicate at an effector-to-target cell (E:T) ratio of 1:10 in a 0.2-ml volume in round-bottom 96-well plates and incubated for 6 h. Cell-free supernatant was collected, and 51Cr release was measured. The percentage of specific lysis was determined as described previously (11, 12). In some experiments, concanamycin A (CMA) was used at 1 μg/ml to selectively block the perforin-based lytic pathway.

sTRAIL and IFN-α/β assays. All reagents were used for their lowest levels of endotoxin contamination. PBMC (2.5 × 10^6/well) were cultured in 24-well plates for 18 h with (i) CpG-2216 (10 μg/ml), (ii) resiquimod (10 μg/ml), (iii) primary autologous CD4+ T cells infected or not with HIV-1, or (iv) AT2-treated virus (300 ng/ml p24 Ag equivalent). Cell-free supernatants and cryopreserved plasma samples from controls and HIV+ subjects were evaluated for soluble TRAIL (sTRAIL) (Diacalone, Stamford, CT), IFN-α (multiplicatypes with no cross-reactivity with human IFN-γ, IFN-β, and IFN-ω), or IFN-β (both IFN-α and IFN-β were from PBL Biomedical Lab, Picatway, NJ) by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer’s specifications. All measurements were based on the average of duplicate samples. Limits of detection for IFN-α/IFN-β and TRAIL were 12 to 27 pg/ml and 120 pg/ml, respectively.

Statistical analysis. Differences between groups were tested using the Mann-Whitney U test. Correlations between variables at specific time points were tested using Spearman’s rank correlation tests. Between-time-point comparisons were performed using parametric or nonparametric analysis of variance depending on the data distribution. All tests were two-tailed, and P values of <0.05 were considered statistically significant (α = 0.05). Analysis was performed with JMP (SAS, Carley, NJ) or Prism (Graphpad Software Inc., San Diego, CA).

RESULTS

Immune activation after therapy interruption or upon chronic viremia is associated with an increase in sTRAIL but not in plasma IFN-α/IFN-β. IFN-α has been shown to induce robust TRAIL expression, and PDC have been proposed to contribute to increased levels of sTRAIL and IFN-α in chronic HIV-1 infection (40). Similar to TNF, biologically active TRAIL can be present as both soluble and membrane-bound forms. To study the relationship between plasma TRAIL and IFN-α/IFN-β in the presence of viral replication during chronic HIV-1 infection, we evaluated plasma levels of TRAIL and IFN-α/IFN-β in a longitudinal cohort (n = 21) before and after ART interruption and viral rebound. As previously reported (60), a 6-week ART interruption resulted in viral rebound (from <50 copies/ml to a range of 824 to 493,000 copies/ml) and activation-induced changes in both CD4 and CD8 T-cell compartments. As shown in Fig. 1A, ART interruption induced an acute increase in TRAIL plasma levels. This increase was noticeable as early as 2 weeks after TI, when viral rebound was minimum, and peaked at week 4, before decreasing at week 6 at maximum viral load. However, no changes in the observed circulating IFN-α or IFN-β plasma levels were detected at any time point tested (Fig. 1B and C). Correlation analysis showed a positive association between TRAIL and viral load (P = 0.023; Spearman’s ρ = 0.493). The change in the frequency of CD8+ HLA-DR+ T cells (used as a marker of viremia-induced immune activation) and the change in soluble TRAIL levels during a 6-week TI were also correlated (as well as 2- and 4-week TI not shown); CD8+ HLA-DR+, P = 0.015, Spearman’s ρ = 0.5353) (Fig. 1D), yet no association was noted between T-cell activation and plasma IFN-α/IFN-β levels (data not shown). The differential regulation between plasma levels of TRAIL and IFN-α/IFN-β was also reconfirmed in a cross-sectional comparison of 69 HIV+ subjects with uninfected controls (Fig. 2A). While plasma levels of TRAIL were significantly higher in HIV+ viremic subjects than in aviremic and uninfected control subjects, no difference in levels of IFN-α (Fig. 2B) or IFN-β was observed (median, 170 pg/ml in controls, n = 24, versus 118 pg/ml in HIV+ subjects, n = 68; difference not significant) (Fig. 2C). Our results clearly demonstrated that, in our cohorts, increased viral replication is associated with increased plasma sTRAIL but not IFN-α or IFN-β.

HIV-1 replication increases mTRAIL expression on circulating PDC in association with lower IFN-7 levels and no increase in TLR-mediated IFN-α production. Having shown a lack of association between soluble TRAIL and soluble IFN-α/IFN-B plasma levels, we evaluated mTRAIL expression on PDC from HIV+ subjects and uninfected controls. A significant increase in the percentage of PDC expressing mTRAIL (as well as MFI) was observed ex vivo in HIV-infected subjects compared to uninfected controls (median, 37.20% in HIV+ subjects versus 16.80% in controls; P = 0.0035) (composite data and representative diagrams are shown in Fig. 3A). Based on several reports, including ours, describing a profound impairment in IFN-α production by PDC during chronic viremia following TLR engagement (13, 14, 49) and because IRF-7 has been identified as the key player in induced type I IFN production (45, 46), we measured levels of intracellular IRF-7 in PDC. Using a polyclonal antibody that recognizes total IRF-7 (phosphorylated and unphosphorylated forms [16, 31, 56]), we observed that the constitutive intracellular levels of IRF-7 were lower in circulating PDC from chronically HIV-1-infected subjects than in those from uninfected control subjects (P < 0.002) (Fig. 3B shows composite data and representative diagrams).

In agreement with our previous work (10), we observed a low intracellular expression of IFN-α and TNF-α following PDC stimulation with resiquimod in HIV-infected PBMC (Fig. 3C). We also evaluated PDC of HIV+ subjects, before and after short-term ART interruption, to determine if PDC became immediately refractory to stimulation with HIV upon viremia. We stimulated PBMC for 18 h with HIV AT2 (300 ng/ml) and tested for the presence of IFN-α by ELISA. As depicted in Fig. 3D and consistent with a partial recovery of PDC function under ART (10), we observed a “net IFN-α response” to HIV AT2 stimulation in samples before ART interruption (median, 52 pg/ml without stimulation versus 409 pg/ml after stimulation) and after 6 weeks of ART interruption (62 pg/ml without stimulation versus 378 pg/ml after stimulation). Levels measured were significantly higher than those during long-term viremia in chronic infection (data not shown) (10) but lower than levels seen in controls (median, 2,100 pg/ml), indicating that, in spite of increases in TRAIL and a partial retention of PDC function upon acute viremia, levels of plasma IFN-α remained unchanged, as shown above.

DR5 expression is not increased in circulating primary CD4+ T cells from chronically infected HIV+ subjects or after in vitro HIV-1 exposure/infection of primary CD4+ T cells. We
then sought to determine whether an increase in CD4+ T cells expressing TRAIL receptor (DR5) was present in HIV-1 infection, allowing for direct effects by TRAIL-expressing PDC as a potential effector mechanism of CD4 T-cell death. To determine the specificity of the anti-DR5 antibody used by others (38) and in this study, we observed that Sup-T1 (infected or not), K562, and WM793 cells expressed high DR5 levels (Fig. 4A). Thus, we used four independent experimental approaches to determine whether HIV infection was associated with DR5 induction in primary CD4+ T cells. First, we measured DR5 expression in circulating CD4+ T cells from a cross-sectional HIV-cohort including subjects with long-term viremia and subjects undergoing an acute viremic episode (i.e., as a result of a 6-week ART interruption) utilized for the studies described above. As shown in Fig. 4B and C, and consistent with results described by others (36, 68), circulating CD4+ T cells from all HIV+ subjects tested showed similar low levels of DR5 expression compared to those from uninfected controls. No increase in DR5 levels (percent positive cells and MFI) was observed during rebound viremia following ART interruption in 3 HIV+ subjects tested at week 6 of ART interruption (data not shown). Second, analysis of in vitro-isolated primary CD4+ T cells infected for 3 to 4 days with HIV-1NL4-3 did not show upregulation of DR5 expression on CD3+CD4+ gated p24 antigen-positive cells (also showing downregulation of CD4 expression), indicating that in vitro infection was not able to induce measurable membrane DR5 expression (Fig. 4D). Similar results were obtained using X4 and R5 HIV-1 isolates (not shown). Third, stimulation of PBMC from control uninfected subjects with a TLR9 agonist or in vitro HIV-infected primary autologous CD4+ T cells for 24 h did not result in an increase in DR5 expression in CD4+ T cells gated from PBMC (Fig. 5A and B) despite the fact that in the same cultures high levels of IFN-γ, indicative of an intact PDC response to activation in uninfected controls, were observed (Fig. 5C and D). Finally, we exposed control uninfected PBMC to conditions that have been reported to induce an increase in DR5 mRNA expression in vitro (40), i.e., infectious HIV-1NL4-3, noninfectious HIV-1NL4-3 (AT2 treated and heat killed), exogenous IFN-γ (10,000 U/ml), and various other stimuli (resiquimod at 10 μg/ml, heat-killed influenza virus PR8 at 10 hemagglutinin (HA)/ml, and lipopolysaccharide [LPS] at 100 ng/ml) for 24 h. As shown in Table 2, DR5 expression within CD4+ T cells did not increase after 24 h of exposure (as well as 48 and 72 h [data not shown]) regardless of stimuli used, although robust secretion of IFN-γ and sTRAIL was observed as described above. Of interest, we have tested several currently available anti-TRAIL-R2/DR5 antibodies (eBioscience [used throughout this study], Biolegend, and R&D Systems), and none of them detected an upregulation in DR5 expression on primary CD4+ T cells, while all of median values for each group, and brackets indicate statistical significance by Wilcoxon signed rank tests. ns, not significant; VL, viremia level (copies of HIV-1 RNA/ml). (D) Association between increased levels of TRAIL and T-cell activation was determined by the Spearman rank correlation. ETI, end of 6-week treatment interruption; BTI, beginning of 6-week treatment interruption.
them detected high levels of DR5 in various cell lines (K562, Sup-T1 infected or not with HIV, and WM793). Taken together, these results failed to document an increased DR5 expression in primary CD4+ T cells in vivo during viral rebound, during chronic HIV-1 viremia, or after in vitro HIV-1 exposure/PDC activation, indicating that PDC activation alone is unlikely to account for major induction of CD4 T-cell apoptosis.

Activated PDC provide accessory help for NK cell-mediated lysis of primary autologous CD4+ T cells. Previous studies have documented the ability of PDC to lyse tumor targets (9), and a recent report by Hardy et al. (37) predicted that PDC could kill autologous HIV-infected targets based on the ability of PDC to lyse transformed cells expressing DR5, such as Sup-T1 cells. In order to understand the lytic ability of PDC, we first evaluated the presence of perforin in highly purified PDC depleted of residual NK cells (Fig. 6A). While PDC expressed variable amounts of TRAIL (Fig. 3A), expression of intracellular perforin was very low or absent compared to that by purified NK cells, used as a positive control (Fig. 6B). While these results make it unlikely that PDC could mediate perforin-dependent lysis, we directly evaluated the cytotoxic potential of purified activated PDC depleted of residual NK cells compared to that of purified NK cells for lytic activity against K562 cells, which express high levels of DR5 but are highly resistant to TNF-α, Fas-L, and TRAIL-induced apoptosis (43). As shown in Fig. 6C, activated PDC (containing ~0.1 to 0.5% contaminating NK cells) exhibited very low to no cytotoxic activity against K562 at an E:T ratio of 10:1 compared to purified NK cells. Of interest, when an E:T ratio of 20:1 was used in preliminary experiments, the percentage of specific killing did not increase and remained similar to the level seen at an E:T ratio of 10:1 (results not shown). Addition of concanamycin A (CMA), a potent inhibitor of perforin-mediated killing, produced a dramatic reduction in NK cell-mediated lysis but had no effect on the activity of PDC (Fig. 6C).

We then analyzed PDC-mediated cytotoxic killing of DR5-expressing Sup-T1 (infected or uninfected) cells. Sup-T1 cells were acutely infected for 3 days and used as targets. Activated NK-depleted purified PDC were incubated with chromium-labeled Sup-T1 cells at an E:T ratio of 10:1 for 6 h. Figure 7A depicts the levels of DR5 and p24 Ag in the infected cells. As shown in Fig. 7B, and consistent with the observations of Hardy et al. (37) describing the killing of Sup-T1 cells by PDC, activated PDC efficiently induced cytolysis of DR5-expressing HIV-infected Sup-T1 cells. In addition, we also show that PDC killing of HIV-infected Sup-T1 cells was dependent on TRAIL, as addition of neutralizing antibodies against TRAIL abolished the killing (Fig. 7B). In parallel experiments and as a control, we show that granule release plays a dominant role in the NK-mediated killing of Sup-T1 cells.

Having shown a different lytic mechanism between PDC and NK cells, we next sought to determine whether PDC activated by in vitro HIV-1-infected autologous primary CD4+ T cells (HIV/aCD4) alone could provide accessory help to activate NK cells and enhance NK cell-mediated cytotoxic killing. As depicted in Fig. 8A, total PBMC and isolated PDC produced high levels of IFN-α when stimulated with HIV/aCD4. HIV/aCD4-stimulated PBMC but not HIV/aCD4-stimulated PDC-depleted PBMC triggered activation of NK cells, as indicated by CD69 upregulation (Fig. 8B) and enhanced lysis of HIV/aCD4 cells (Fig. 8C). The latter results extended our previously reported data using TLR-9 agonist-activated PDC (72).

Because the lysis of physiologically relevant targets such as autologous CD4 primary T cells by PDCs has not been fully investigated, we tested whether the interaction between activated PDC that expressed TRAIL and in vitro HIV-infected autologous CD4 T-cell targets could result in CD4+ T-cell...
lysis. Acutely infected autologous CD4+ T cells containing 30 to 60% p24 Ag+ cells were used as targets as described by Tomescu et al. (72). As a positive control and in agreement with our previous report (72), PDC-activated NK cells were cytotoxic for autologous infected targets (Fig. 9) through a perforin-mediated killing mechanism (not shown) (76). Unlike what was found for Sup-T1 cells and as depicted in Fig. 9, PDC failed to show any cytotoxic activity against in vitro HIV-1-infected primary autologous CD4+ T cells (Fig. 9). In two different experiments, the use of a higher E:T ratio (20:1) did not increase the level of cell-mediated killing by PDC (not shown).

Taken together, our data are consistent with the lack of activity by PDC to mediate TRAIL/DR5-dependent lysis of HIV/aCD4 cells, while reconfirming in the same autologous system the potential for IFN-α-producing PDC to activate functional NK cells to induce lysis of primary HIV/aCD4+ T cells.

**DISCUSSION**

In this report, we provide the first evidence that plasma levels of sTRAIL and its expression on the PDC membrane are associated with HIV-1 replication in vivo, in the absence of detectable plasma IFN-α or IFN-β. We also provide new data on the lack of activity by PDC to mediate CD4 T-cell apoptosis by showing that PDC activated following interaction with HIV-infected primary CD4 T cells do not directly lyse HIV-infected autologous CD4+ T cells via TRAIL/DR5 but rather enhance NK cell-mediated cytotoxicity.
The activation of innate and adaptive responses following acute HIV-1 infection would predict that TRAIL and IFN-β could be positively correlated if responses by PDC remained functional in spite of continued viral replication. Indeed, recent data from acute infection by simian immunodeficiency virus in sooty mangabeys (SIVsm) show a relationship between increasing IFN-β plasma levels and acute viremia, yet IFN-β levels subsequently decrease in spite of sustained viremia (19). Studies of acute infection in humans have also shown a severe loss of circulating PDC, with conflicting data regarding sustained plasma IFN-β levels, which are expected to be transiently increased, as observed in SIV infection. Once chronic infection is established, a decrease in IFN-β production from PDC in spite of increased accumulation of IFN-β mRNA in the remaining circulating PDC has been described (50). With regard to TRAIL expression, we document its induction in PDC and association with viral replication yet do not document a relationship with increasing type I interferon plasma levels (IFN-β or IFN-γ). Contrary to the report by Herbeuval et al. (39), and in line with our previous results (10, 11), we were unable to document a difference in IFN-β or IFN-β plasma levels in controls versus HIV-positive subjects during the chronic phase or during the acute viral rebound in all HIV-positive subjects tested. Of interest, we further document that, even with retained partial PDC function during a short-term period of viremia, no change in plasma IFN-β/IFN-γ or increase in IFN-β expression was noted in PDC upon viral replication. With chronic viremia, an increasingly impaired PDC response is evidenced by a decrease in IRF-7 as a central transcriptional mediator of IFN production. Decreased IRF-7 levels further indicate an absence of a positive feedback of IFN receptor activation, leading to increases of IRF-7, as otherwise expected if PDC were continually responding to type I receptor activation and in return had the potential for higher IFN-β production upon activation.

As suggested by Kim et al. (48), in human and nonhuman primates the presence of significantly high levels of sTRAIL but only very low levels of IFN-β in HIV+ subjects tested suggests that IFN-β alone is likely not a major factor sustaining elevated TRAIL expression upon chronic viremia. Besides apoptosis induction, TRAIL has also been shown to have antiapoptotic (prosurvival), regulatory, and proliferative functions (7, 21, 50, 64, 73), and its role in HIV infections is likely to be more complex than proposed. Interestingly, a recent report by Shepard et al. clearly showed the beneficial effect of TRAIL by demonstrating a reduction in viral load without significant death of bystander cells (65).

Because binding of TRAIL to its cognate receptors induces
apoptosis, we analyzed in depth the expression of DR5 during HIV infection using several experimental approaches. Although Herbeuval et al. reported an increase of DR5 mRNA expression (38, 40, 41), we were unable to document an increase in DR5 surface expression in primary cell cultures exposed to or following in vitro infection with HIV-1 to establish TRAIL/DR5 as a major virally induced mechanism of lymphocyte depletion. Most importantly, we failed to demonstrate an ability of activated PDC to lyse primary autologous CD4 T-cell targets whether infected or not with HIV-1, although we observed TRAIL-dependent lysis of a DR5-expressing Sup-T1 cell line (infected or not with HIV). Our inability to document direct lysis stands in contrast to a recent report by Stary and colleagues showing an increase in DR4 expression in CD4+ T cells from HIV+ subjects compared to controls (5% in viremic subjects, 2% in ART-treated subjects, and 1% in controls), where approximately 30% killing of autologous CD4+ T cells by PDC was shown (68). Data in the above-mentioned report that remain unexplained include the high level of killing along with a low level of DR4 expressed on autologous CD4+ T cells, which we have also confirmed is similar to the level of DR5, as DR4 is not upregulated in infected T-cell targets (data not shown).

While our study clearly showed that circulating CD4 cells do not express higher levels of DR5 as a result of exposure to increasing levels of viremia following therapy interruption, it remains to be determined whether surface DR5 or another death receptor is expressed in tissue lymphocytes from HIV-1-infected patients (68). With regard to tissue distribution, PDC in tissues have not been consistently reported as highly prevalent, as reported by Biancotto et al., who used LN of HIV+ subjects compared to controls (5% in viremic subjects, 2% in ART-treated subjects, and 1% in controls), where approximately 30% killing of autologous CD4+ T cells by PDC was shown (68). Data in the above-mentioned report that remain unexplained include the high level of killing along with a low level of DR4 expressed on autologous CD4+ T cells, which we have also confirmed is similar to the level of DR5, as DR4 is not upregulated in infected T-cell targets (data not shown).

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**FIG. 6.** Purified PDC do not express perforin and cannot kill K562 target cells. (A) PDC were enriched to high purity from PBMC by negative selection with magnetic beads and stained with monoclonal antibodies to BDCA-2/4, lineage (LIN) cocktail mix, and CD56. The frequencies of PDC before and after purification and the frequency of contaminating CD56+ NK cells are shown. (B) Perforin expression in CD56- CD3+ gated NK cells and BDCA-2+ 4 Lin- gated PDC. (C) Purified PDC and NK cells were used as effector cells in a chromium release assay with DR5-expressing K562 targets in the presence or absence of 1,000 nM concanamycin A (CMA) at a 10:1 E:T ratio for 6 h (PDC) or 4 h (NK). Means ± standard errors of 4 different experiments are shown. DMSO, dimethyl sulfoxide.
Gendelman’s group and others (8, 29, 30, 69), demonstrated biological differences in IFN-α/H9251 proteins produced during advanced HIV infection compared to those of the controls. A limitation of our study is that the HIV/H11001 cohort analyzed does not include patients with opportunistic infections or with end stage disease (i.e., CD4 counts < 50 copies/ml), which may represent an immunopathogenic situation different from those described in the present study, with steady-state viremia and higher CD4 counts.

Our data strongly support a predominant antiviral role for PDC and IFN-α in the pathogenesis of HIV-1, although their possible contribution to the induction of factors associated with immunodeficiency such as indoleamine 2,3-dioxygenase (IDO), as shown in vitro (5), remains to be established in vivo. Furthermore, recent work by the Bhardwaj laboratory (53) elegantly described a dichotomy in the response of PDC to HIV: enhancing innate and adaptive responses to HIV and at the same time limiting chronic immune activation through generation of regulatory T cells (Tregs). While the in vivo activation of IFN-inducible genes in circulating PBMC during chronic HIV infection suggests the activation of IFN-mediated signaling, described in several reports (reviewed in reference 47), it remains to be determined to what extent this IFN signature reflects the effect of IFN-α alone rather than IFN-γ, known to be produced by activated CD8 T cells in the lymphoid organs of HIV-1-infected patients (17, 22). Our findings do not support a sustained role for PDC through TRAIL expression and IFN-α/IFN-β secretion as being responsible for a progressive T-cell loss over the extended period of chronic infection but instead indicate that PDC are decreased in number and impaired in function without a potential to mediate CD4 T-cell loss through TRAIL. Our data instead support the hypothesis that, if PDC are functional and activated in the presence of functional NK cells, as observed in long-term non-progressors (67), a predominant antiviral response, including activation of NK cell-mediated responses to limit viral replication, would be expected.

HIV-induced dysregulation of dendritic cell (DC)/NK cell cross talk may represent a major mechanism through which HIV
escapes immune surveillance (14, 61, 62), and several observations support a role for PDC/NK cell responses in HIV-1 control (10, 27, 66, 72). Our data predict that, in contrast to blocking systemic production of IFN-α, as recently suggested (42, 71, 74), IFN-α would mediate viral control in the presence of ART-mediated immune reconstitution, as already suggested by the decreased HIV titer observed in HIV/hepatitis C virus (HCV)-coinfected subjects treated with IFN-α/ribavirin (59). Current clinical studies sponsored by our laboratory in which patients on ART are treated with pegylated IFN-α2a upon ART interruption are directly testing this hypothesis (http://www.clinicaltrials.org/identifier: NCT00594880). Taken together, our findings challenge the hypothesis of a detrimental role for PDC in AIDS pathogenesis and suggest an IFN-α-independent pathway for the progressive loss of circulating CD4 T cells during HIV infection.

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J.C., E.P., and C.T. contributed equally to this work.

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FIG. 9. PDC lack lytic activity against HIV-infected primary autologous CD4+ T cells. (A) Purified NK cells were incubated in the presence or absence of autologous PDC and the TLR9 agonist CpG-2216 for 18 h at a 10:1 NK cell/PDC ratio. Cells were then incubated with chromium-labeled primary HIV-1-infected aCD4+ T cells at a 10:1 E/T ratio for 6 h in a standard chromium release assay. (B) Resting or activated PDC or activated NK cells were incubated with chromium-labeled primary HIV-1-infected aCD4+ T cells at a 10:1 E/T ratio for 6 h. Means ± standard errors of 4 to 6 different experiments are shown.
apoptosis markers and morphology in peripheral lymph node of HIV-infected individuals. Infection 36:120–129. 


Table 1: A summary of the key findings and conclusions from the article. 

<table>
<thead>
<tr>
<th>Key Finding</th>
<th>Conclusion</th>
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<tbody>
<tr>
<td>HIV infection leads to the activation of type I interferon</td>
<td>HIV-positive individuals have increased type I interferon production.</td>
</tr>
<tr>
<td>Interferon-alpha (IFN-α) induction leads to apoptosis</td>
<td>Interferon-alpha induces apoptosis in lymphoid tissue of HIV-infected patients.</td>
</tr>
<tr>
<td>Interferon-gamma (IFN-γ) increases cell survival</td>
<td>Interferon-gamma increases the survival of lymphoid cells.</td>
</tr>
<tr>
<td>Interferon-beta (IFN-β) affects cell proliferation</td>
<td>Interferon-beta affects cell proliferation.</td>
</tr>
<tr>
<td>Interferon-α/β induces apoptosis in CD4+ T cells</td>
<td>Interferon-α/β induces apoptosis in CD4+ T cells.</td>
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<tr>
<td>Interferon-γ induces proliferation in CD8+ T cells</td>
<td>Interferon-γ induces proliferation in CD8+ T cells.</td>
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<tr>
<td>Interferon-β induces cell survival in CD19+ B cells</td>
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<tr>
<td>Interferon-α/β induces cell proliferation in monocytes</td>
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Figure 1: A schematic representation of the role of interferons in HIV infection. 

The figure shows the following: 

1. Interferon-alpha (IFN-α) induces apoptosis in HIV-infected patients. 
2. Interferon-beta (IFN-β) increases cell survival in HIV-infected patients. 
3. Interferon-gamma (IFN-γ) induces proliferation in HIV-infected patients. 
4. Interferon-alpha and beta (IFN-α/β) induce apoptosis in CD4+ T cells. 
5. Interferon-gamma (IFN-γ) induces proliferation in CD8+ T cells. 
6. Interferon-beta (IFN-β) induces cell survival in CD19+ B cells. 
7. Interferon-alpha and beta (IFN-α/β) induce cell proliferation in monocytes. 
8. Interferon-gamma (IFN-γ) induces cell survival in monocytes. 
9. Interferon-alpha, beta, and gamma (IFN-α/β/γ) induce cell proliferation in dendritic cells. 

Figure 2: A graphical representation of the effects of interferons on cell proliferation. 

The figure shows the following: 

1. Interferon-alpha (IFN-α) induces apoptosis in HIV-infected patients. 
2. Interferon-beta (IFN-β) increases cell survival in HIV-infected patients. 
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8. Interferon-gamma (IFN-γ) induces cell survival in monocytes. 
9. Interferon-alpha, beta, and gamma (IFN-α/β/γ) induce cell proliferation in dendritic cells. 

Figure 3: A bar chart showing the differential expression of IFN-alpha and TRAIL/DR5 in lymphoid tissue of HIV-infected patients. 

The bar chart shows the following: 

1. Differential expression of IFN-alpha and TRAIL/DR5 in lymphoid tissue of HIV-infected patients. 
2. IFN-alpha expression is increased in HIV-infected patients. 
3. TRAIL/DR5 expression is decreased in HIV-infected patients. 
4. IFN-alpha expression is decreased in HIV-infected patients. 
5. TRAIL/DR5 expression is increased in HIV-infected patients. 

Figure 4: A flow cytometry analysis of the effects of interferons on cell survival. 

The flow cytometry analysis shows the following: 

1. Interferon-alpha (IFN-α) induces apoptosis in HIV-infected patients. 
2. Interferon-beta (IFN-β) increases cell survival in HIV-infected patients. 
3. Interferon-gamma (IFN-γ) induces proliferation in HIV-infected patients. 
4. Interferon-alpha and beta (IFN-α/β) induce apoptosis in CD4+ T cells. 
5. Interferon-gamma (IFN-γ) induces proliferation in CD8+ T cells. 
6. Interferon-beta (IFN-β) induces cell survival in CD19+ B cells. 
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