Blood Myeloid Dendritic Cells from HIV-1-Infected Individuals Display a Proapoptotic Profile Characterized by Decreased Bcl-2 Levels and by Caspase-3+ Frequencies That Are Associated with Levels of Plasma Viremia and T Cell Activation in an Exploratory Study

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Reduced frequencies of myeloid and plasmacytoid dendritic cell (DC) subsets (mDCs and pDCs, respectively) have been observed in the peripheral blood of HIV-1-infected individuals throughout the course of disease. Accumulation of DCs in lymph nodes (LNs) may partly account for the decreased numbers observed in blood, but increased DC death may also be a contributing factor. We used multiparameter flow cytometry to evaluate pro- and antiapoptotic markers in blood mDCs and pDCs from untreated HIV-1-infected donors, from a subset of infected donors before and after receiving antiretroviral therapy (ART), and from uninfected control donors. Blood mDCs, but not pDCs, from untreated HIV-1-infected donors expressed lower levels of antiapoptotic Bcl-2 than DCs from uninfected donors. A subset of HIV-1-infected donors had elevated frequencies of proapoptotic caspase-3+ blood mDCs, and positive correlations were observed between caspase-3+ mDC frequencies and plasma viral load and CD8+ T-cell activation levels. Caspase-3+ mDC frequencies, but not Bcl-2 expression, were reduced with viral suppression on ART. Apoptosis markers on DCs in blood and LN samples from a cohort of untreated, HIV-1-infected donors with chronic disease were also evaluated. LN mDCs displayed higher levels of Bcl-2 and lower caspase-3+ frequencies than did matched blood mDCs. Conversely, LN pDCs expressed lower Bcl-2 levels than their blood counterparts. In summary, blood mDCs from untreated HIV-1-infected subjects displayed a proapoptotic profile that was partially reversed with viral suppression, suggesting that DC death may be a factor contributing to blood DC depletion in the setting of chronic, untreated HIV disease.

Dendritic cells (DCs) are antigen-presenting cells (APCs) found throughout the body that are central in bridging innate and adaptive immune responses (5, 6). Immature DCs typically detect the presence of microbes through the binding of conserved molecular patterns to pattern recognition receptors (PRRs) such as C-type lectins and Toll-like receptors (TLRs) (32, 55). DCs exposed to microbial products then undergo a regulated maturation process, migrate to lymph nodes (LNs) and stimulate naive T cells and other immune cells to induce appropriate adaptive immune responses (5, 6). Although shown to be the most potent of the APCs in activating naive T cells, DCs have also recently been shown to play a crucial role in reactivating memory T cells (43, 95, 99) and in maintaining peripheral tolerance to self-antigens (91, 92).

Human DCs can be divided into myeloid DC (mDC) and plasmacytoid DC (pDC) subsets that differ in ontogeny, phenotype, and function (19, 50, 51, 53, 54, 61, 77–79, 87, 94). Specifically, pDCs are considered to be involved in antiviral immunity, are capable of sensing viral single-stranded RNA (ssRNA) through TLR7 and bacterial CpG DNA through TLR9, and predominantly produce type I interferons (IFNs). Conversely, mDCs sense both bacterial and viral pattern motifs through a broader range of TLRs (TLR1 to TLR8) and are critically dependent on mitochondria. Although initiation of DC death was also noted (18).

Apoptosis, a form of programmed cell death, results from a series of tightly controlled complex pathways (3, 29). These signaling cascades are typically initiated either extrinsically, through death receptors on the surfaces of cells, or intrinsically, through disruption in intracellular homeostasis, a process critically dependent on mitochondria. Although initiation of these pathways differs, there is significant cross talk and con-
Increased pDC migration to LNs and increased pDC apoptosis in vitro showed increased susceptibility of blood CD4+/H11001 independent of the initial induction point (3, 24). The intrinsic pathway and a characteristic of apoptosis that is, therefore, effector caspases are common to both the extrinsic and intrinsic pathway (e.g., caspase-3, -6, and -7) and cell death (3, 24). Activation of specific initiator cysteine aspartate-specific proteases (caspases), ultimately leading to activation of effector caspases (e.g., caspase-3, -6, and -7) and cell death (3, 24). Effector caspases are common to both the extrinsic and intrinsic pathway and a characteristic of apoptosis that is, therefore, independent of the initial induction point (3, 24).

Early studies of T-cell dysfunction during HIV-1 infection showed increased susceptibility of blood CD4+ and CD8+ T cells to apoptosis in vitro (35). Subsequent in vivo studies demonstrated apoptotic T cells and B cells within the LNs of HIV-1-infected individuals (13, 74). Increased blood T-cell apoptosis has been associated with disease progression, (26, 34, 73, 98), and the expression of markers of apoptosis is predictive of adverse clinical events (96).

A number of studies have demonstrated a role for DCs in the induction of T-cell apoptosis (20, 45–47, 58, 68, 82, 89). However, despite multiple observations of decreased frequencies of blood DCs during the course of HIV-1 infection, investigations into DC apoptosis in the setting of HIV-1 disease have been limited. Increased HIV-1 replication in pDCs and pDC death has been shown to occur following CD40 ligation in vitro (84), and a recent study by Myers et al. demonstrated increased pDC apoptosis in response to exposure to HIV-1 in vitro, a process likely mediated through pDC–HIV-1 fusion (72). Few studies have addressed the impact of HIV-1 infection on DC survival in vivo. Brown et al. demonstrated that both increased pDC migration to LNs and increased pDC death within the LNs contributed to depletion of pDCs in a monkey model of acute SIV infection (18).

We hypothesized that reduced frequencies of blood DCs were, in part, related to increased DC apoptosis during HIV-1 infection. In this present study, we investigated whether blood DCs from HIV-1-infected individuals, evaluated directly ex vivo or after overnight in vitro culture, displayed altered levels of pro- and antiapoptotic markers compared to those of uninfected donor DCs. We also evaluated associations between expression of apoptotic markers and markers of clinical HIV-1 disease progression, including plasma viral load, CD4 count, and level of T-cell activation. Further, we investigated whether viral suppression with antiretroviral therapy (ART) modulated the apoptotic profile of blood DCs. We also compared blood and LN DC apoptotic profiles in HIV-1-infected individuals. Lastly, we evaluated how innate signaling contributed to the survival of blood DCs by stimulating total peripheral blood mononuclear cells (PBMC) with viral and bacterial TLR ligands (TLRLs) in vitro and assessing apoptotic markers on mDC and pDC subsets.

### MATERIALS AND METHODS

**Study participants.** For PBMC studies, blood samples were obtained from 23 HIV-1-infected subjects (Table 1) who were receiving care at the University of Colorado Infectious Disease Group Practice, University of Colorado Hospital (Aurora, CO). Inclusion criteria included being ART naïve or on no ART for at least 6 months at the time of screening. Subjects coinfected with hepatitis C were excluded from the study. To assess the effect of ART, a second blood sample was obtained from 11 of these 23 HIV-1-infected donors after having received at least 3 months of ART. Blood samples were also obtained from 19 healthy adults, self-identifying as HIV-1-uninfected (seronegative), and used as controls.

Inguinal LNs were collected from HIV-1-infected donors that were ART naive or not receiving ART for 3 months (n = 6; Table 1) as previously described in detail (25). Autologous blood samples were collected at the same time as collection of LNs.

**Isolation of PBMC and LN cells.** PBMC from seronegative and HIV-1-infected donors were isolated from heparinized blood by gradient density centrifugation using previously described standard techniques (25). To prevent unin-
tended cell death, all blood samples were processed within 3 h after drawing of blood, and no statistical difference was found in the median time between blood draw and processing of the blood samples from seronegative or HIV-1-infected donors (data not shown).

Inguinal LNs from HIV-1-infected donors were processed as described previously (25). All LNs and matched PBMC samples were processed within 1 h. For these studies, disaggregated LN cells and matched PBMC were cryopreserved in freezing medium consisting of RPMI 1640 medium (Invitrogen, Carlsbad, CA) plus 1% penicillin-streptomycin-glutamine (Invitrogen), 45% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), and 10% dimethyl sulfoxide (Fisher Scientific, Pittsburgh, PA) and stored in liquid nitrogen. LN cells and PBMC were thawed in RPMI 1640 medium, 1% penicillin-streptomycin-glutamine, 10% FBS, 10 μg of DNase I (Sigma-Aldrich)/ml prior to undergoing flow cytometry staining and analysis as detailed below.

Evaluation of activation and apoptosis levels of blood DCs. Previous work evaluating apoptosis in total PBMC demonstrated minimal differences in apoptosis of PBMC from seronegative and HIV-1-infected donors directly ex vivo but, after overnight in vitro culture, significantly higher frequencies of apoptotic cells in PBMC from HIV-1-infected donors relative to seronegative donors were observed (7b). Therefore, we also measured apoptosis markers on blood DCs in PBMC both directly ex vivo and after culturing total PBMC in vitro for 13 to 20 h. PBMC were immediately stained with monoclonal antibodies to enumerate DC subsets and determine their activation and apoptosis status and were also cultured overnight with or without various TLR ligands and apoptosis states determined.

Monoclonal antibodies and apoptotic markers. The following antibodies were used to identify blood or LN DCs directly ex vivo or blood DCs after overnight in vitro culture: fluorescein isothiocyanate (FITC)-labeled anti-lineage (Lin) cocktail (BD Biosciences), CD11c/CD123 (BD Biosciences), and APC-labeled CD123 (IL-3R; Miltenyi Biotec, Auburn, CA). Plus 1% penicillin-streptomycin-glutamine (Invitrogen), 45% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), and 10% dimethyl sulfoxide (Fisher Scientific, Pittsburgh, PA) and stored in liquid nitrogen. LN cells and PBMC were thawed in RPMI 1640 medium, 1% penicillin-streptomycin-glutamine, 10% FBS, 10 μg of DNase I (Sigma-Aldrich)/ml prior to undergoing flow cytometry staining and analysis as detailed below.

Monoclonal antibodies and apoptotic markers. The following antibodies were used to identify blood or LN DCs directly ex vivo or blood DCs after overnight in vitro culture: fluorescein isothiocyanate (FITC)-labeled anti-lineage (Lin) cocktail (BD Biosciences), CD11c/CD123 (BD Biosciences), and APC-labeled CD123 (IL-3R; Miltenyi Biotec, Auburn, CA). DC subsets were defined as follows: mDCs were Lin−CD11c+CD123+CD34−CD123low. The surface expression of CD40 was measured on biotinylated CD40 (Ancell, Bayport, MN) with streptavidin-FITC (BD Biosciences), and matched PBMC samples were washed with 4 ml of cold DPBS (Invitrogen), and held overnight (25). All LNs and matched PBMC samples were processed within 1 h. For assessment of Bel-2 and caspase-3 expression after overnight in vitro culture of PBMC, similar staining procedures were followed except the cells were surface stained, fixed in Fix/Perm, washed twice with Perm/Wash buffer, stained with Bel-2 or caspase-3, and acquired immediately on the FACSArray.

For assessment of T-cell activation, freshly isolated PBMC were surface stained with CD3, CD4, CD8, and CD38 and HLA-DR or matched isotype controls, washed in FACS buffer, and fixed in 1% PFA. The cells were held overnight at 4°C prior to acquisition on the FACSArray.

FcR blocking reagent (Miltenyi Biotec) was included in all initial incubations to limit nonspecific antibody binding through Fc receptors.

Annexin V and 7-AAD staining. After overnight culture, total PBMC were collected, washed, and surface stained as described above for T-cell activation. Cells were then washed twice in cold phosphate-buffered saline (PBS) and resuspended in annexin binding buffer (BD Biosciences) containing annexin V and 7-AAD. Cells were gently vortexed and incubated 15 min at room temperature. The cells were then washed in annexin binding buffer plus 20 μg of actinomycin D (Sigma-Aldrich)/ml and subsequently fixed in 1% PFA prepared in annexin binding buffer plus 20 μg of actinomycin D/ml. The addition of actinomycin D to the wash buffer and fixative prevents further 7-AAD staining within fixed cells. The cells were acquired on the FACSArray within 1 h.

In vitro cultures and TLR stimulation of total PBMC. PBMC were isolated as described above and resuspended in RPMI 1640 medium with 10% human AB serum (Gemini Bioproducts, West Sacramento, CA) at 2 × 106 cells/ml. PBMC were stimulated overnight at 37°C and 5% CO2 with TLR7L (Imiquimod; TLR7L; 10 μg/ml; InvivoGen, San Diego, CA) or with LPS (0.001 ng/ml; from Salmonella enterica serotype Minnesota (10 μg/ml; Sigma-Aldrich), with Imiquimod (TLR7L; 10 μg/ml; InvivoGen, San Diego, CA) or with LPS, a derivative of the imidazoquinoline compound R848 (TLR7/8L: 5 μg/ml, InvivoGen). After 13 to 20 h, PBMC were collected, and the assessment of DC activation and the expression of apoptotic markers was performed as described above.

Flow cytometry acquisition and analysis. To control for the accuracy and precision of measurements taken over the course of the study, routine quality control was performed on the FACSArray using the Cytometer Setup & Tracking (CS&T) feature within BD FACSdiva software version 6.1.2 (BD Biosciences). Voltage, laser delay, and area scaling were determined using standardized CS&T beads (BD Biosciences), and the settings were tracked over time to verify the laser delay and area scaling determined by CS&T, a manual quality control using rainbow beads was performed daily. To further control for quantitative comparisons of levels of expression of surface and intracellular molecules, the collection of data from seronegative donors was interspersed throughout the study period with the acquisition of data from HIV-1-infected donors.

Up to seven color flow cytometry was performed to identify mDCs (Lineage−CD34−HLA-DR+CD123+CD11c+) and pDCs (Lineage−HLA-DR−CD34+CD123+CD11c−) within PBMC from HIV-1-infected donors. For assessment of Bel-2 and caspase-3 was assessed within each of the DC subsets. In addition, the expression of the death receptor Fas, and its corresponding ligand (FasL), a manual quality control using rainbow beads was performed daily. To further control for quantitative comparisons of levels of expression of surface and intracellular molecules, the collection of data from seronegative donors was interspersed throughout the study period with the acquisition of data from HIV-1-infected donors.

Data analysis was performed using BD FACSdiva software version 6.1.2 on total cells. The average numbers of events collected for mDCs and pDCs from seronegative donors were 2,591 for mDCs and 2,115 for pDCs, and the fewest events collected were 776 for mDCs and 278 for pDCs. On average, 1,713 mDC and 702 pDC events were collected from HIV-1-infected donors (minimum number of mDCs, 361; pDCs, 155). Values were typically expressed as mean fluorescence intensity (MFI) minus the background isotype staining (net MFI). Due to the distinctive bimodal expression of caspase-3 by DCs (Fig. 1), caspase-3 expression is shown as the fraction of DCs within the total DC population that are positive for this apoptotic marker. In addition, the frequency of T cells
coexpressing CD38 and HLA-DR within the total T-cell population are shown with background staining removed (net %).

Statistical analysis. Nonparametric statistics were used due to small sample sizes. No adjustments were made for multiple comparisons due to the exploratory nature of the present study. Comparisons between independent groups were made by using the Mann-Whitney $t$ test, and paired analysis was performed using the Wilcoxon signed-rank test. A $P$ value of $<0.05$ was considered to be of exploratory significance. The Spearman test was used to determine associations between two variables with correlations considered to be of exploratory significance when $r > 0.3$ and $P < 0.05$. All statistical analyses were performed by using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Untreated, HIV-1-infected donors have reduced frequencies of blood mDCs and pDCs compared to seronegative donors. In agreement with our previous studies (9, 25) and those of others (28, 30, 37, 75, 88), fewer mDCs and pDCs were observed in PBMC from 23 untreated, HIV-1-infected donors (mDCs: median, 0.26% of total cells; range, 0.11 to 0.47%; pDCs: 0.10%; range, 0.05 to 0.28%) compared to 19 seronegative donors (mDCs: 0.41%; range, 0.17 to 0.72%, $P = 0.006$; pDCs: 0.31%; range, 0.12 to 0.57%, $P = 0.0001$).

Blood mDCs from untreated, HIV-1-infected donors express lower levels of Bcl-2 than mDCs from seronegative donors. In fresh PBMC, mDCs from HIV-1-infected donors ($n = 18$) had statistically lower expression levels of Bcl-2 than did mDCs from seronegative donors ($n = 14$) (Fig. 2A). No significant associations of blood mDC Bcl-2 expression with viral load ($r = -0.21$, $P = 0.4$) or with CD4 T-cell count ($r = -0.34$, $P = 0.17$) were noted among HIV-1-infected donors. Evaluation of Bcl-2 expression by blood mDCs after overnight in vitro culture also showed that HIV-1-infected donors ($n = 17$) expressed statistically lower levels of Bcl-2 compared to mDCs from seronegative donors ($n = 14$) (Fig. 2B). Similar to observations made with Bcl-2 expression directly ex vivo, no significant correlations with viral load or CD4 T-cell count were identified for Bcl-2 expression among HIV-1-infected donors after overnight in vitro culture (viral load: $r = -0.07$, $P = 0.78$; CD4 T-cell count: $r = -0.30$, $P = 0.25$).

Expression of caspase-3 by blood mDCs correlates with viral load. The median percentage of caspase-3$^+$ mDCs from HIV-1-infected donors ($n = 23$; median net caspase-3$^+$ mDCs within total mDCs 3.1%; range, 0.4 to 13.1%) was only slightly higher than that of seronegative donors ($n = 18$; median 2.7%; range, 1.0 to 5.1%; $P = 0.43$) (Fig. 2C). However, a greater range of caspase-3$^+$ mDC frequencies was noted among HIV-1-infected donors, with $>25\%$ of subjects having higher caspase-3$^+$ mDC percentages than the highest percentage observed among seronegative donors (Fig. 2C). Among HIV-1-infected donors, a significant positive correlation was observed between the percentages of caspase-3$^+$ mDCs and plasma viral load ($r = 0.51$, $P = 0.01$; Fig. 2D). No significant association between caspase-3$^+$ mDC frequency and CD4 count was noted.
When percentages of caspase-3 mDCs were evaluated after overnight in vitro culture, no statistical differences were noted between HIV-1-infected (n = 22; median, 11.4%; range, 2.7 to 35.2%) and seronegative donors (n = 18; median, 11.7%; range, 3.6 to 22.7%).

Frequency of caspase-3 mDCs from untreated, HIV-1-infected donors is associated with CD8 T-cell activation levels. Increased levels of CD4+ and CD8+ T-cell activation, defined as either (i) total CD38 expression (assessed as MFI) or (ii) as the percentage of each T-cell subset coexpressing CD38 and HLA-DR, were observed in HIV-1-infected donors compared to seronegative donors (data not shown). CD4 count correlated inversely with both measures of CD4+ and CD8+ T-cell activation and a positive correlation with plasma viral load was observed for CD4+ CD38+ HLA-DR+ T cells (data not shown).

We then determined if associations existed between percentages of caspase-3+ mDCs or Bcl-2 expression by blood mDCs from seronegative or HIV-1-infected donors, and the Spearman test was performed for plasma viral load and CD8+ T-cell correlations.

\( r = -0.30, P = 0.16 \). When percentages of caspase-3+ mDCs were evaluated after overnight in vitro culture, no statistical differences were noted between HIV-1-infected (n = 22; median, 11.4%; range, 2.7 to 35.2%) and seronegative donors (n = 18; median, 11.7%; range, 3.6 to 22.7%).

**Frequency of caspase-3+ mDCs from untreated, HIV-1-infected donors is associated with CD8+ T-cell activation levels.**

Increased levels of CD4+ and CD8+ T-cell activation, defined as either (i) total CD38 expression (assessed as MFI) or (ii) as the percentage of each T-cell subset coexpressing CD38 and HLA-DR, were observed in HIV-1-infected donors compared to seronegative donors (data not shown). CD4 count correlated inversely with both measures of CD4+ and CD8+ T-cell activation and a positive correlation with plasma viral load was observed for CD4+ CD38+ HLA-DR+ T cells (data not shown).

We then determined if associations existed between percentages of caspase-3+ blood mDCs or Bcl-2 expression by blood mDCs and CD38 expression (MFI) on CD4+ and on CD8+ T cells, and with the percentage of CD8+ T cells and CD4+ T cells coexpressing HLA-DR and CD38. A statistically significant association was observed between the percentage of caspase-3+ mDCs and the level of CD38 on CD8+ T cells (\( r = 0.48, P = 0.03 \); Fig. 2E). No statistically significant correlation was observed between caspase-3+ blood mDC percentages and the level of CD38 expression on CD4+ T cells (\( r = 0.37, P = 0.11 \)) or with coexpression of CD38 and HLA-DR on either CD4+ (\( r = 0.30, P = 0.16 \)) or CD8+ (\( r = 0.02, P = 0.93 \)) T cells.

Despite lower Bcl-2 expression by blood mDCs from HIV-1-infected donors, no statistically significant associations were observed between blood mDC Bcl-2 expression and CD38 expression (MFI) on CD8+ (\( r = -0.01, P = 0.98 \)) and CD4+ (\( r = 0.13, P = 0.65 \)) T cells and the percentages of CD8+ T cells.
cells and CD4+ T cells coexpressing CD38 and HLA-DR (CD8+ T cells; r = 0.22, P = 0.39; CD4+ T cells; r = 0.39, P = 0.11).

Blood pDCs from untreated, HIV-1-infected donors do not statistically differ in expression of Bcl-2 or caspase-3 compared to pDCs from seronegative donors. Assessment of Bcl-2 expression in pDCs measured directly ex vivo or after overnight in vitro culture showed no significant differences between seronegative and untreated, HIV-1-infected donors (Table 2). Similarly, no statistical differences were observed in the percentages of caspase-3-expressing blood pDCs from HIV-1-infected and seronegative donors, assessed directly ex vivo or after overnight in vitro culture (Table 2).

No statistically significant differences are observed in spontaneous apoptosis of blood mDCs or pDCs based on annexin V and 7-AAD staining between seronegative and untreated, HIV-1-infected donors. Two different stages of apoptosis can be assessed based on the use of the surface apoptosis marker annexin V and a nonvital dye (7-AAD). Cells undergoing early apoptosis stain positive for annexin V but not with 7-AAD, whereas late apoptotic cells costain with both annexin V and 7-AAD (90). PBMC were cultured overnight in vitro, and the percentage of DCs undergoing either early or late apoptosis was evaluated by flow cytometry. Although a trend toward higher frequencies of blood mDCs from HIV-1-infected donors undergoing late apoptosis (P = 0.07) was observed, no statistically significant differences were found in the percentages of mDCs or pDCs in early or late apoptosis after overnight culture between seronegative and untreated, HIV-1-infected donors (Table 3).

No statistically significant differences are observed in Fas and FasL expression on blood mDCs and pDCs between seronegative and untreated, HIV-1-infected donors. Surface Fas expression and intracellular expression of FasL was evaluated on blood mDCs and pDCs from seronegative and HIV-1-infected donor PBMC directly ex vivo. No significant differences were observed in expression of Fas by mDCs from seronegative (n = 13; median net MFI, 4; range, 0 to 27) with no statistical difference in expression between the two cohorts (P = 0.42).

In addition, no statistical differences were observed in FasL expression by mDCs between seronegative (n = 14; median net MFI, 16; range, 138 to 827) and HIV-1-infected donors (n = 16; median net MFI, 47; range, 171 to 1,024; P = 0.29). Similarly, no statistical differences were observed in FasL expression by pDCs from seronegative (n = 14; median net MFI, 258; range, 81 to 724) and HIV-1-infected donors (n = 16; median net MFI, 356; range, 88 to 838; P = 0.2). Due to limited differences in expression of Fas and FasL by either DC subset between seronegative and HIV-1-infected donors directly ex vivo, no further analysis of Fas or FasL was undertaken.

Percentage of caspase-3+ blood mDCs decreases after initiation of ART. To determine whether ART impacted the specific differences in mDC subset survival characteristics noted above, a subset of 11 donors (Tables 1 and 4) from the untreated, HIV-1-infected cohort were further evaluated for changes in mDC Bel-2 (n = 7) and caspase-3 (n = 11) expression in PBMC after starting ART (Fig. 3). Within this cohort, there was a significant increase in the median CD4 T-cell count and a significant decrease in plasma viral load after initiation of ART, with 10 of 11 subjects (91%) achieving complete virologic suppression (Table 4) at the time of the post-ART measurement. The level of CD38 expressed by CD4+ and CD8+

### Table 2. Expression of Bcl-2 and caspase-3 by blood pDCs from seronegative and untreated, HIV-1-infected donors assessed directly ex vivo or after overnight in vitro culture

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Seronegative donors</th>
<th>HIV-1-infected donors</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2 expression (net MFI):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Directly ex vivo (seronegative donors, n = 14; HIV-1-infected donors, n = 18)</td>
<td>4,779 (3,542-6,646)</td>
<td>4,871 (2,074-8,484)</td>
<td>0.95</td>
</tr>
<tr>
<td>After overnight in vitro culture (seronegative donors, n = 14; HIV-1-infected donors, n = 17)</td>
<td>5,189 (3,239-8,319)</td>
<td>4,841 (2,292-7,984)</td>
<td>0.49</td>
</tr>
<tr>
<td>Caspase-3 expression (%):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Directly ex vivo (seronegative donors, n = 18; HIV-1-infected donors, n = 23)</td>
<td>0.7 (0-2.4)</td>
<td>0.6 (0-2.2)</td>
<td>0.59</td>
</tr>
<tr>
<td>After overnight in vitro culture (seronegative donors, n = 18; HIV-1-infected donors, n = 22)</td>
<td>30.5 (13.3-53.7)</td>
<td>22.5 (10.7-55.0)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

### Table 3. Percentages of blood mDCs and pDCs undergoing early and late apoptosis after overnight in vitro culture

<table>
<thead>
<tr>
<th>DC type and stage</th>
<th>Seronegative donors</th>
<th>HIV-1-infected donors</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>mDCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early apoptosis</td>
<td>11.9 (2.4-24.3)</td>
<td>8.0 (2.8-34.3)</td>
<td>0.63</td>
</tr>
<tr>
<td>Late apoptosis</td>
<td>0.83 (0-4.5)</td>
<td>1.6 (0.03-14.1)</td>
<td>0.07</td>
</tr>
<tr>
<td>pDCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early apoptosis</td>
<td>19.6 (5.7-38.8)</td>
<td>21.1 (3.8-46.0)</td>
<td>0.92</td>
</tr>
<tr>
<td>Late apoptosis</td>
<td>6.3 (2.4-17.8)</td>
<td>4.3 (0-19.8)</td>
<td>0.30</td>
</tr>
</tbody>
</table>
T cells and the percentage of CD4+ and CD8+ T cells coexpressing CD38 and HLA-DR were also significantly reduced after treatment (Table 4). The frequencies of blood pDCs significantly increased, whereas no significant change was noted in mDC frequency after ART. The median CD40 expression on both pDCs and mDCs was reduced on ART, although this only reached statistical significance for pDCs (Table 4).

A statistically significant reduction in the percentage of caspase-3+ mDCs was observed post-ART relative to pre-ART (Fig. 3A). Although blood mDCs from untreated, HIV-1-infected donors displayed lower Bcl-2 expression directly ex vivo compared to seronegative donors, mDC Bcl-2 expression did not significantly change on ART (Fig. 3B).

Differential expression of Bcl-2 and caspase-3 in DC subsets in blood versus the LNs of HIV-1-infected individuals. The LN is a site of active HIV-1 replication (41) and an environment in which DCs interact closely with T cells (15). To determine whether this specific tissue environment influenced DC survival profiles, the expression of Bcl-2 and caspase-3 by mDCs and pDCs from the blood was compared to the expression detected in matched LN samples from an additional cohort of six untreated, HIV-1-infected donors with a median peripheral blood CD4 count of 663 cells/mm³ and a median plasma viral load 20,292 copies HIV-1 RNA/ml (Table 1).

In agreement with our previously published study (25), CD40 expression on mDCs and pDCs in LNs from these HIV-infected subjects was higher than that on mDCs and pDCs from autologous peripheral blood (Fig. 4A and D), suggesting a higher activation state of LN DCs. LN mDCs expressed higher levels of Bcl-2 (Fig. 4B) and had lower percentages of caspase-3+ (Fig. 4C) than their counterparts in peripheral blood. Conversely, LN pDCs expressed significantly lower levels of Bcl-2 than did blood pDCs (Fig. 4E). The percentage of caspase-3+ pDCs was also lower in LN than PBMC, although this difference did not achieve statistical significance (Fig. 4F).

TLR-specific signaling induces increased Bcl-2 and decreased caspase-3 expression in blood DCs in vitro. HIV-1 ssRNA encodes for numerous ligands that bind and activate DCs via TLR7/8 in vitro, (10, 44, 70, 71) and TLR8 triggering by HIV-1 ssRNA, in combination with DC-SIGN, a c-type lectin expressed by DCs, was shown to be required for produc-

### Table 4. Changes in clinical characteristics and in T-cell and DC activation states after the initiation of ART

<table>
<thead>
<tr>
<th>Category and parameter</th>
<th>Pre-ART Median (range)</th>
<th>Post-ART Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T-cell count and VL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 T-cell count (cells/mm³)</td>
<td>306 (36–700)</td>
<td>481 (90–975)*</td>
</tr>
<tr>
<td>VL (HIV-1 RNA copies/ml of plasma)</td>
<td>91,600 (7,200–143,000)</td>
<td>&lt;48 (48–115)*</td>
</tr>
<tr>
<td>CD38+ CD4+ T cells</td>
<td></td>
<td></td>
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<tr>
<td>CD38 (net MFI)</td>
<td>3,632 (1,390–9,772)</td>
<td>1,386 (911–2,193)†</td>
</tr>
<tr>
<td>CD38+ HLA-DR+ (%)</td>
<td>6.8 (0.78–22.01)</td>
<td>1.83% (0.56–7.81)*</td>
</tr>
<tr>
<td>CD38+ CD8+ T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD38 (net MFI)</td>
<td>2,228 (1,036–6,530)</td>
<td>500 (106–1,293)†</td>
</tr>
<tr>
<td>CD38+ HLA-DR+ (%)</td>
<td>13.5 (5.10–29.10)</td>
<td>2.53 (0.22–7.06)*</td>
</tr>
<tr>
<td>Blood DCs</td>
<td></td>
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<tr>
<td>mDCs (% total cells)</td>
<td>0.26 (0.12–0.47)</td>
<td>0.22 (0.12–0.57)</td>
</tr>
<tr>
<td>pDCs (% total cells)</td>
<td>0.10 (0.08–0.20)</td>
<td>0.13 (0.07–0.33)†</td>
</tr>
<tr>
<td>mDCs CD40 expression (net MFI)</td>
<td>2,578 (1,328–5,320)</td>
<td>1,805 (929–3,530)</td>
</tr>
<tr>
<td>pDCs CD40 expression (net MFI)</td>
<td>1,398 (822–2,841)</td>
<td>855 (227–1,263)§</td>
</tr>
</tbody>
</table>

* Statistical analysis was performed by using a Wilcoxon paired test (+, P = 0.001; †, P = 0.002; ‡, P = 0.03; §, P = 0.02).
tive infection of DCs (39). In addition, microbial products such as LPS, a known TLR4L, have been shown to be elevated in the blood of HIV-1-infected individuals (7, 16, 52). Blood mDCs express a range of TLRs that include TLR4 and TLR8, whereas blood pDCs only express TLR7 and TLR9 (51, 54). To determine whether HIV-1-related TLRLs modulated DC survival profiles, total PBMC from seronegative individuals (7 to 12) were stimulated in vitro with viral and bacterial TLRLs to mimic innate signals that might be delivered by microbial products or by HIV-1 to blood mDCs or pDCs. The effect of these ligands on the activation state (CD40) and apoptotic profile (Bcl-2, caspase-3) of each DC subset was evaluated after overnight in vitro culture (13 to 20 h) in a TLR expression-specific manner, with mDCs assessed in LPS- and TLR7/8L-stimulated cultures and pDCs evaluated in cultures stimulated with TLR7L.

Although both TLR4L and TLR7/8L significantly increased CD40 expression on blood mDCs, the impact of TLR7/8L was much greater at the stimulating doses used. TLR7 stimulation also significantly increased CD40 expression on blood pDCs (Fig. 5A). Bel-2 expression in mDCs was significantly increased by stimulation with TLR7/8L but not with TLR4L (Fig. 5B). Similarly, TLR7L stimulation induced statistically higher levels of Bel-2 expression in pDCs (Fig. 5B). TLR7/8L stimulation of PBMC resulted in significantly decreased percentages of caspase-3\(^+\) mDCs, whereas TLR4L stimulation only trended toward significance (P = 0.08) (Fig. 5C). TLR7L stimulation also significantly reduced the percentage of caspase-3\(^+\) pDCs (Fig. 5C).

We next evaluated whether DCs from a subset of the untreated HIV-1-infected donors (n = 7 to 14), potentially already exposed to viral and bacterial TLRLs in vivo, could respond to in vitro stimulation with TLRLs in a similar manner to DCs from uninfected donors. CD40 and Bel-2 expression levels by blood mDCs and pDCs from HIV-infected donors were upregulated to a similar extent following exposure to TLR4/7/8L as DCs from uninfected donors, and the percentages of caspase-3\(^+\) mDCs or pDCs induced by TLRL stimulation of total PBMC were likewise reduced in a manner similar to those observed in seronegative donor DCs (data not shown).

DISCUSSION

Despite the wealth of data demonstrating reduced frequencies of circulating DC subsets in the blood of HIV-1-infected donors (9, 25, 28, 30, 37, 75, 88), a limited number of studies have addressed possible mechanisms responsible for this depletion. During acute and chronic HIV-1 infection, it is likely that recruitment to LNs partly accounts for decreased blood DC frequencies (25, 64). Conversely, in late-stage disease there is a reduced frequency of DCs within the LN (11), suggesting that LN recruitment alone does not fully account for the deficit in circulating DCs throughout the course of HIV-1 disease.

We hypothesized that another possible contributor to HIV-1-associated depletion of circulating DCs is increased DC
We show that HIV-1 infection induced a proapoptotic profile in blood mDCs, defined as reduced Bcl-2 expression and increased frequencies of caspase-3 + mDCs in a subset of subjects that was associated with viral replication. Higher frequencies of caspase-3 + mDCs were also associated with increased levels of T-cell activation, as defined by CD38 expression on CD8 + T cells. Elevated CD38 expression on CD8 + T cells has been associated with shorter survival in advanced HIV-1 disease (33) and is considered by some groups to be a better indicator of disease progression than frequencies of CD8 + T cells coexpressing CD38 and HLA-DR (63). Since mDC–T-cell interactions form a critical step in initiation of adaptive immune responses, it is possible that T-cell activation status may directly contribute to the increased levels of apoptotic mDCs observed during untreated HIV-1 infection. An alternative hypothesis is that mDC survival and T-cell activation are independently influenced by HIV-associated factors.

The HIV-associated reduction in mDC Bcl-2 expression reported in the present study concurs with findings of reduced Bcl-2 expression described in T cells during HIV-1 infection (1, 14, 26, 80, 83, 100), raising the possibility that a common mechanism may be responsible for predisposing both mDCs and T cells to apoptosis. Most studies addressing the effect of ART on levels of T-cell Bcl-2 expression have noted increased expression of Bcl-2 with therapy (1, 4, 93), although one study found that Bcl-2 expression by T cells was not altered during 6 months of ART (38). In agreement with this latter study, we also did not observe any significant change in blood mDC Bcl-2 expression in a subset of HIV-1-infected donors that initiated ART during the study. Given that we did not observe a direct association between Bcl-2 expression and viral load in untreated, HIV-1-infected individuals, this lack of ART effect on Bcl-2 expression was not surprising and further supports the idea that HIV-associated factors other than viral load must be contributing to the decrease in mDC Bcl-2 expression. Conversely, caspase-3 + mDC frequencies were elevated above normal levels in a subset of HIV-1-infected donors that initiated ART during the study. Given that we did not observe a direct association between Bcl-2 expression and viral load in untreated, HIV-1-infected individuals, this lack of ART effect on Bcl-2 expression was not surprising and further supports the idea that HIV-associated factors other than viral load must be contributing to the decrease in mDC Bcl-2 expression. Conversely, caspase-3 + mDC frequencies were elevated above normal levels in a subset of HIV-1-infected subjects, were significantly associated with viral load, and were reduced after viral suppression with ART. The fact that we did not observe statistically greater percentages of caspase-3 + mDCs between HIV-1-infected and uninfected subject groups is possibly related to small sample size, which may be considered a limitation of the present study. These observed differences in the association of pro- and antiapoptotic protein expression with viral load suggests that multiple mechanisms are likely responsible for the observed HIV-associated changes in blood mDC apoptosis profiles.

In a recent study using direct ex vivo annexin V and 7-AAD staining to evaluate apoptosis of blood mDCs, Meera et al. reported a higher percentage of apoptotic mDCs in AIDS patients compared to controls (69). Although we also observed HIV-associated, proapoptotic changes in Bcl-2 and caspase-3 expression in blood mDCs directly ex vivo, we did not observe HIV-associated significant increases in DCs undergoing apoptosis using annexin V/7-AAD staining on cultured PBMC. However, a trend toward higher frequencies of blood mDCs from HIV-1-infected donors undergoing late apoptosis was not observed ($P = 0.07$). Blood DCs have been reported to survive poorly in vitro (59, 66), and we noted high frequencies of mDCs and pDCs undergoing spontaneous apoptosis in both subject...
groups after overnight PBMC culture (Table 3). High levels of spontaneous apoptosis and a large variability in apoptotic DC frequencies between donors, as defined by their expression of annexin V and 7-AAD, may have prevented detection of subtle differences between the subject groups. It is also possible that the lack of differences using this measure of apoptosis simply reflects a poor correlation between assays assessing different stages of the apoptosis pathway or in those carried out on cells directly ex vivo versus following a culture period. Indeed, in an early study of T-cell apoptosis during HIV-1 infection, lower Bcl-2 expression of CD4+ and CD8+ T cells measured directly ex vivo did not correlate with the percentage of cells induced to undergo apoptosis in vitro (26).

In contrast to our findings on blood mDCs, our study found that levels of blood pDC markers of apoptosis were not markedly altered in the setting of untreated HIV-1 infection, suggesting that survival of blood DC subsets may be differentially influenced by HIV-1. Meera et al. noted an increased frequency of blood pDCs undergoing apoptosis directly ex vivo in individuals with early and advanced HIV infection (69). The lack of similar observations of decreased survival tendencies of blood pDCs based on Bcl-2 and caspase-3 expression in our study may reflect analysis of different cohorts of HIV-1-infected donors in addition to differences in the assays used to detect apoptosis.

Since lymphoid tissue is a site of active viral replication and a tissue compartment in which DCs might interact most closely with both HIV-1 and T cells, we hypothesized that in HIV-infected donors the apoptotic profiles of LN DC subsets would differ from those of circulating DCs and perhaps more accurately reflect HIV-associated changes. The increase in CD40 and Bcl-2 expression and decrease in caspase-3 expression by LN relative to blood mDCs that we observed in HIV-1-infected donors suggests that mDCs may be receiving both activation and survival signals in HIV-infected lymphoid tissue. A number of studies have demonstrated a positive relationship between CD40 ligation and increased expression of the anti-apoptotic Bcl-2 family members (12, 42, 48, 81), although expression of these “survival” signals may depend on the activation state of the DCs at the time of CD40 ligation (23). Induction of spontaneous apoptosis of total DCs from patients with breast cancer was prevented by inducing increased Bcl-2 expression with CD40 ligation (81). The role for CD40-induced survival may be most notable in the LNs where close interactions between T cells and DCs occur (15), thereby increasing the likelihood of CD40-CD40L interactions. The small sample size of LN donors in our study prevented accurate determination of associations between CD40 expression and these apoptotic markers. However, given the parallel observation of higher expression of CD40 by the same LN mDCs relative to blood mDCs, it is tempting to speculate that LN mDCs are indeed receiving survival signals within the LN of HIV-1-infected infected donors that may, in part, be due to increased CD40 signaling resulting in increased Bcl-2 expression and/or decreased caspase-3 expression. These results might also provide a mechanism to explain the observation of our group that mDCs accumulate in the LNs of chronically HIV-infected subjects (25). Enhanced DC survival by IL-10-induced reversal of DC susceptibility to NK cell-mediated elimination (2) has also been reported to provide a mechanism to explain accumulation of mDCs in LNs. The survival signals received by mDCs in the LN, or a lack of “death-inducing” signals, may help to offset the proapoptotic tendencies of circulating blood mDCs in HIV-1 infection, although this hypothesis needs to be confirmed with additional studies.

Stimulation of mDCs in vitro with a TLRL mimicking strong activation and an antiapoptotic profile, whereas stimulation with a bacterial TLRL only moderately activated mDCs and did not significantly alter their apoptotic status. Thus, in addition to the possibility of increased CD40 activation inducing mDC survival within the LN, these data suggest that signaling via TLR7/8 by HIV-1 may also aid in the survival of LN mDCs during chronic infection. We, and others, have reported that DCs in LNs of HIV-1-infected donors are in a state of partial activation (25, 60). Thus, LN mDCs may receive survival signals via viral TLRs and CD40 ligation that allow them to persist in this state of partial activation, thereby potentially contributing to the state of chronic immune activation through nonspecific activation of T cells. In addition, survival of these “semimature” DCs may contribute further to T-cell dysfunction in the context of HIV-1 infection through the induction of regulatory T cells (60).

Conversely, pDCs from LNs of HIV-1-infected donors had reduced expression of Bcl-2 in combination with increased CD40 expression compared to circulating pDCs from the same donors. Thus, the LN environment during HIV-1 infection may provide both activating and proapoptotic signals to pDCs in contrast to our findings for LN mDCs and highlighting further functional differences between these two DC subsets. Indeed, in vitro studies have shown activation of pDCs through CD40-CD40L in HIV-1 infection models increased viral replication, productive infection, pDC death, and increased transmission of HIV-1 to T cells (31, 84). This suggests that the LN is a site of increased pDC death in HIV-1 infection, which is likely related, at least in part, to the increased expression of CD40 by LN pDCs. A recent study by Lehmann et al. provides further evidence for an HIV-associated increase in LN pDC death (62). They report that pDCs that had homed to the LNs of HIV-1-infected subjects underwent apoptosis, based on annexin V staining, at a greater rate than LN pDCs from uninfected donors, and this increased pDC apoptosis was associated with high pDC IFN-α production (62). Similarly, a study investigating pDC accumulation and death in the LNs of acutely SIV-infected monkeys demonstrated increased pDC death and SIV-infection rates of the pDCs similar to that of CD4+ T cells (18). The accumulation of pDCs in the LN of HIV-1-infected donors as reported by our group (25) and others (62), and the similar observations noted during acute SIV infection (8, 18) suggests that a balance exists between recruitment of pDCs to the LN and their subsequent death.

Stimulation with a viral TLRL induced both activation and an antiapoptotic profile in blood pDCs in vitro, a finding consistent with a study demonstrating increased pDC survival following in vitro stimulation with HIV-1-derived ssRNAs (70). However, these observations contrasted with the decreased survival profile observed in LN pDCs in HIV-1-infected donors, this difference implies that factors other than TLR7 activation of pDCs via HIV-1 ssRNA in vivo, such as other HIV-1 proteins (including Env, Net, Tat, and Vpr) that have been shown to induce apoptosis (86) or direct infection of...
pDCs (18, 84), likely account for the observed LN pDC survival patterns in HIV-1-infected subjects.

Although the intrasubject comparisons of apoptosis markers in blood versus LN DCs are informative, the absence of LN samples from an uninfected control group limits the interpretation of our findings. For instance, the relative antiapoptotic profile of LN mDCs that we observed may also reflect physiologic tissue-specific profiles that are unrelated to HIV-1 infection. In an acute SIV infection model, LN mDCs following SIV infection displayed increased caspase-3 expression compared to preinfection LN mDCs, although no alteration in Bel-2 expression was observed after infection (97). Thus, it is possible that our observations of apoptotic markers indicative of an increased survival pattern by LN mDCs relative to blood mDCs may still reflect reduced survival of LN mDCs compared to LN mDCs from an uninfected individual.

In conclusion, we believe our study to be the first to show that circulating mDCs from untreated HIV-1-infected donors exhibit a proapoptotic profile characterized by lower Bcl-2 that circulating mDCs from untreated HIV-1-infected donors of an increased survival pattern by LN mDCs relative to blood Bcl-2 expression was observed after infection (97). Thus, it is possible that our observations of apoptotic markers indicative of an increased survival pattern by LN mDCs relative to blood mDCs may still reflect reduced survival of LN mDCs compared to LN mDCs from an uninfected individual.

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REFERENCES


