Shift in Monocyte Apoptosis with Increasing Viral Load and Change in Apoptosis-related ISG/Bcl2-family Gene Expression in Chronic HIV-1 Infected Subjects

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Keywords: Macrophage, Myeloid, Pathogenesis, Activation, Viremia.

Running Title: Monocyte Apoptosis and Gene Expression in HIV-1

Abstract Word Count: 223 of 250 allowed.
Importance Abstract Word Count: 94 of 150 allowed.

Manuscript (without references, figure legends) Word Count: 3,649.
ABSTRACT

Although monocytes and macrophages are targets of HIV-1-mediated immunopathology, the impact of high viremia on activation-induced monocyte apoptosis relative to monocyte and macrophage activation changes remains undetermined. Here, we determined constitutive and oxidative stress-induced monocyte apoptosis in uninfected and HIV(+) individuals across a spectrum of viral load (n=35, range: 2,243 to 1,355,998 HIV-1 RNA copies/mL) and CD4 count (range: 26 to 801 cells/mm³). Both constitutive and oxidative stress-induced apoptosis were positively associated with viral load and negatively associated with CD4 with an elevation in apoptosis occurring in patients with greater than 40,000 (4.6 log) copies/mL. As expected, expression of Rb1 and interferon-stimulated genes (ISGs), plasma sCD163 concentration, and the proportion of CD14+/CD16+ intermediate monocytes were elevated in viremic patients compared to uninfected controls. Although most ISG expression, sCD14, sCD163, and CD14+/CD16+ frequencies were not directly associated with a change in apoptosis, sCD14 and ISG expression showed an association with increasing viral load. Multivariable analysis of clinical values and monocyte gene expression identified changes in IFI27, IFITM2, Rb1, and Bcl2 expression as determinants of constitutive apoptosis (p=3.77×10⁻⁵, adjusted-R²=0.5983), while changes in viral load, IFITM2, Rb1, and Bax expression were determinants of oxidative stress-induced apoptosis (p=5.59×10⁻⁵, adjusted-R²=0.5996). Our data demonstrate differential activation states in monocytes between levels of viremia in association with differences in apoptosis that may contribute to greater monocyte turnover with high viremia.
IMPORTANCE

This study characterizes differential monocyte activation, apoptosis, and apoptosis-related gene expression in low versus high level viremic HIV-1 patients, suggesting a shift in apoptosis regulation that may be associated with disease state. Using single and multivariable analysis of monocyte activation parameters and gene expression, we support the hypothesis that monocyte apoptosis in HIV disease is a reflection of viremia and activation state with contributions from gene expression changes within the ISG/Bcl2-gene families. Understanding monocyte apoptosis response may inform HIV immunopathogenesis, retention of infected macrophages, and monocyte turnover in low or high viral load states.

INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1) infection targets CD4-positive T-cells, macrophages, and other myeloid cells using CD4 and a chemokine co-receptor (CCR5/CXCR4) (1). While CD4 T-cell loss is observed in tissue (2) and peripheral blood (3, 4) during disease, loss of monocytes and macrophages is less evident. However, monocyte activation, reflected in TLR/ISG-expression (5-9), monocyte subsets (10-12), and soluble markers of activation (plasma sCD14, sCD163) (12-17) has been described in both early and advanced HIV-1 disease. The impact of HIV-1 viremia on monocyte activation, gene expression (8, 18-20), and function (9-11, 13, 21, 22) are largely independent of productive monocyte infection as only an estimated 0.03 to 0.1% of circulating monocytes harbor integrated HIV-1 DNA (11, 23, 24). However, exposure to viral particles and proteins, microbial products, and host products (such as cytokines)
can modulate monocyte function (8, 19, 20, 25-28) and apoptosis (18, 29-31) during
disease.

Unlike CD4 T-cells, the apoptotic fate of monocytes during chronic and elevated
HIV/SIV viremia is less clear, as different *ex vivo* and *in vitro* studies have reported both
anti-apoptotic (18, 31-33) and pro-apoptotic (30, 34, 35) mechanisms and outcomes.
Gene expression studies have identified a predominant anti-apoptosis gene signature in
monocytes during chronic disease (18), and multiple studies have described anti-
apoptosis mechanisms *ex vivo* and *in vitro* including: engagement of CCR5 and pro-
survival signaling (p38/ERK/MAPK pathway expression) (18), elevated intracellular zinc
content, metallothionein expression (32), elevated Rb1 protein activity (33), and M-CSF
mediated protection from TRAIL-induced apoptosis (31). Based on oxidative stress (36)
and increased expression of apoptotic ligands (37-39) in HIV-1 disease, resistance to
apoptosis has been tested by multiple induction mechanisms (CdCl2, sFasL) in cross-
sectional studies (18, 31-33). By contrast, non-human primate SIV studies have focused
on longitudinal changes from early to late disease under high viremic settings and show
higher monocyte turnover and elevated activation markers in animals with rapid disease
progression and neuropathogenesis (12, 40, 41), suggesting a functional shift in
monocyte viability upon the onset of advanced disease. Other SIV and *in vitro* HIV-1
studies have demonstrated pro-apoptotic outcomes of viral exposure based on
modulation of Bcl2 family genes (Bax, Bak, Mcl-1), downregulation of anti-apoptotic
cFLIP proteins, and downregulation of TRAIL decoy receptors (30, 34). While the
impact of rising viremia on monocyte activation has been characterized, no HIV-1 study,
to date, has measured macrophage activation with concurrent measurements of
monocyte apoptosis to identify functional changes relative to one another and viral load. In addition, reports on anti-apoptotic responses in monocytes examined a limited range of viral load (median viral load: 14,822 and 13,468 copies/mL, (18, 33), respectively), thus requiring a more extensive analysis across a wider viral load distribution.

Herein, we reconcile data between independent reports of pro- and anti-apoptotic gene expression and functional apoptosis outcomes by the *ex vivo* characterization of circulating monocytes from a broad clinical spectrum of HIV-1 viremia in the absence of anti-retroviral therapy.

**MATERIALS AND METHODS**

**Donor population.** HIV-1 seropositive patients (*n*=35, 11.4% female, median age=43.0 years) were recruited from the Hospital of the University of Philadelphia (HUP) and Philadelphia Field Initiating Group for HIV-1 Trials (FIGHT) and were not on therapy at the time of the single draw. Viremic patients showed a broad spectrum of viremia (viral load range: 2,243-1,359,041 copies/mL; CD4 range 7-801 cells/mm³) (Table 1). Viral load and CD4 count are reported from the most recent clinical visit (at most 3 months). HIV-1 seronegative donors (*n*=33, 24.2% female, median age 43.5 years) were recruited from the University of Pennsylvania Human Immunology Core and the Wistar Donors Program (Supplemental Table 1). Assays data showing less than the total recruited HIV(-) or HIV(+) population reflect limited patient material. All participants provided informed consent prior to blood draw, and all protocols were approved by the institutional review boards of the National Institutes of Health and The Wistar Institute.
Monocyte and CD4 T-cell isolation and immunophenotyping. Heparinized blood (70-200 mL) was processed within 1-3 hours of draw. Peripheral blood mononuclear cells (PBMC) and plasma samples were obtained using Ficoll-Paque (Amersham Pharmacia Biotech) gradient separation. HIV(-) and HIV(+) donor CD4 T-cells were obtained from the University of Pennsylvania's Center for AIDS Research Human Immunology Core obtained by Rosette Sep (Stemcell Technologies) isolation from leukapheresis-PBMC. Monocytes were isolated by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec, 139-091-153) with a mean biological purity greater than 85%. PBMC and isolated monocytes from each sample were stained with CD4-V450, CD14-APC, CD16-APCH7, CD3-PerCPCy5.5, and CD8-FITC for determination of purity, T-cell subsets, and monocyte subsets. All flow cytometry antibodies were purchased from BD Biosciences and used per the manufacturer's instructions.

Plasma activation markers. HIV(-) and HIV(+) plasma samples were assayed for sCD14 (R&D Systems) and sCD163 (Trillium Diagnostics), using 1:500 dilutions, according to the manufacturers’ instructions. Plates were read on a BioTek Synergy HT microplate reader at 450 nm with the appropriate wavelength corrections.

Quantitative real-time PCR. Total RNA was isolated from 2-5 million cells using the RNeasy Plus II Kit (Qiagen) per the manufacturer’s protocol. Up to 250 ng of RNA was used with the iScript cDNA synthesis kit (BioRad) as instructed. Quantitative real-time
PCR was performed in a 20 μL reaction using Power SYBR Green PCR Master Mix (Life Technologies) and custom designed primers (Integrated DNA Technologies) (Table 2) using the AB 7000 Sequence Detection System. The QuantumRNA Universal 18s Internal Standard (Ambion) was used due to its robust and consistent expression across all samples. All primer sets were assayed by serial dilution PCR across multiple donors to allow for absolute RNA quantification by the standard curve method. The mean of three replicates of each primer-sample pair was input in the standard curve method and normalized to the sample’s 18s value to generate an absolute RNA expression units value. (Expression units defined as relative expression value to 18s after standardization.)

**Apoptosis induction assay.** Purified monocytes and CD4 T-cells were incubated overnight (18-22 hours) in non-adherent conditions (monocytes in Teflon pots to prevent attachment) in the presence of RPMI 1680 media with 1% Penicillin/Streptomycin (Corning), 1% L-Glutamine (Corning), and 10% Human Serum (Gemini Bio-Products) with or without the addition of 20 μM CdCl2 (Sigma). All staining was performed at room temperature (22°C). Between 500,000 and 1 million cells were washed and incubated with FACS wash (DPBS (Life Technologies) containing 3 mM NaN3 (Fisher), 1% Bovine Serum Albumin (Sigma), 5% Human Serum (Gemini Bio-Products)) as an Fc-receptor block. Cells were stained with CD3-Pacific Blue, CD14-APC and 7-aminoactinomycin D for 15 minutes. Cell fixation/permeabilization was performed using the BD Cytofix/Cytoperm kit per the manufacturer’s instructions and washed in 1× PermWash buffer. Cells were then stained with anti-active caspase 3-PE (BD Biosciences),
washed, and resuspended in 300 μL 1× PermWash buffer. Apoptosis was also assayed at baseline isolation in select samples. Up to 100,000 events were analyzed on a 14-color LSR II (BD Biosciences).

**Gating strategy.** Flow cytometry data analysis was performed with FlowJo software (TreeStar) (Supplemental Figure 1). Singlets were gated using forward scatter height and area. Live cells were gated, excluding debris and aggregates, with forward and side scatter. To characterize monocyte subsets in PBMC, monocytes were gated based on distinct forward and side scatter parameters and then CD14+ or CD16+ cells within this population. Monocytes were then divided into classical (CD14++/CD16-), intermediate (CD14+/CD16+), and non-classical (CD14+/CD16++) subsets. In the apoptosis analysis, all events were gated on singlets, intact cells, and either lymphocyte or monocyte forward/side scatter parameters. Cells were analyzed using 7-AAD and caspase-3, and all cells in the early apoptotic and late apoptotic quadrants were counted when determining the percent of apoptotic cells within the population.

**Statistical analysis.** All dot, box and whisker, and scatter plots were constructed and statistically tested using Prism 4 (GraphPad, La Jolla, CA). All group-wise comparisons were performed using Mann-Whitney test with p-value displayed. Correlations between two variables were performed using Spearman’s Correlation (Rho) test with p and Rho (noted as R) values displayed. All tests were two-sided and p<0.05 was considered significant. The Spearman correlation matrix was created with JMP10 software. Based on the analysis of the primary variable (monocyte apoptosis), HIV(+) patient measured
variables are presented below and above a viral load cutoff of 40,000 copies/mL (Log_{10} 4.60).

**Multivariate linear regression modeling.** A stepwise multiple linear regression analysis was performed to model constitutive and induced apoptosis using the combination of clinical and gene expression variables. We conducted log transformation on the independent variables and confirmed lack of multi-co-linearity in the final models within independent variables by means of the variance inflation factor (VIF). The Cook’s distance was used in outlier detection and removal of each independent model. The model selection package “leaps” (Lumley and Miller 2009) in R was utilized to identify the best subset of clinical/gene expression parameters that predicted constitutive and induced apoptosis in the linear regression. The regsubsets function within “leaps” was used to find the best subset of predictive parameters. The adjusted R-squared statistic was used as the criterion for selecting the best model with four or less variables per total input variable size. Various diagnostic plots were used to check the assumptions for the multiple linear regressions (data not shown). Q-Q plot was used to examine normality. Residuals plot was used to examine the equal variance assumption.

**RESULTS**

**Monocyte apoptosis is elevated in HIV-1 subjects with higher viremia.**

Monocytes constitutive and induced apoptosis was examined in a cohort of viremic patients (Table 1) that included a wide range of viral load (2,243-1,355,998 copies/mL) and CD4 count (26-801 cells/mm³) (Figure 1, 2). As expected, CD4 count
was negatively associated with viral load ($p=0.0012$, $R=-0.5255$).

Both constitutive and oxidative stress-induced apoptosis ($20 \mu M \text{CdCl}_2$) (42-45) showed a higher frequency of caspase-3 positive cells at viral loads above 40,000 (Log$_{10}4.60$) copies/mL (constitutive $p=0.0034$, induced $p=0.0002$) when compared to lower viremic subjects (Figure 2A). Irrespective of viral load, increased T-cell apoptosis was observed in all HIV(+) patients as compared to uninfected donors (data not shown). Across all HIV(+) patients, constitutive and induced apoptosis were positively associated with viral load ($p=0.0256$, $R=0.3883$ and $p=0.0002$, $R=0.5974$, respectively), and inversely associated with CD4 count, ($p=0.0128$, $R=-0.4288$ and $p=0.0464$, $R=-0.3491$, respectively) (Figure 2B, 2C). Supporting a threshold effect of viral load, the correlation of viral load with apoptosis was not present if restricting to only patients lower or higher than 40,000 copies/mL (all $p$-values $>0.05$). Our data shows higher viral load is associated with increased constitutive and induced apoptosis in circulating monocytes.

Markers of innate immune activation are increased with HIV(+) infection but do not associate with change in monocyte apoptosis

Previous findings have described CD14+/CD16+ intermediate monocytes (10-12, 46) and the plasma factors sCD14 (13, 14, 17, 47) and sCD163 (15, 16, 40, 48) as biomarkers of monocyte/macrophage activation and pathogenesis in SIV/HIV-1 disease. Therefore, we investigated the relationship between these variables and change in monocyte apoptosis with increasing viral load. We found the CD14+/CD16+ intermediate subset and plasma sCD163 concentrations were elevated in the HIV(+).
group compared to controls (Figure 3A, columns 1, 3), but did not detect differences
within the HIV(+) groups (Figure 3B) or an association between these variables with
viral load (Figure 3C), monocyte apoptosis (Figure 3D), or CD4 count (Supplemental
Figure 2). Conversely, plasma sCD14 concentration was associated with viral load
(Figure 3C) and CD4 count (Supplemental Figure 2A), but not monocyte apoptosis
(Figure 3D, p=0.1070). Below a CD4 count of 500 cells/mm³ (Supplemental Figure 2B),
CD4 count demonstrated a strong negative correlation with sCD14 (p<0.0001) and
trended with sCD163 (p=0.0657). Taken together, data support the presence of
monocyte activation with persistent sCD163 and CD14+/CD16+ monocytes (and an
association of sCD14 with viremia and CD4) that is independent of changes in
monocyte apoptosis with viral load.

Apoptosis-related ISG family gene expression is altered in association with viral
load.

Based on prior reports identifying key genes associated with monocyte
apoptosis, such as Rb1 (18, 33), and type I interferon gene expression in monocytes
from highly viremic HIV-1 patients (7, 8, 19), we focused on testing if functional changes
in monocyte apoptosis were associated with changes in p53 (33, 49, 50),
Bcl2/cytochrome C (51-55), and ISG (56-63) family expression due to their known
apoptosis regulation functions (Table 2). RT-qPCR analysis at time of isolation showed
significantly higher Rb1 expression in the total HIV(+) group versus HIV(-) group
(p=0.0100) but no association with viral load, consistent with reports of Rb1
contributions to apoptosis resistance (33) in circulating monocytes during HIV infection.
Within the Bcl2/cytochrome C genes analyzed, the HIV(+) group (relative to the HIV(-) group) was characterized by lower Bcl-w (p=0.0169) and higher cytochrome C gene expression (p=0.0392), both with no association of gene expression with viral load. In contrast, Mx1 and the apoptosis-associated ISG12-members (IFI6, IFI27, IFITM2) showed higher expression in the HIV(+) group relative to the HIV(-) group, and IFI27 and IFITM2 showed higher expression in the HIV(+) group above 40,000 copies/mL relative to the HIV(+) group below 40,000 copies/mL (Figure 4A). The apoptosis-related ISGs (IFI6, IFI27, IFITM2) were also positively associated with viral load (Figure 4B), consistent with published data (7, 8, 19). Taken together, our data demonstrate a shift in monocyte gene expression with HIV-1 infection and increasing viral load consistent with apoptosis modulation.

**Multivariate analysis identifies genes contributing to changes in constitutive and induced apoptosis in HIV infection.**

Although expression of genes within target families was correlated (p53, pro-apoptotic Bcl2-genes, and ISGs) (Figure 5), only IFITM2 was associated with constitutive apoptosis, indicating that single gene expression analysis could not reflect change in both constitutive and induced apoptosis function. Multivariable stepwise regression modeling was applied to evaluate if the combination of viral load, CD4 count, and gene expression variables could generate a model for the distribution of constitutive and induced apoptosis responses observed in HIV-infected subjects. The adjusted R-squared rank method identified IFI27, IFITM2, Rb1, and Bcl2 expression as the best model to describe variance in constitutive apoptosis (p=3.77×10^-5, adjusted-R²=0.5983).
(Table 3, Figure 6A). A reduction of this model showed the key variables were IFI27 and Bcl2 accounting for 96.4% of the adjusted R-squared. For oxidative stress-induced apoptosis, the model system identified log viral load, IFITM2, Rb1, and Bax expression ($p=5.59 \times 10^{-5}$, adjusted-$R^2=0.5996$) (Table 3, Figure 6B) with log viral load and Rb1 gene expression, representing 83.1% of the adjusted R-squared. Taken together, the shift in functional monocyte apoptosis observed with increasing viral load was associated with a multi-gene contribution including changes in Rb1, ISG, and Bcl-2 expression.

**DISCUSSION**

Here we show for the first time that monocyte apoptosis is elevated in patients with greater than 40,000 copies/mL and negatively associated with CD4 count (Figure 2). Our data suggest that a viral load below this threshold can modulate monocytes and macrophages activation as indicated by change in sCD163 and CD14+/CD16+ monocytes, yet does not result in a rise in apoptosis until higher viral loads, clarifying conflicting reports of monocyte apoptosis in HIV infection (18, 30-33, 35, 41).

Interestingly, our data suggests the presence of at least two distinct phases of monocyte/macrophage activation relative to viremia: (1) below 40,000 copies/mL, characterized by increases in plasma sCD163, the CD14+/16+ intermediate monocyte subset, Rb1 expression, and cell viability (as described in (18, 32, 33)), and (2) above 40,000 copies/mL, described here, characterized by increasing plasma sCD14 with viral load, high ISG expression, and elevated apoptosis.
Although monocyte type-1 interferon-stimulated gene expression (7, 8, 19) and monocyte activation markers (10-17, 40, 46-48) have been described as modulated during HIV-1/SIV infection, the present study extends this data by showing that an elevation in apoptosis at higher viremia can be detected in spite of active mechanisms of apoptosis resistance (i.e. higher zinc content and Rb1 expression) (18, 31, 32).

Change in ISG expression and increasing sCD14 with viremia may evidence increasing microbial translocation, as microbial products were associated with interferon-α activation in HIV-1 viremic patients (17) and may contribute to the shift in monocyte ISG expression and apoptosis induction (64, 65).

Previous studies have demonstrated elevated sCD14 in multiple HIV(+) to HIV(-) donor comparisons (14, 16, 17, 66-68), and while we demonstrate an association with viral load and CD4 count (Figure 3C and Supplemental Figure 2), we do not detect a significant increase from the HIV(-) to HIV(+) group (Figure 3A), which may reflect differences in sample size and the characteristics of the HIV(+) cohort. Interestingly, others have observed biphasic CD163 expression on CD14+/CD16+ monocytes relative to CD4 count (negative correlation below, positive correlation above 500 cells/mm^3) (69). Although we did not measure membrane CD163 expression, we observed a negative trend of CD4 count with sCD163, as well as a strong negative correlation with sCD14, in patients below 500 cells/μL (Supplemental Figure 2) supporting these observations. We interpret the reversal of monocyte activation upon ART-mediated suppression may vary qualitatively and temporally, as indicated by resolution of Rb1 protein expression (33), decreased CD14+/CD16+ monocyte levels when treated in early infection but not if treated in chronic infection (15), persistence of plasma sCD14
(16, 66, 68), and partial, but incomplete plasma sCD163 resolution (16, 70). Future longitudinal studies will be needed to establish the temporal relationship between monocyte activation variables described here in the context of disease progression and ART-mediated viral suppression.

In addition to the main study conclusion per impact of viral load, multivariable analysis highlighted candidate ISG and Bcl2 genes as potential contributors to the functional apoptosis shift when added to rising viral load and sustained Rb1 expression (Table 3, Figure 6). Members of the ISG12 family (56, 62) examined here, including IFI6 (57, 58), IFI27 (59), and IFITM2 (60), have described apoptosis-regulation functions. Of interest, IFITM2 was the only gene associated with apoptosis outcome at the single gene level (Figure 5) and IFITM2 and Rb1 were present in both models (Table 3, Figure 6), supporting specific candidate roles of these genes as determinant of monocyte apoptosis in HIV-infected subjects. The impact of HIV-1 on Bcl2-mediated apoptosis in the CD4 compartment is also well described (reviewed in (39)), and the effect on non-CD4 cell types, namely CD8 T-cells (71), dendritic cells (72), and now monocytes, suggests a global impact of HIV-1 viremia and immune activation on Bcl2-mediated apoptosis regulation. In addition, it has been established that Rb1 can override p53-mediated apoptosis induction \textit{in vitro} (50), yet it remains to be determined whether Bax and ISG12 family expression can override anti-apoptotic Rb1 expression as suggested by our data. The multiple genes measured \textit{ex vivo} as contributing to apoptosis regulation makes it highly unlikely the functional phenotype can be accounted by any one gene. However, future work could determine how the described pro-apoptotic
(IFI27, IFITM2, Bax) and anti-apoptotic (Rb1, Bcl2) proteins exert regulation of apoptosis when co-expressed to defining overall dominance or an additive effect.

Our data does not address the potential impact of age and gender on monocyte apoptosis (14, 73, 74). Both HIV(-) and HIV(+) groups represent similar gender (no more than 25% female) and age distributions, but our cohort is limited in the representation of females and aged individuals (Supplemental Table 1). Future studies will need to address the impact of age and gender on monocyte apoptosis and apoptosis-gene expression. Despite lower median apoptosis levels in the <40,000 copies/mL HIV(+) group when compared to the HIV(-) group, no significant difference was detected in contrast to previous studies (18, 32, 33). We interpret this difference to reflect methods used as the current study’s apoptosis assay was based on overnight incubations compared to 6 hours post-isolation, as done previously.

Although we interpret a direct role of target genes to contribute to apoptosis, a limitation of this study is the lack of single- to multiple-gene knockdowns in monocytes to establish the conclusive role of target genes. However, the well-established functional role of pro-apoptotic genes identified here as contributing to apoptosis lends support to the notion that their increased expression may impact apoptosis. Also, while we focused on the relationship between apoptosis and apoptosis-related gene families expressed within cells (p53, ISG, Bcl2), our data does not exclude contributions by added external mechanisms in vivo affecting total monocyte numbers such as de novo production/turnover described in SIV infection (40, 41) or external factors such as soluble-TRAIL in viremic serum (35, 38) or increased TRAIL-mediated apoptosis (30, 34, 37, 75).
Our data joins an increasing body of evidence suggesting that high viral load and low CD4 count may be associated with changes in monocyte functional responses including greater monocyte turnover (associated with plasma sCD163) (40, 41), tissue migration (40, 41, 76), and macrophage apoptosis in tissue (41). This study complements studies in SIV-infected macaques (using in vivo BrdU labeling) in which disease progression (i.e., high viral load) was associated with increased monocyte turnover which may associate with higher apoptosis (40, 41). Indeed, an increase in monocyte apoptosis with higher viremia may associate with the onset of pathology as suggested by observations of high constitutive monocyte apoptosis and pro-apoptotic Bcl2 family expression in SIV-infected macaques during pathogenic SIV-macaque infection, but not in non-pathogenic African Green Monkey infection (30).

In summary, we establish a shift in monocyte functional apoptosis and gene expression in association with high HIV-1 viral load and low CD4 count, which may bear on mechanisms for immunodeficiency, HIV replication, and/or HIV reservoir retention in HIV-1 disease.

ACKNOWLEDGMENTS
This study was supported by U.S. National Institutes of Health grant R01 AI073219, the Philadelphia Foundation (Robert I. Jacobs Fund), Henry S. Miller, Jr., J. Kenneth Nimblett, the Commonwealth Universal Research Enhancement Program, the Pennsylvania Department of Health, the Penn Center for AIDS Research P30AI045008, and the NIH-T32 HIV Pathogenesis Training Grant through the University of Pennsylvania. Support for Shared Resources utilized in this study was provided by
Cancer Center Support Grant (CCSG) P30CA010815 to The Wistar Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We thank the Wistar Genomics Facility, Jeffery Faust (Wistar Flow Cytometry) for help with experiments, and Wistar Phlebotomist Deborah Davis for blood donor recruitment.
REFERENCES


FIGURE LEGENDS

Figure 1: Monocyte apoptosis characterization. Representative 7-AAD/caspase-3 dot plots of purified monocytes from uninfected and HIV(+) (14,000 and 439,508 copies/mL) patients. Plots represent overnight constitutive apoptosis (first row) and oxidative stress induced apoptosis (20 μM CdCl₂) (second row).

Figure 2: Monocyte apoptosis is elevated in patients above 40,000 copies/mL and associated with viral load and CD4 T-cell count. (A) Group-wise comparison of constitutive (left) and induced (right) apoptosis in HIV(-) versus HIV(+) individuals and HIV(+) patients below versus above 40,000 (Log₁₀4.6) copies/mL. (B-C) Spearman ranked correlation of viral load (B) and CD4 count (C) versus monocyte apoptosis levels in HIV(+) patients. The line on the y-axis represents the median constitutive (8.48%) and induced (14.82%) apoptosis levels of the HIV(-) group. Group-wise comparison displays median and interquartile range using Mann-Whitney test (two-tailed) with p<0.05 considered significant. Spearman (two-tailed) Rho and p-values displayed with p<0.05 considered significant.

Figure 3: Monocyte/macrophage activation in HIV(+) patient cohort. (A-B) Group-wise comparison of percentage of intermediate CD14+/CD16+ monocytes (left), plasma sCD14 (middle), and plasma sCD163 (right) in (A) HIV(-) versus HIV(+) individuals and (B) HIV(+) patients below versus above 40,000 (Log₁₀4.6) copies/mL. (C-D) Spearman ranked correlation of viral load (C) and induced apoptosis (D) versus monocyte/macrophage activation metrics. Group-wise comparison displays median,
interquartile range (box), and range (whiskers) using Mann-Whitney test (two-tailed) with p<0.05 considered significant. Spearman (two-tailed) Rho and p-values displayed with p<0.05 considered significant.

Figure 4: Monocyte ISG12 family expression is elevated in patients above 40,000 copies/mL and associated with viral load. (A) Group-wise comparison IFI6, IFI27, and IFITM2 gene expression in HIV(+) patients below versus above 40,000 (Log_{10}4.6) copies/mL. (B) Spearman ranked correlation of viral load versus monocyte gene expression. Group-wise comparison displays median, interquartile range (box), and range (whiskers) using Mann-Whitney test (two-tailed) with p<0.05 considered significant. Spearman (two-tailed) Rho and p-values displayed with p<0.05 considered significant.

Figure 5: Gene expression correlation of p53, Bcl2, and ISG family genes in monocytes of HIV(+) donors. Spearman ranked correlation matrix of clinical parameters (brown), p53 (pink), Bcl2 (blue), and ISG (green) family genes, and constitutive/induced apoptosis (purple). Spearman test (two-tailed, unadjusted) with p<0.05 considered significant (bolded/red) and Spearman Rho is displayed. (n = 30).

Figure 6: Multivariate analysis of monocyte apoptosis in HIV-1 viremic patients. Top models generated showing (fitted) versus observed (A) constitutive and (B) induced apoptosis by multivariate stepwise regression of clinical parameters and apoptosis-gene expression.
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**Table 1: HIV-1 patient population.**
<table>
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<tr>
<th>Gene Symbol</th>
<th>Apoptotic Impact</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
<th>HIV(-), n=19 Median (IQR)</th>
<th>HIV (+), n=30 Median (IQR)</th>
<th>p-value HIV(-) versus HIV(+)</th>
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<tr>
<td>Rb1</td>
<td>Anti-Apoptotic</td>
<td>GCC TCT CGT CAG GCT TGA G</td>
<td>TCA TCT AGG TCA ACT CGT GGA A TCT GTC TCA CTA ATT GCT TCT CT</td>
<td>2.559 (1.729-8.421)</td>
<td>2.559 (1.729-8.421)</td>
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<tr>
<td>MDM2</td>
<td></td>
<td>GAA TCA TCG GAC TCA GGT ACA TC</td>
<td></td>
<td>1.595 (0.7060-3.316)</td>
<td>1.595 (0.7060-3.316)</td>
<td>n.s.</td>
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<tr>
<td>Bcl2</td>
<td>Anti-Apoptotic</td>
<td>TTG CCA GCC GGA GCC GAG TCC ACA GCT CTA TAC</td>
<td>CCA AGG CGA CCA CCA GCA ATG ATA</td>
<td>0.1108 (0.08957-0.3244)</td>
<td>0.1108 (0.08957-0.3244)</td>
<td>n.s.</td>
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<tr>
<td>Mcl-1</td>
<td>Anti-Apoptotic</td>
<td>TGC TTC GGA AAC TGG ACA TCA A</td>
<td>TAG CCA CAA AGG CAC CAA AAG</td>
<td>0.8846 (0.5637-1.242)</td>
<td>0.8846 (0.5637-1.242)</td>
<td>n.s.</td>
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<tr>
<td>Bax</td>
<td>Anti-Apoptotic</td>
<td>CCC GAG AGG TCT TTT TCC GAG</td>
<td>CCA GCC CAT GAT GGT TCT GAT</td>
<td>0.4709 (0.4939-1.030)</td>
<td>0.4709 (0.4939-1.030)</td>
<td>n.s.</td>
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<tr>
<td>Bak1</td>
<td>Anti-Apoptotic</td>
<td>GTT TTC CCG AGC TAC GTT TTT G</td>
<td>GCA GAG GTA AGG TGA CCA TCT C</td>
<td>1.846 (1.338-3.304)</td>
<td>1.846 (1.338-3.304)</td>
<td>n.s.</td>
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<td>Bik</td>
<td>Anti-Apoptotic</td>
<td>GAC CTG GAC CCT ATG GAG GAC</td>
<td>CCT CAG TCT GGT CTT AGT AGA TGA</td>
<td>0.6919 (0.3536-1.256)</td>
<td>0.6919 (0.3536-1.256)</td>
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<tr>
<td>CypS</td>
<td>Pro-Apoptotic</td>
<td>TCT TGG GCG GAA GAC AGG TC</td>
<td>TTA TTG GCG GCT GTG TAA GAG</td>
<td>0.8745 (0.4069-1.408)</td>
<td>0.8745 (0.4069-1.408)</td>
<td>n.s.</td>
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<tr>
<td>Mx1</td>
<td>Anti-Apoptotic</td>
<td>GGT TCC GAA GTG GAC ATC GCC</td>
<td>CCA TCA AGT GAT TAA GGA TGG GA</td>
<td>0.1703 (0.1163-0.3839)</td>
<td>0.1703 (0.1163-0.3839)</td>
<td>n.s.</td>
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<td>IFI6</td>
<td>Anti-Apoptotic</td>
<td>GGT CTG CGA TCC TGA ATG GG</td>
<td>TCA CTA AGT TAA TGG AGA TAC TCG ACG GAG</td>
<td>0.8497 (0.4130-1.969)</td>
<td>0.8497 (0.4130-1.969)</td>
<td>n.s.</td>
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<td>IFITM2</td>
<td>Pro-Apoptotic</td>
<td>TGG CTC TCC GGT AGT TTT T</td>
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<td>0.3359 (0.4130-0.8334)</td>
<td>0.3359 (0.4130-0.8334)</td>
<td>n.s.</td>
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</table>

**Table 2:** Apoptosis related p53, Bcl2/cytochrome C, and ISG family genes measured by absolute quantification RT-PCR.
### Constitutive

| Coefficient | Estimate | Std. Error | 95% CI | t value | Pr(>|t|) |
|-------------|----------|------------|--------|---------|----------|
| (Intercept) | 6.445281 | 2.2119471  | 1.8695—11.0210 | 2.914 | 7.82E-03 |
| IFI27       | 0.003723 | 0.0008659  | 0.001932—0.005514 | 4.299 | 2.67E-04 |
| IFITM2      | -1.24797 | 0.001932—0.005514 | -1.825 | 0.081066 |
| Rb1         | 0.393577 | 0.3363558  | -0.3022—1.0894 | 1.17 | 2.54E-01 |
| Bcl2        | 1.811856 | 0.4172257  | 0.9488—2.6750 | 4.343 | 2.49E-04 |

Multiple R-squared: 0.6578
Adjusted R-squared: 0.5983

F-statistic: 11.05 on 4 and 23 DF
p-value: 3.77E-05

### Induced

| Coefficient | Estimate | Std. Error | 95% CI | t value | Pr(>|t|) |
|-------------|----------|------------|--------|---------|----------|
| (Intercept) | -98.354  | 21.986     | -143.9495—-52.7577 | -4.473 | 1.90E-04 |
| Log Viral Load | 19.890  | 3.532      | 12.5647—27.2153 | 5.631 | 1.16E-05 |
| IFITM2      | -5.899   | 4.277      | -14.7694—2.9718 | -1.379 | 0.18173 |
| Rb1         | 15.037   | 5.332      | 3.978022—26.0957065 | 2.820 | 0.00997 |
| Bax         | -16.529  | 8.200      | -33.534222—-0.4762045 | -2.016 | 0.0562 |

Multiple R-squared: 0.6612
Adjusted R-squared: 0.5986

F-statistic: 10.73 on 4 and 22 DF
p-value: 5.59E-05

**Table 3: Multivariate analysis of HIV-1 monocyte apoptosis.**
Figure 1: Monocyte apoptosis characterization. Representative 7-AAD/caspase-3 dot plots of purified monocytes from uninfected and HIV(+) (14,000 and 439,508 copies/mL) patients. Plots represent overnight constitutive apoptosis (first row) and oxidative stress induced apoptosis (20 μM CdCl₂) (second row).
Figure 2: Monocyte apoptosis is elevated in patients above 40,000 copies/mL and associated with viral load and CD4 T-cell count. (A) Group-wise comparison of constitutive (left) and induced (right) apoptosis in HIV(-) versus HIV(+) individuals and HIV(+) patients below versus above 40,000 (Log$_{10}$4.6) copies/mL. (B-C) Spearman ranked correlation of viral load (B) and CD4 count (C) versus monocyte apoptosis levels in HIV(+) patients. The line on the y-axis represents the median constitutive (8.48%) and induced (14.82%) apoptosis levels of the HIV(-) group. Group-wise comparison displays median and interquartile range using Mann-Whitney test (two-tailed) with p<0.05 considered significant. Spearman (two-tailed) Rho and p-values displayed with p<0.05 considered significant.
Figure 3: Monocyte/macrophage activation in HIV(+) patient cohort. (A-B) Group-wise comparison of percentage of intermediate CD14+/CD16+ monocytes (left), plasma sCD14 (middle), and plasma sCD163 (right) in (A) HIV(-) versus HIV(+) individuals and (B) HIV(+) patients below versus above 40,000 (Log_{10}4.6) copies/mL. (C-D) Spearman ranked correlation of viral load (C) and induced apoptosis (D) versus monocyte/macrophage activation metrics. Group-wise comparison displays median, interquartile range (box), and range (whiskers) using Mann-Whitney test (two-tailed) with p<0.05 considered significant. Spearman (two-tailed) Rho and p-values displayed with p<0.05 considered significant.
Figure 4: Monocyte ISG12 family expression is elevated in patients above 40,000 copies/mL and associated with viral load. (A) Group-wise comparison IFI6, IFI27, and IFITM2 gene expression in HIV(+) patients below versus above 40,000 (Log_{10} 4.6) copies/mL. (B) Spearman ranked correlation of viral load versus monocyte gene expression. Group-wise comparison displays median, interquartile range (box), and range (whiskers) using Mann-Whitney test (two-tailed) with p<0.05 considered significant. Spearman (two-tailed) Rho and p-values displayed with p<0.05 considered significant.
Figure 5: Gene expression correlation of p53, Bcl2, and ISG family genes in monocytes of HIV(+) donors. Spearman ranked correlation matrix of clinical parameters (brown), p53 (pink), Bcl2 (blue), and ISG (green) family genes, and constitutive/induced apoptosis (purple). Spearman test (two-tailed, unadjusted) with p<0.05 considered significant (bolded/red) and Spearman Rho is displayed. (n = 30).
Figure 6: Multivariate analysis of monocyte apoptosis in HIV-1 viremic patients. Top models generated showing (fitted) versus observed (A) constitutive and (B) induced apoptosis by multivariate stepwise regression of clinical parameters and apoptosis-gene expression.
Correction for Patro et al., Shift in Monocyte Apoptosis with Increasing Viral Load and Change in Apoptosis-Related ISG/Bcl2 Family Gene Expression in Chronically HIV-1-Infected Subjects

Sean C. Patro, Sharmistha Pal, Yingtao Bi, Kenneth Lynn, Karam C. Mounzer, Jay R. Kostman, Ramana V. Davuluri, Luis J. Montaner

The Wistar Institute, HIV Immunopathogenesis Laboratory, Philadelphia, Pennsylvania, USA; University of Pennsylvania Perelman School of Medicine, Department of Microbiology, Philadelphia, Pennsylvania, USA; The Wistar Institute, Center for Systems and Computational Biology, Philadelphia, Pennsylvania, USA; UPENN-Presbyterian Medical Center, Philadelphia, Pennsylvania, USA; Philadelphia FIGHT, The Jonathan Lax Treatment Center, Philadelphia, Pennsylvania, USA

Volume 89, no. 1, p. 799–810, 2015. Page 800, Table 1, row 2 from bottom, column 3: For the median age, “44” should read “43.”

Page 801, Table 2, column 2, lines 6, 7, and 8: “Antiapoptotic” should read “Proapoptotic” for the Bax, Bak1, and Bik genes.

Page 804, Fig. 3B, left panel: “n/H11005 11” and “n/H11005 17” should read “n/H11005 9” and “n/H11005 16,” respectively.

Page 807, column 1, line 26: “(15)” should read “(16).”

Page 807, column 1, line 27: “(16, 66, 68)” should read “(16, 66, 70).”

Page 807, column 1, line 28: “(16, 70)” should read “(15, 16, 68, 70).”

Page 807, Acknowledgments, paragraph 3, line 1: “Jeffery” should read “Jeffrey.”

Page 809: Reference 68 should read as follows.