Shared monocyte subset phenotypes in HIV-1 infection and in uninfected subjects with acute coronary syndromes

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Running title: Monocyte phenotypes in HIV disease or ACS

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Abstract

The mechanisms responsible for increased cardiovascular risk associated with HIV-1 infection are incompletely defined. Using flow cytometry, we examined activation phenotypes of monocyte subpopulations in patients with HIV-1 infection or acute coronary syndromes (ACS) to find common cellular profiles. Non-classical (CD14+CD16++) and Intermediate (CD14++CD16+) monocytes are proportionally increased and express high levels of tissue factor (TF) and CD62P in HIV-1 infection. These proportions are related to viremia, to T cell activation, and to plasma levels of IL-6. In vitro exposure of whole blood samples from uninfected control donors to LPS increased surface TF expression on all monocyte subsets, but, exposure to HIV-1 resulted in activation only of non-classical monocytes. Remarkably, the profile of monocyte activation in uncontrolled HIV-1 disease mirrors that of acute coronary syndromes (ACS) in uninfected persons. Thus, drivers of immune activation and inflammation in HIV-1 disease may alter monocyte subpopulations and activation phenotype, contributing to a pro-atherothrombotic state that may drive cardiovascular risk in HIV-1 infection.
Introduction

Immune activation and inflammation are recognized predictors of HIV-1 disease progression and mortality\(^1\text{-}^3\). Human immunodeficiency virus (HIV-1) infection is also associated with an increased risk of venous and arterial thrombosis\(^4\text{-}^8\). In a recent clinical trial, plasma levels of the pro-inflammatory cytokine interleukin-6 (IL-6), C-reactive protein, D-dimers, and soluble CD14 (sCD14), were independent predictors of mortalities, that included deaths related to cardiovascular events \(^2\text{-}^3\text{,}^9\). Patients randomized to receive intermittent antiretroviral therapy were at greater risk particularly for cardiovascular events, suggesting that dynamic changes in levels of inflammation associated with viral replication increase the risk of atherothrombosis in HIV-1 infected patients.

Inflammation is an important contributor to atherosclerosis and coronary artery disease (CAD)\(^10\text{-}^{11}\). Previous work suggests that systemic immune activation may be particularly relevant in patients who transition from stable coronary artery disease to unstable acute coronary syndromes (ACS)\(^12\text{-}^{17}\). While chronic immune activation and inflammation are associated with progressive HIV-1 disease\(^1\), the drivers of inflammation and morbidity, and their mechanisms in HIV-1 disease, are incompletely defined.

Blood monocytes and tissue macrophages have been implicated in the initiation, progression, and thrombotic complications of atherosclerosis, as they can mediate inflammation, are found within atherosclerotic plaques, and can drive coagulation
through mechanisms that include the expression of tissue factor (TF)\textsuperscript{10,11,18,19}.

Monocytes are not a uniform cell population and can be segregated by CD14 and CD16 expression levels into three distinct subsets. Intermediate monocytes express high levels of CD14 and CD16 (CD14\textsuperscript{++}CD16\textsuperscript{+}) and are functionally distinguishable from classical CD14\textsuperscript{+}CD16\textsuperscript{−} monocytes based on homing marker expression, antigen presentation capabilities, and by the levels of proinflammatory cytokines produced following stimulation with bacterial Toll-like receptor (TLR) ligands\textsuperscript{20-23}. In contrast, non-classical (CD14\textsuperscript{+}CD16\textsuperscript{++}) monocytes can recognize viral products and can home to the vascular endothelium via expression of CX3CR1\textsuperscript{22,24}.

Increased proportions of CD16 expressing monocytes have been reported in patients with inflammatory conditions, such as sepsis\textsuperscript{25} and rheumatoid arthritis\textsuperscript{26,27} Here, we report that the proportions of CD14\textsuperscript{+}CD16\textsuperscript{++} and CD14\textsuperscript{++}CD16\textsuperscript{+} monocytes are significantly increased in HIV-1 infection, as are proportions of each monocyte subset expressing the procoagulant tissue factor (TF). In HIV-1 infected patients, the proportions of both intermediate and non-classical monocytes are related to the magnitude of viremia, to markers of T cell activation (CD38 and HLA-DR), and to plasma IL-6 levels. We also demonstrate that non-classical, but not intermediate or classical monocytes, can be activated by HIV-1 to express TF \textit{in vitro}. Plasma levels of D-dimer products of fibrinolysis and the bacterial lipopolysaccharide (LPS) receptor sCD14 were directly related to the proportion of intermediate (CD14\textsuperscript{++}CD16\textsuperscript{+}) monocytes in HIV-1 infection and, in patients with controlled viremia, plasma LPS levels
correlated directly with the proportion of intermediate monocytes. Finally, in HIV-1 uninfected patients with acute coronary syndromes (ACS), the proportions of intermediate and non-classical monocytes, and the levels of TF on these cells, are increased in patterns similar to those seen in HIV-1+ patients with uncontrolled viremia. Monocyte activation, with resultant procoagulant expression, is linked to cardiovascular disease in HIV-1 uninfected persons and to predictors of morbidity and mortality in HIV-1 infected persons. These activated monocytes may play an important role in cardiovascular disease risk in both HIV-1 infected and uninfected persons.

**Materials and Methods**

**Patients**

These studies were performed in compliance with policies of the Institutional Review Board at Case Western Reserve University/University Hospitals- Case Medical Center. Blood samples were obtained following informed consent in accordance with the Declaration of Helsinki. HIV-1 negative subjects (N=23) were healthy donors from the general CWRU/UH population. HIV-1 infected donors (N=57) were recruited from the Special Immunology Unit of University Hospitals/Case Medical Center and were divided into two populations based upon viremia (VL<400 copies/mL and VL>400 copies/mL). ACS patients (N=10) had unstable symptoms consistent with cardiac ischemia and either elevated serum levels of cardiac troponins or dynamic electrocardiographic changes. They were recruited from the in-patient cardiology service and blood was drawn prior to cardiac catheterization. Stable CAD controls (N=16) had two or more conventional cardiac risk factors, but did not have clinically apparent CAD. The majority
(13/16) had evidence of coronary artery calcification by computed tomography suggesting subclinical CAD. Age and cholesterol levels between ACS and stable CAD patients did not significantly differ. Framingham risk scores could be computed for 25 HIV+ patients with controlled viremia, 7 HIV+ patients with uncontrolled viremia, all ACS and 10/16 stable CAD patients using gender, age, total cholesterol, high density lipoprotein (HDL) level, diabetes, and smoking status as determined by chart review. To avoid the use of arbitrary blood pressure measurements, patients with hypertension were considered to have a blood pressure between 140-149/90-99 mmHg and patients without hypertension were considered to have a blood pressure between 120-129/80-84 mmHg. This Framingham-based risk score was calculated using the online calculator at www.mdcalc.com and is expressed as the risk of incident coronary heart disease within a 10 year period. Framingham-based risk scores were not different for any of the 4 groups on which we were able to calculate them (P>0.43 for each). Demographics for all patients in this study are listed in Table 1.

**Cell preparation and incubations**

Blood was drawn into EDTA coated tubes. Whole blood (250µl) was incubated in 15 ml polypropylene Falcon tubes (Becton Dickinson Labware, Franklin Lakes, NJ) for 3 hours with individual Toll-like receptor ligands. Lipopolysaccharide (LPS, from *E.Coli*, 50ng/ml), imiquimod (5 µg/ml), and single stranded Poly U complexed with the cationic lipid LyovecR (ssPolyU 10 µg/ml) were purchased from Invivogen (San Diego CA).

Aldrithiol-2 (AT-2) treated HIV-1 was kindly provided by Jeffrey Lifson (NCI, Frederick...
HIV-1 (MN) CL.4/SUPT1 lot P4095 is a CXCR4 tropic HIV-1 strain and HIV-1 ADA-M/SUPT1-CCR5 CL.30 lot P4101 is a CCR5 tropic virus. Each virus was tested at 3 capsid concentrations: 50ng/mL, 150ng/mL, and 500ng/ml, and the 150ng/mL concentration was found to be optimal for TF induction. Empty vesicles, at a total protein concentration that was equal to the total protein in the viral preps, were used as a control.

**Flow Cytometry**

Monocytes and T cells were identified by size, granularity, and by expression of CD14 and CD16, or CD3 and CD8 or CD4, respectively. CD14 expression was based on an isotype and population gating strategy; with both the lymphocyte population and the isotype serving as the lower limit for determining which cells were CD14+ versus CD14-. The upper limit of CD14+ and lower limit for CD14++ expression is based on the CD14++ population. Expression of CD16 was based on a conservative isotype gating strategy and was confirmed by fluorescence intensity of the CD16- lymphocyte population. Fluorescence minus 1 and isotype gating strategies were used to identify expression of activation markers.

Cell surface molecule expression was monitored by staining cells with the following fluorochrome-labeled antibodies: anti-Tissue Factor fluorescein isothiocyanate (FITC) (American Diagnostica, Stamford, CT), anti-CD14 Pacific Blue, anti-CD16 phycoerythrin (PE), anti-CD62P-Selectin PE-cy5 (BD Pharmingen, San Diego, CA), anti-CX3CR1 allophycocyanin (APC) (BioLegend, San Diego, CA) anti-CD69 PE-cy7, and anti-HLA-
DR APC-cy7 (BD Biosciences, San Jose, CA). Positive expression of these surface proteins was determined based on isotype control staining. In order to assure that monocyte populations were not contaminated by lymphocytes, preliminary experiments using an exclusion-gate that included anti-CD3 FITC, anti-CD20 FITC, anti-CD56 FITC (BD Pharmingen), were performed.

T cell activation was measured using anti-CD38 PE, anti-HLA-DR FITC, anti-CD3 APC (BD Biosciences) anti-CD8 allophycocyanin-cy7 (APC-cy7), anti-CD4 Pacific Blue (BD Pharmingen) and appropriate isotype control monoclonal antibodies.

Whole blood samples were incubated for 15 minutes on ice with FACS Lyse buffer (BD Biosciences) and then washed in wash buffer (phosphate buffered saline with 1% bovine serum albumin and 0.1% sodium azide). Cells were then stained for 30 minutes in the dark on ice and then washed in wash buffer, fixed in 1% formaldehyde, and analyzed using a Miltenyi MACS Quant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). MACS Quant software (version 2.21031.1, Miltenyi Biotec), and Prism 5.0 Graphpad software (La Jolla, CA) were used to organize and analyze the data.

**Measurement of D-dimers, IL-6, sCD14, and CRP**

Fresh whole blood samples were collected in EDTA containing tubes and were centrifuged for 15 minutes at 495x g. Plasma was removed and frozen at -80°C until thawed once and analyzed in batch. Levels of D-dimers were measured using the
Asserachrom D-DI immunoassay (Diagnostica Stago, Asnieres France). Soluble CD14, C-reactive protein, and IL-6 levels were measured using Quantikine ELISA kits (R&D Systems Minneapolis MN).

Measurement of LPS
Plasma samples were diluted to 10% or 20% with endotoxin free water and then heated to 85°C for 15 minutes to denature plasma proteins. We then quantified plasma levels of LPS with a commercially available Limulus Amebocyte Lysate (LAL) assay (QCL-1000, Lonza, Walkersville, MD) according to the manufacturer’s protocol. Samples were run in triplicate; backgrounds were subtracted, and mean values were reported.

Statistical Methods
We used conventional measures of central location and dispersion to describe the data. We performed pairwise comparisons using Mann-Whitney’s U test, and evaluated correlations between pairs of continuous variables using Spearman’s rank correlation. Given considerable differences in conventional risk factors between each of the HIV-infected groups and the healthy control group, we fitted multiple linear regression models that included age, race (coded as Caucasian/non-Caucasian), sex, and current smoking status as covariates in order to assess the independent contribution of subject group to the differences observed in the distribution of total and tissue factor-positive monocytes. Analyses were done using SPSS, v. 20.0 (IBM Corp., Armonk, NY) and Stata MP, v. 11.2 (StataCorp, College Station, TX). All comparisons are two-sided.
without formal correction for multiple comparisons, and p values <0.05 were considered statistically significant.

Results

We have demonstrated previously that monocyte expression of TF is increased in HIV-1 infection \(^{29}\). The current studies were designed to explore further the role of monocyte activation and TF expression as potential mediators of increased cardiovascular disease (CVD) risk. Since three monocyte subsets have been delineated, distinguishable by their responsiveness to bacterial and viral products\(^{20-23}\), we assessed the proportional representation of these subsets and their relative state of activation in HIV-1 disease. Initial studies were performed on fresh whole-blood samples obtained from 57 HIV-1 infected patients and 23 controls not known to be HIV-1 infected. We divided the HIV-1-infected population into two groups; one with controlled viremia (plasma HIV-1 RNA<400copies/mL, N=39) and one with uncontrolled viremia (plasma HIV-1 RNA >400c/mL, N=18). The median CD4+ T cell count was 578 cells/uL for the patients with controlled viremia and 398/uL for the uncontrolled group. All patients with controlled viremia and 44% of the uncontrolled viremia patient group were receiving antiretroviral therapy. A more complete description of these groups is included in Table 1.

Monocytes and their subsets were identified by flow cytometry based on forward and side scatter characteristics and by expression of CD14 and CD16\(^{22}\). All monocyte
subsets expressed high levels of HLA-DR. Based on previous reports\textsuperscript{20-23} and preliminary experiments that contained a lymphocyte exclusion gate (CD3, CD20, and CD56), the populations were assured to be monocytes. The gating strategy used to identify classical (CD14\textsuperscript{++}CD16\textsuperscript{−}), intermediate (CD14\textsuperscript{++}CD16\textsuperscript{+}), and non-classical monocytes (CD14\textsuperscript{−}CD16\textsuperscript{++}), is shown in Figure 1A. Representative dotplots from 1 control and 3 HIV-1+ patients (Figure 1A) and summary figures (1B) are shown. The proportions of non-classical monocytes (CD14\textsuperscript{−}CD16\textsuperscript{++}) were significantly increased in HIV-1+ patients with (uncontrolled viremia (median and interquartile range (IQR) =17.1\%, 13.0\%-27.5\%, P < 0.001) but not in patients with controlled viremia (9.3\%, 6.0\%-15.2\%, P = 0.05) when compared to these proportions in healthy controls (8.1\%, 5.5\%-9.9\%). After controlling for age, sex, race, and smoking, the difference between aviremic patients and controls was also not significant (adjusted P=0.07).

The proportion of intermediate (CD14\textsuperscript{++}CD16\textsuperscript{+}) monocytes was increased in the viremic group (38.8\%, 29.6\%-59.8\%, P<0.0001), but not in the controlled viremia group (20.7\%, 15.6\%-28.2\%) when compared to healthy controls (20.2\%, 13.6\%-24.1\%). When adjusted for age, race, sex and smoking, both the viremics (P = 0.001) and the controlled viremia group (P = 0.014) had increased proportions of intermediate monocytes.

CVD risk in HIV-1 infection is inversely related to CD4+ T cell count\textsuperscript{30} and is increased in patients who experience uncontrolled viral replication during intermittent antiretroviral therapy\textsuperscript{2}, potentially linking the pathogenesis of CVD to immune deficiency and viral
replication. We therefore compared monocyte subset proportions to CD4+ T cell counts, plasma HIV-1 RNA levels and expression of the activation markers CD38 and HLA-DR on CD8+ T lymphocytes that has been linked to disease course 1. In all HIV-1 infected patients, the proportion of intermediate (CD14++CD16+) monocytes correlated directly with viremia (r=0.59, P<0.0001) and the proportion of activated CD8+ T cells (r=0.67, P<0.0001) and inversely with CD4+ T cell count (r= -0.424, P= 0.001). Also in all patients, the proportion of non-classical CD14+CD16++ monocytes correlated directly with viremia (r=0.372, P=0.004) and the proportion of activated CD8+ T cells (r= 0.317, P=0.023) but not with CD4+ T cell counts (r=-0.218, P=0.104). As viremia, CD4+ count, and T cell activation are often correlated, we asked if any of these indices were related to the proportions of monocyte subsets independently. The proportion of CD14++CD16+ intermediate monocytes in these patients was independently associated with the magnitude of viremia and the proportions of activated CD8+ T cells (P=0.013, and P=0.004, respectively). Also, the proportion of non-classical CD14+CD16++ monocytes was independently associated with the proportion of activated CD8+ T cells (P=0.027).

Monocytes can home to the vascular endothelium utilizing a number of surface receptors, including the fractalkine receptor (CX3CR1) 20,22,24. We confirmed that CX3CR1 is most often and most highly expressed on the CD14+CD16++ monocyte subset, with comparatively less expression on the CD14++CD16+ subset and lower levels on the CD14++CD16− subset (Figure 2A). Viremic patients had proportionally more CD14+CD16− cells that expressed CX3CR1 than did uninfected controls (41.0%, 25.8- 60.3% versus 27.3%, 18.4-35.4%, P= 0.034). Since monocyte subsets
differentially home to the vascular endothelium where they may contribute to thrombus formation, we next asked if any of these subsets were enriched for surface expression of the procoagulant tissue factor (TF) or the adhesion molecule P-selectin (CD62P). When compared to findings among healthy controls, the proportions of intermediate and non-classical monocytes that express TF are increased in both the controlled (P < 0.001 for both) and uncontrolled (P <0.02 and P<0.003) viremia patient populations (Figure 2B), although the difference in TF+ intermediate monocytes between viremic HIV-positive patients and controls became non-significant after controlling for age, sex, race, and smoking history (adjusted P= 0.096). In both patient populations, the non-classical monocytes had the greatest proportion of TF positive cells.

The proportion of cells that expressed CD62P was significantly higher in the non-classical monocyte populations from patients with controlled (median=25.7%, 13.4-36.7%) and uncontrolled viremia (30.0%, 24.9-38.8%) when compared to this proportion in controls (11.7%, 5.8-19.5, P=0.025 and 0.001, respectively, Figure 2C). Monocytes that expressed TF also frequently expressed CD62P (Figure 2D); and not surprisingly, the proportions of non-classical monocytes that were both TF+ and CD62P+ were significantly increased in patients with controlled (15.3%, 7.8-20.6%) and uncontrolled (15.0%, 11.3-37.2%) viremia when compared to the proportion in controls (7.6%, 4.4-11.4%, P=0.02 and 0.002, respectively). The increased proportional representation and activated, procoagulant phenotype of non-classical monocytes in HIV-1 disease, in patients with controlled and uncontrolled viremia, may provide insights into the increased risk for thrombosis in these patients.
Soluble CD62P (sCD62P) is shed from activated platelets and endothelial cells\textsuperscript{31}, can induce monocyte TF expression in vitro\textsuperscript{32}, and is a strong independent predictor of venous thrombosis in HIV-1 infected patients\textsuperscript{33}. Plasma levels of sCD62P were higher in the controlled viremia (median, IQR= 75.9ng/ml, 61.2-88.9ng/mL, P<0.0001) and the uncontrolled viremia (59.2ng/ml, 53.1-87.5 ng/mL, P=0.01) patients than among controls (48.4ng/ml, 41.5-58.3ng/mL, Figure 2E). Among the HIV-1+ patients, there was a strong correlation between the plasma levels of sCD62P and the proportions of intermediate (\(r=0.515\) P=0.007) and non-classical (\(r=0.534,\) P=0.005) monocytes that expressed CD62P.

Increased plasma levels of the inflammatory cytokine IL-6, the acute phase protein C-reactive protein (CRP), and D-dimer products of fibrinolysis are known to predict CVD risk in HIV-1 negative populations\textsuperscript{34} and were also predictive of all-cause and cardiovascular related mortality in HIV-1 infected patients in the SMART study\textsuperscript{2}. As expected, plasma levels of IL-6 (Sup Figure 1A) and D-dimers (Sup Figure 1B) were significantly higher in both HIV-1 infected patient populations than among healthy controls. CRP levels were increased only in the uncontrolled viremia population and not in the controlled viremia patients, when compared to levels in controls (Sup Figure 1C). The proportion of intermediate monocytes in HIV-1 infected patients was correlated with plasma levels of D-dimers (\(r= 0.369,\) P=0.005) and CRP (\(r=0.283,\) P=0.033). Plasma IL-6 levels correlated with both the proportion of CD14\textsuperscript{++}CD16\textsuperscript{+} monocytes (\(r=0.294\) P=0.026) and the proportion of CD14\textsuperscript{+}CD16\textsuperscript{++} monocytes (\(r= 0.268,\) P=0.043) in HIV-1
infected patients.

We also measured levels of sCD14, an LPS coreceptor shed from monocytes upon activation, as these levels also predict mortality in HIV-1 infection. Levels of sCD14 were significantly higher in both patient populations than levels in controls (P<0.0001 for both, Sup Figure 1D). Plasma levels of D-dimers and sCD14 were directly correlated in the HIV-1 infected population (r=0.304, P=0.021), again suggesting a link between monocyte activation and coagulation in HIV-1 infection.

Microbial translocation has been linked to inflammation and immune activation in HIV-1 disease. Since classical and intermediate monocyte subsets produce high levels of IL-8, IL-6, and IL-10, in response to LPS exposure, we assessed the relationship between plasma LPS levels and monocyte activation. Plasma levels of LPS were significantly increased in both the controlled viremia (median 81.6pg/mL, 61.8-96.1pg/mL) and uncontrolled viremia patients (87.2pg/mL, 65.1-117.4pg/mL) compared to levels of LPS in the plasma of controls (38.1pg/mL, 20.3-62.4pg/mL, P<0.0001 for both comparisons, Figure 3A). We found a strong correlation between plasma LPS levels and CD8+ T cell activation (r= 0.393, P=0.004) in the HIV-1-infected population, consistent with a previous report. Levels of plasma LPS were not significantly correlated with proportions of CD14++CD16+ monocytes in the entire HIV-1+ patient population (r=0.216, P=0.106, Figure 3B). In patients with controlled viremia, however, there was a significant correlation between plasma LPS levels and the proportion of intermediate monocytes (r=0.336, P=0.037, Figure 3C), but this correlation was not
seen in patients with uncontrolled viremia \( (r=0.005, \ P=0.984, \ \text{Figure 3D}) \), suggesting that in patients with controlled viremia, systemic translocation of microbial products may drive intermediate monocyte expansion, while in subjects with sustained HIV-1 replication, other factors such as HIV-1 itself contribute more to this phenotype.

Monocyte subsets are differentially responsive to TLR stimulation with intermediate monocytes especially activated by bacterial TLR ligands (e.g., LPS) and non-classical monocytes by viral products through TLR 7 and 8 ligation\(^{22}\). Because both viral replication and systemic translocation of bacterial elements are characteristic of chronic HIV-1 infection, we assessed the relative responsiveness of these subsets to HIV-1 and other microbial elements \textit{in vitro}. Whole blood samples from healthy, uninfected donors were incubated for three hours alone, or in the presence of individual TLR ligands (LPS: TLR4, imiquimod:TLR7, or single stranded PolyU:TLR8) or aldrithio-2 (AT-2) inactivated HIV-1 (MN4095 or P4101). Exposure to LPS resulted in significant increases in TF expression on all monocyte subsets (Figure 4), while only CD14\(^+\)CD16\(^++\) monocytes increased TF expression after incubation with imiquimod or single stranded PolyU (Figure 4). Likewise, exposure of whole blood samples to either the CXCR4 using HIV-1 MN4095 or the CCR5 tropic HIV-1 P4101 induced TF only on CD14\(^+\)CD16\(^++\) monocytes (Figure 4).

Persons with atherosclerosis, are at risk for the acute coronary syndrome (ACS), in which active atherothrombosis occurs at a site of atherosclerotic plaque rupture or superficial endothelial cell erosion. Activation of monocytes/macrophages is thought to
be central to the pathogenesis of atherosclerosis and appears to play an important role in the progression to ACS. 17,39-41

Therefore, to provide a context in which the degree of monocyte activation in HIV-1+ patients could be judged, we next examined monocyte subsets and their activation phenotype in patients presenting to the cardiac catheterization laboratory with ACS. None of these subjects was known to be HIV-1 infected. We also studied a second control cohort comprised of subjects with stable coronary artery disease CAD (none known to be HIV-1 infected) with two or more known cardiac risk factors. The majority of these patients had evidence of subclinical CAD (coronary calcification by cardiac CT scan), but none had a clinical history of CAD or symptoms of ACS. Although individual cardiac risk factors varied, ACS and stable CAD patients were similar in age and background cardiovascular risk as computed by Framingham risk score assessment (Table 1).

The proportions of both intermediate and non-classical monocytes were increased in the ACS patient group (N= 10, median=37.4%, 28.1-39.7%, and 14.0%, 10.8-18.4%, respectively) compared to the proportions in the Stable CAD controls (N=16, median=19.1%, 17.5-23.3%, and 8.1%, 6.0-9.8%, P<0.001 and 0.002 respectively), and proportions were similar to those in HIV-1-infected subjects with uncontrolled viremia (P=0.23 and P=0.29, respectively, Figure 5A). The proportions of intermediate and non-classical monocytes that expressed TF were also increased in the ACS subjects (median =20.0%, 19.1-29.6% and 38.8%, 25.2-45.0%, respectively) compared
to the proportions of TF+ cells in the Stable CAD controls (median= 5.1%, 3.0-9.2%, and 5.7%, 4.6-10.2%, respectively, P<0.0001 for both, Figure 5B). The proportions of TF expressing cells in the intermediate (P=0.001) and non-classical (P=0.002) monocyte subsets in the ACS patients were higher than among the patients with both controlled (P= 0.003, and 0.0006) and uncontrolled viremia (P=0.001 and P = 0.002). Importantly, markers of T cell activation (CD38 and HLA-DR) were low on CD8+ lymphocytes in the ACS patients (7.9%, 5.3-11.7%), clearly distinguishing these subjects from viremic HIV-1 infected patients (39.9%, 29.7-48.9% P<0.0001) and suggesting that despite a similar monocyte phenotype, the underlying determinants of T cell activation status are different in these two groups.

Discussion

As HIV-1 infected patients are now living longer, CVD is becoming more prevalent in this population. Inflammation is a recognized contributor to atherosclerosis and as immune activation and inflammation are increased in HIV-1 disease, it is plausible that these processes may accelerate the progression of cardiovascular disease in HIV-1 infected patients.

Immune activation in HIV-1 disease is likely driven by multiple mechanisms, including HIV-1 itself, increased replication of other viral copathogens such as cytomegalovirus 42-44 in the setting of HIV-1-related immune deficiency, and systemic translocation of microbial products across a damaged gut mucosal barrier. We and others have found increased levels of LPS and bacterial DNA in the plasma of HIV-1-infected...
persons\textsuperscript{37,38,45}. These microbial products can activate innate immune receptors, such as Toll-like receptors (TLRs), resulting in production of inflammatory cytokines and cellular activation \textsuperscript{46}. We have correlated high levels of LPS and bacterial DNA directly with indices of immune activation and inversely with CD4+ T cell restoration after administration of combination antiretroviral therapy\textsuperscript{37,38}, linking microbial translocation to both immune activation and impaired immune homeostasis in HIV-1 infection.

Monocytes and tissue macrophages, which express multiple TLRs, have been implicated in cardiovascular disease as they can mediate inflammation, plaque destabilization, and coagulation\textsuperscript{10,11,18,19}. Recently, in a substudy of SMART, the monocyte activation marker sCD14 was an independent predictor of mortality in HIV-1 infection \textsuperscript{3}. We have earlier demonstrated that monocytes of HIV-1 infected patients express high levels of the procoagulant tissue factor (TF), and that TF expression is related to viremia and to plasma levels of both sCD14 and D-dimer products of fibrinolysis\textsuperscript{29}. Tissue factor can initiate the extrinsic clotting pathway\textsuperscript{47-49}, potentially linking monocyte activation to coagulation and thrombosis in HIV-1 disease.

Three monocyte subsets, that are functionally and phenotypically distinguishable, have been identified\textsuperscript{22}. Here, we report striking alterations in the proportions of these monocyte subsets in HIV-1 infection, expanding on previous reports\textsuperscript{50,51}, and demonstrating, that proportions of non-classical (CD14\textsuperscript{+}CD16\textsuperscript{++}) monocytes correlate directly with viremia and markers of T cell activation. We also show for the first time that both intermediate and non-classical monocytes from HIV-1 infected patients are enriched for cells that express TF, readily distinguishable from findings among healthy
controls. Proportions of non-classical monocytes that express TF are increased in patients with controlled or uncontrolled viremia, suggesting that even during successful antiretroviral therapy, these vascular homing monocytes may increase risk for coagulation in HIV-1 disease. We also report for the first time that expression of CD62P is increased on CD14⁺CD16⁺⁺ monocytes from HIV-1 infected donors. This may play a role in transition of these monocytes into foam cells, as has previously been reported for the monocytic cell line U937 upon exposure to ox-LDL.52

Intermediate monocytes produce high levels of IL-6 and IL-8 in response to TLR4 stimulation.22 We find that in HIV-1 infection, the proportions of intermediate monocytes are related to plasma levels of LPS, suggesting that microbial translocation may, at least partially, drive expansion of these cells. Proportions of these CD14⁺⁺CD16⁺ monocytes were directly related to plasma levels of D-dimers in our HIV-1 infected population, suggesting a link between intermediate monocytes and intravascular coagulation. Plasma levels of sCD14 were also directly correlated with D-dimer levels; also linking monocyte activation and coagulation.

Non-classical monocytes tended to have the highest levels of TF expression among all monocyte subsets, a novel and potentially clinically important observation since these monocytes also readily home and become tethered to the vascular endothelium via CX3CR1. Thus, we speculate this subpopulation may be particularly prothrombotic in vivo.22 We also show, for the first time, that CD14⁺CD16⁺⁺ monocytes, but not other monocyte subsets, can be directly activated by HIV-1 to express surface TF. We
suspect that HIV-1 mediated induction of TF is not HIV-1 coreceptor mediated, but likely though innate immune receptor recognition, as all monocyte subsets express CXCR4 and CCR5 \(^{24}\), but only non-classical monocytes increased TF expression following HIV-1 exposure. Experiments with small molecule inhibitors of CCR5 (maraviroc) or CXCR4 (AMD3100) indicate that interaction between R5 or X4 using HIV-1 and coreceptors is necessary for induction of TF on non-classical monocytes (not shown). This same monocyte subpopulation is also responsive to imiquimod and sspolyU (Figure 4) - ligands for TLR7,8 that also can be activated by genomic sequences of HIV-1 \(^{53}\). Alternatively and in our view, less likely, non-classical monocytes might be uniquely responsive to a soluble mediator induced by HIV-1 exposure. Additional studies are needed to identify the precise mechanisms of TF upregulation by HIV-1.

Our data therefore suggest that HIV-1 infection results in a potentially prothrombotic state of monocyte activation. Activation of non-classical monocytes by HIV-1 may directly result in an increased risk of clot formation. Also, in both controlled and uncontrolled HIV-1 infection, systemic translocation of microbial products such as lipopolysaccharide may activate and increase expression of the procoagulant tissue factor on all circulating monocyte subsets perhaps contributing to the increased cardiovascular risks seen even in HIV-1 infection controlled with antiretroviral therapy \(^{2,54}\).

The pattern of altered monocyte homeostasis in HIV-1 disease may be mechanistically relevant to increased cardiovascular risk. In contrast to uninfected patients with known
cardiovascular risk factors, patients with ACS display a profile of TF expression and increased proportions of intermediate and non-classical monocytes similar to that of HIV-1 infected patients with uncontrolled viremia. The upstream drivers of inflammation and monocyte activation in HIV-1 disease and ACS may be distinguishable, but the resulting alterations in monocyte subpopulations and tissue factor expression are very similar. The cross-sectional nature of this study prevents us from making strong mechanistic conclusions about the involvement of monocyte subsets in ACS; but, future longitudinal studies measuring monocyte subset activation and inflammatory biomarkers in patients after MI and percutaneous coronary intervention (PCI), or following therapeutic intervention that reduces immune activation, viral replication, or microbial translocation may provide insights into the drivers of monocyte activation in ACS and in HIV-1 disease.

A limitation of this study is related to the heterogeneous nature of our patient and control populations. Thus, we cannot completely exclude a role for differences in other CVD risk factors on monocyte phenotypes. We tried to account for differences in age, sex, race, and smoking status by using multiple linear regression models, and after controlling for these variables, HIV-1 infection appears associated with significant abnormalities of monocyte activation and coagulation phenotype.

These data have important implications for understanding the mechanisms that lead to ACS. Despite advances in our understanding of atherosclerosis, the mechanisms that lead to conversion from stable CAD to ACS remain elusive. This study is the first to directly compare the inflammatory state associated with ACS to that of HIV-1 disease.
Despite differences in the pathogenesis of HIV-1 and ACS, these two diseases lead to a common profile of prothrombotic monocyte activation. Thus, further studies are required to distinguish the upstream initiators of monocyte activation in these two settings.
Acknowledgements

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Author Contributions and Competing Interests

N.T.F. A.L. C.S. J.M. performed experiments. D.Z. M.C. D.S. B.R. and M.L. obtained patient samples. B.R. provided statistical support. All authors contributed to experimental design, data analysis, and writing of the manuscript.

The authors have no competing financial interests.

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References


Table 1. Demographic information on HIV-1 infected patients, healthy uninfected controls and on HIV-1 uninfected patients with ACS and Stable CAD.

<table>
<thead>
<tr>
<th></th>
<th>HIV-1 uninfected controls</th>
<th>HIV-1 infected patients (VL&lt;400c/mL)</th>
<th>HIV-1 infected patients (VL&gt;400c/mL)</th>
<th>Stable Coronary Artery Disease (CAD) Controls</th>
<th>Acute Coronary Syndrome (ACS) patients</th>
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<tr>
<td>Number</td>
<td>(N=23)</td>
<td>(N=39)</td>
<td>(N=18)</td>
<td>(n=16)</td>
<td>(n=10)</td>
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<tr>
<td>Age</td>
<td>Median= 40 years</td>
<td>Median= 46 years</td>
<td>Median= 43 years</td>
<td>Median= 62 years</td>
<td>Median= 67 years</td>
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<tr>
<td>Gender (% female)</td>
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<td>15%</td>
<td>39%</td>
<td>38%</td>
<td>70%</td>
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<td>Range = 25-73</td>
<td>Range = 23-55</td>
<td>Range= 37-73</td>
<td>Range= 37-84</td>
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<tr>
<td>Race (% non-caucasian)</td>
<td>30%</td>
<td>57%</td>
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<td>CD4+ T cell Count</td>
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<td>Median = 398 cells/µL</td>
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<td>Median = 48 copies/mL</td>
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<tr>
<td>On HAART (%)</td>
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<tr>
<td>Diabetes</td>
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<td>45%</td>
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<td>Current Smoker (%)</td>
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<td>61%</td>
<td>6%</td>
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N.A. = Not applicable  
N.R. = Not reported  
* > 3 days prior to analysis  
# Based on available data (10 patients)
Figure 1. Proportions of monocyte subsets are altered in HIV-1 disease.
Whole blood samples were obtained from 57 HIV-1 infected donors and 23 healthy controls and the relative proportions of monocyte subsets were analyzed by flow cytometry. A) Three monocyte subsets were identified by size and granularity and by CD14 and CD16 expression and representative dotplots showing monocyte subsets from 1 healthy control and 3 HIV-1 infected patients are displayed (patients i, ii, and iii). All three patients were male and were receiving antiretroviral therapy. Patients i and ii had VL < 400c/mL and CD4+ T cell counts of 857 and 456 cells/µl, respectively. Patient iii was viremic (333,706 c/mL) and had a CD4+ T cell count of 132 cells/µl. B) Summary data of monocyte subsets among healthy controls and HIV-1 infected patients with controlled (<400 copies/mL) or uncontrolled (>400copies/mL) viremia.

Figure 2. Non-classical and intermediate monocytes from HIV-1 infected patients are enriched for cells that express tissue factor and P-selectin. Monocyte subsets were identified in whole blood samples from HIV-1 infected patients and uninfected controls. Surface expression of CX3CR1, tissue factor, and CD62P-selectin was measured on monocyte subsets by flow cytometry. B) Summary data of tissue factor expression. C) Summary data of CD62P expression. D) Representative dot plots of TF and CD62P expression. E) Plasma samples from all donors were thawed and levels of soluble CD62P were measured in batch by ELISA.

Figure 3. Plasma levels of LPS are increased in HIV-1 infected patients and correlate with the proportion of circulating intermediate monocytes. Plasma
samples from all donors were thawed and levels of LPS were measured using the limulus lysate assay.  A) Plasma levels of LPS are significantly increased in HIV-1 infected patients with controlled (<400 copies/mL) and uncontrolled (>400 copies/mL) viremia compared to levels in uninfected controls.  B) There was a modest and not significant correlation between plasma LPS levels and the proportion of CD14++CD16+ monocytes in the entire HIV-1 infected population.  C) There is a direct and significant correlation between plasma levels of LPS and the proportion of CD14++CD16+ intermediate monocytes in patients with controlled viremia, but this correlation was not seen in patients with uncontrolled viremia (D).

Figure 4. Exposure of whole blood samples to HIV-1 results in increased surface expression of TF on non-classical (CD14+CD16++) monocytes, but not on the intermediate (CD14++CD16+ CD14+CD16+) or CD14++CD16- monocytes. Whole blood was obtained from HIV-1 uninfected subjects and was exposed to lipopolysaccharide (LPS, 50 ng/mL), imiquimod (5 µg/mL), ssPolyU (10 µg/mL), or AT-2 inactivated HIV-1 (X4 or R5 tropic, 150 ng/mL) for 3 hours. Surface expression of tissue factor (TF) was measured on monocyte subsets by flow cytometry. Exposure to LPS resulted in a significant increase in TF on all monocyte subsets, but exposure to imiquimod, ssPolyU, or HIV-1, resulted in increased TF expression on only the CD14+CD16++ subset.
Figure 5. HIV-1 Uninfected Patients with acute coronary syndromes (ACS) also have altered monocyte subset proportions and increased monocyte expression of TF. Whole blood samples were collected from 10 HIV-1-uninfected ACS patients and 16 patients without ACS, but with a similar CV risk profile (Stable CAD controls). Proportions of A) monocyte subsets and B) monocytes that express TF were analyzed by flow cytometry. Proportions of intermediate and non-classical monocytes are increased in ACS patients compared to controls; TF expression is also increased on these cells.
Shared monocyte subset phenotypes in HIV-1 infection and in uninfected subjects with acute coronary syndromes

Nicholas T. Funderburg, David A. Zidar, Carey Shive, Anthony Lioi, Joseph Mudd, Laura W. Musselwhite, Daniel I. Simon, Marco A. Costa, Benigno Rodriguez, Scott F. Sieg and Michael M. Lederman

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