THE ASSOCIATION OF PERIPHERAL BLOOD MONOCYTES TO
CARDIOMETABOLIC COMPLICATIONS IN HIV-INFECTED INDIVIDUALS
ON STABLE ANTIRETROVIRAL THERAPY

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE
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OF THE REQUIREMENTS FOR THE DEGREE OF
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IN
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Chapter 1

Introduction
NATURAL HISTORY OF HIV INFECTION

Prior to the introduction of antiretroviral therapy (ART), the typical course of human immunodeficiency virus (HIV) infection in humans involved persistent viral replication and immune activation, which led to the deterioration of the immune response and the development of acquired immune deficiency syndrome (AIDS), leaving the host susceptible to opportunistic infections that would normally be controlled by a functional immune response [1, 2]. HIV mainly targets activated CD4 T cells and achieves entry through the interaction of CD4 and chemokine co-receptors, CCR5 or CXCR4, on the host cell surface. Other cells bearing CD4 and chemokine co-receptors CCR5 or CXCR4 can also be infected by HIV including resting CD4 T cells, dendritic cells, monocytes and macrophages. A founder virus that preferentially enters target cells utilizing CCR5, rather than CXCR4, in conjunction with CD4 interaction, is observed to establish transmission into the host [3].

Soon after HIV transmission, acute viremia is detected in the plasma and the virus is widely spread throughout the body, primarily to the lymphoid tissues [1, 2, 4, 5]. With the spike in HIV viremia, there is also a marked reduction of circulating CD4 T cells that is detected in the peripheral blood. During this time, the infected individual may experience a clinical syndrome with variable severity [6-8]. The clinical syndrome associated with primary HIV infection includes fever, sore throat, skin rash, splenomegaly, lymphadenopathy, myalgia, and arthritis. Less often, meningitis may occur during primary HIV infection. These symptoms are non-specific to HIV infection and may be an important reason why HIV-infected individuals do not report symptoms of primary HIV infection to a physician.
Rapid replication of HIV during primary infection is partially contained by the host immune response, which includes innate and adaptive immune mechanisms. Induction of inflammatory cytokines and chemokines are observed as an immediate response to the rapid viral replication of HIV during primary infection. This pro-inflammatory response is in stark contrast to the initial immune responses seen for other chronic viral infections, such as hepatitis B or hepatitis C [9]. Innate cellular responses to HIV are largely mediated by natural killer cells, and are shown to be crucial for viral control. HIV-specific CD8 T cell responses are seen soon after infection, which is involved in the specific killing of HIV-infected host cells. Specific HLA types, such as in individuals who have the HLA-B57 allele, can produce an effective immune response against HIV. This response is characterized by having HIV-specific T cells with high avidity, polyclonality, and capacity to proliferate against both immunodominant and escaped HIV peptides [10, 11]. However, in the majority of individuals infected with HIV, progressive exhaustion of HIV-specific T cells occur due to HIV-associated chronic immune activation. High expression of programmed death 1 (PD-1) on both total and HIV-specific T cells have been characterized and shown to result in loss of effector function [12]. Neutralizing antibodies that are specific for HIV arise later, approximately 2-3 months after the transmission of the virus.

The onset of a robust immune response leads to a decrease in HIV viremia detected in the peripheral blood, as well as a recovery of CD4 T cell counts, though usually lower than counts observed prior to HIV infection. However, the virus is not completely eliminated and a state of chronic, persistent viral replication ensues, leading to the deterioration of the host immune function as measured by the progressive
decrease of CD4 T cell counts. As CD4 T cell counts continue to decrease overtime due to the effects of persistent HIV replication, without treatment the host ultimately expires from immune susceptibility and inability to resolve opportunistic infections or AIDS-related illnesses including atypical herpes simplex virus disease, cryptosporidiosis, cytomegalovirus disease, pneumocystis pneumonia, and HIV-associated dementia.

**HIV INFECTION AS A CHRONIC DISEASE IN THE ERA OF ART**

Since 1995, there have been drastic improvements in the care provided to individuals infected with HIV. The development and availability of different classes of antiretroviral drugs targeting different points of the HIV life-cycle provided options for clinicians to better treat HIV-infected individuals. Improvements in guidelines for HIV infection management have also shown to be beneficial, such as the implementation of the combination of different classes of antiretroviral drugs in a single regimen to reduce the development of antiretroviral drug resistance by the virus [13]. With these improvements, ART has been shown to effectively suppress HIV viremia and preserve immune function as measured by increased CD4 T cell counts. As a result, incidences of opportunistic infections are dramatically decreased, as well as overall mortality among HIV-infected individuals on ART [14]. As long as those who are on ART remain compliant to their antiretroviral regimens and have life long access to their antiretroviral drugs, risk of developing opportunistic infections or AIDS-related illnesses is not a major concern. However, HIV-infected individuals on suppressive ART don’t fully restore their health [15].
Currently observed among HIV-infected individuals on ART is the increased risk of non-AIDS comorbidities as compared to age-matched HIV-uninfected individuals. Cardiovascular disease, liver and kidney complications, metabolic abnormalities, osteoporosis, and neurocognitive diseases are observed to be common among individuals who are infected with HIV, despite being on suppressive ART regimens [16]. There are several factors that may contribute to the increased risk of non-AIDS comorbidities including treatment toxicities from HIV and non-HIV drugs and traditional risk factors such as physical inactivity and obesity [17]. Chronic inflammation could also contribute to the prevalence of non-AIDS comorbidities in HIV-infected individuals on ART. Elevated frequencies of activated CD38+ HLA-DR+ CD8 T cells and CD16+ monocytes and increased levels of pro-inflammatory cytokines have been reported in the HIV-infected individuals as compared to the general population [18-21].

**Insulin resistance in chronic HIV infection**

Insulin resistance (IR) is defined as a condition in which increased levels of insulin are required to exert the normal biologic response to transport glucose from the extracellular space into the cell to be utilized. IR is a hallmark of type 2 diabetes mellitus (T2DM), in that it precedes and predicts the onset of disease for several years. Although IR is associated with increased fasting insulin levels, a clinically relevant threshold has not been defined. Thus, IR is usually clinically suspected in the setting of elevated fasting blood glucose levels or impaired glucose tolerance from the oral glucose tolerance test (OGTT). In research, there are three well described indices of IR that utilize blood glucose and insulin values in either a fasting state.
assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI)] or from a OGTT (Matsuda index) [22-24].

HIV-infected individuals on stable suppressive ART have increased rates of abnormal glucose metabolism. A 35% prevalence of impaired glucose tolerance has been reported in HIV-infected individuals on ART, as compared to 5% in matched uninfected controls [25]. The Multicenter AIDS Cohort Study (MACS) showed a 14% incidence of T2DM in HIV-infected individuals, which was shown to be 4x higher than rates in the general population [26]. In addition to IR preceding and predicting the onset of T2DM, IR has been shown to be associated with worsening measures of sub-clinical atherosclerosis in HIV-infected individuals on ART [27].

**Albuminuria in chronic HIV infection**

The presence of excessive albumin in urine is a result of renal dysfunction of either glomerular filtration or proximal tubular reabsorption [28]. Albuminuria is strongly linked to adverse renal and cardiovascular outcomes. In a meta-analysis of nearly 850,000 participants of the general population, it was reported that the risk of end stage renal disease, increased continuously as albumin excretion increased [29]. In addition, as albuminuria increased, so did the risk of progression of CKD and incidence of acute kidney injury (AKI). As for cardiovascular outcomes, individuals with albuminuria have more than twice the risk of severe coronary artery disease. Albuminuria was shown to be associated with increased carotid intima-media thickness, a surrogate marker of sub-clinical atherosclerosis [30]. In a meta-analysis including more than 1 million patients
from the general population, increasing albuminuria was associated with an increase in all-cause mortality [31].

In HIV-infected individuals on ART, there is a higher prevalence (4-20%) of albuminuria as compared to the general population (2%) [32]. It is observed that CKD and AKI are more common among individuals who are infected with HIV. HIV infection has been found to have a strong and independent association with albuminuria [33, 34]. Furthermore, albuminuria among HIV-infected individuals has been found to be strongly associated with adverse renal and cardiovascular events [35].

REFERENCES


Chapter 2

Thesis scope
BACKGROUND OF RESEARCH QUESTION

Since the introduction of combination ART, HIV-infected individuals who are compliant to their daily antiretroviral regimens suppress HIV viremia and see improvement in immune function as measured by CD4 T cell counts. Though the risk of developing AIDS-related complications is nearly eliminated, restoration of health in ART-compliant individuals is not fully attained. HIV-infected individuals have higher risk of developing cardiovascular, liver, kidney, bone, and neurologic diseases as compared to the general population. The etiologies of these non-AIDS comorbidities are multifactorial and include side effects of ART and increased rates of traditional risk factors such as physical inactivity and obesity. Increased inflammation driven by chronic HIV infection may also play an important role in the development of these comorbidities. While studies have traditionally focused on CD8 T cells in HIV-induced immune activation, monocytes have been implicated to have a role in HIV-associated inflammation and the development of non-infectious complications such as atherosclerosis and cognitive impairment. However, the link between monocytes and the development of metabolic and renal complications such as insulin resistance and albuminuria, during chronic HIV infection remains poorly understood.

OBJECTIVE AND HYPOTHESIS

The objective of this study is to evaluate the relationships of peripheral blood monocyte cytokine responses and monocyte subpopulations to clinical measures of insulin resistance and albuminuria in HIV-infected individuals on ART. We hypothesize that in the context of ART-treated chronic HIV infection, elevated pro-inflammatory
cytokine responses by peripheral blood monocytes are associated with increased insulin resistance. Furthermore, elevated CD16⁺ peripheral blood monocyte subpopulations are associated with worsening albuminuria and increased urine pro-inflammatory and pro-fibrotic biomarkers.

**SPECIFIC AIMS**

*Specific aim 1: To evaluate the relationship of peripheral blood monocyte-derived cytokine responses to insulin resistance in HIV-infected individuals on ART.*

**Hypothesis:** Elevated pro-inflammatory cytokine responses by peripheral blood monocytes are associated with increased insulin resistance as measured by homeostatic assessment of insulin resistance (HOMA-IR) in HIV-infected individuals on ART.

**Approach:**

- Collect and analyze demographic, clinical, and cardio-metabolic data from HIV-infected participants of the Hawaii Aging with HIV Cohort-Cardiovascular Disease (HAHC-CVD) study and HIV-uninfected participants of similar age, gender, and cardiovascular risk as measured by Framingham risk score.
- Calculate HOMA-IR values using fasting blood glucose and insulin measures for HIV-infected and –uninfected participants.
- Analyze intracellular IL-1β, IL-6, IL-8, and TNF-α production at both basal levels and after stimulation in peripheral blood monocytes isolated from HIV-infected and –uninfected participants by multi-parametric flow cytometry.
• Comparison analyses between HIV-infected and –uninfected participants will be calculated using Mann-Whitney U and Chi-square tests for continuous and categorical variables, respectively. Pearson product-moment correlation and multivariable linear regression will be utilized to assess associations between clinical and cardio-metabolic parameters to monocyte cytokine responses.

Specific aim 2: To evaluate the relationship of peripheral blood monocyte subpopulations to albuminuria and urine pro-inflammatory and pro-fibrotic biomarkers in HIV-infected individuals on ART.

Hypothesis: Elevated levels of CD16+ monocyte subpopulations in peripheral blood are associated with worsening albuminuria as measured by urine albumin-to-creatinine ratio and increased urine pro-inflammatory and pro-fibrotic biomarkers.

Approach:
• Collect and analyze demographic, clinical, and cardio-metabolic data from HIV-infected participants of the HAHC-CVD study. Participants will be separated into two groups, HIV-infected participants with and without albuminuria, based on measured urine albumin-to-creatinine ratio.
• Analyze subpopulations of peripheral blood monocytes (based on CD14 and CD16 expression) and activated CD8 T cells (based on CD38 and HLA-DR expression) isolated from HIV-infected participants with and without albuminuria by multi-parametric flow cytometry.
• Analyze urine collected from HIV-infected individuals with and without albuminuria for pro-inflammatory (IP-10, MCP-1, and IL-18) and pro-fibrotic
(TGF-β₁, TGF-β₂, TGF-β₃, Collagen IV, and TIMP-1) biomarkers utilizing Luminex technology.

• Comparison analyses between HIV-infected participants with and without albuminuria will be calculated using Mann-Whitney U and Chi-square tests for continuous and categorical variables, respectively. Pearson product-moment correlation and multivariable linear regression will be utilized to assess associations among clinical and cardio-metabolic parameters, cellular immune subpopulations, and urine pro-inflammatory and pro-fibrotic biomarkers.

**SIGNIFICANCE**

The long-term goal of this study is to understand the role of monocytes in the pathogenesis of non-AIDS comorbidities including insulin resistance and albuminuria to direct preventative/therapeutic strategies for these complications in HIV-infected individuals on ART.
Chapter 3

IL-1β monocyte responses are associated with insulin resistance and duration of infection among HIV-infected individuals on suppressive antiretroviral therapy
ABSTRACT

Monocytes are potent secretors of inflammatory cytokines and may contribute to chronic HIV-associated inflammation. We investigated the relationship between monocyte inflammatory properties and insulin resistance (IR) among HIV-infected participants in the setting of HIV viral suppression. Cross-sectional analysis of 33 HIV-infected participants age ≥40 years and on stable ART ≥3 months were compared to 14 HIV-uninfected participants of similar age, gender, and cardiovascular disease risk. Homeostatic model assessment-insulin resistance (HOMA-IR) was calculated from fasting blood glucose and insulin measurements. Peripheral blood monocytes were stimulated with oxidized low-density lipoproteins (oxLDL) and percentages of monocyte intracellular IL-1β, IL-6, IL-8, or TNF-α responses were determined by flow cytometry. We observed higher percentages of monocytes producing IL-1β and IL-8 after oxLDL stimulation were associated with higher IR as measured by HOMA-IR (all p<0.005) in HIV-infected participants on ART (median age 53 years, 87% males) but not in HIV-uninfected participants. Longer duration since HIV diagnosis and longer duration since antiretroviral therapy (ART) initiation were both associated with higher basal monocyte cytokine responses (all p<0.05). These data suggest elevated pro-inflammatory responses by monocytes, in particular IL-1β and IL-8, may contribute to the pathogenesis of IR during chronic HIV disease in the setting of viral suppression. Duration since HIV diagnosis or since ART initiation may contribute to basal monocyte cytokine responses. The role of monocyte cytokine responses in HIV disease progression and in cardio-metabolic complications warrants further study.
INTRODUCTION

HIV-infected individuals on antiretroviral therapy (ART) have increased rates of insulin resistance (IR) and type 2 diabetes mellitus (T2DM) [1], which is associated with higher risk of cardiovascular disease (CVD) [2-4]. Inflammation and immune dysfunction driven by chronic HIV infection during suppressive ART may play a role in the prevalence of these metabolic conditions [5].

Recently, monocytes have been implicated to have a role in HIV pathogenesis [6, 7] and in the development of non-AIDS comorbidities, such as atherosclerosis [8-10] and cognitive impairment [11-15]. We have previously shown that an increase in total blood monocytes is associated with elevated homeostatic model assessment of insulin resistance (HOMA-IR) in HIV-infected participants on potent ART, independent of HIV immuno-virologic and traditional T2DM risk factors [16]. We have also shown that HIV-infected participants on ART regimens have higher percentages of monocytes producing intracellular IL-1β and IL-8 in both the basal state and upon stimulation as compared to HIV-uninfected participants similar in age, gender, and CVD risk [17]. In this study, we sought to investigate the relationships of monocyte cytokine responses to HIV-associated immunologic and cardio-metabolic parameters in HIV-infected participants on ART as compared to HIV-uninfected participants.

METHODS

Study participants and study design

The 33 HIV-infected and 14 HIV-uninfected participants were previously characterized and reported by Jalbert et al [17] in terms of percentages of monocytes
producing pro-inflammatory cytokines. Monocyte cytokine responses presented in this report were further analyzed in reference to additional clinical, HIV immuno-virologic and cardio-metabolic data from these participants obtained from the parent study, the Hawaii Aging with HIV Cohort-Cardiovascular Disease (HAHC-CVD) study [18]. HIV-infected participants who entered into the HAHC-CVD study were ≥40 years old, and were receiving stable ART ≥3 months. Clinical measures and specimen collections were obtained in the morning at entry while in a fasting state (consumed nothing but water for 12 hours). Supine blood pressures, weight, height, and waist measurements were obtained in triplicate and averaged, and body mass index (kg/m²) was calculated. Subjects were assessed for history of tobacco use. A commercial College of American Pathologists (CAP)–certified laboratory (Diagnostic Laboratory Services, Inc.) assessed T cell subsets, plasma HIV RNA, CBC, and metabolic labs (glucose, insulin, directly measured LDL, HDL and total cholesterol, and triglycerides). HOMA-IR was used as an index of insulin resistance [19] and calculated using the formula: 

\[
\frac{\text{fasting glucose}}{18} \times \frac{\text{fasting insulin}}{22.5}
\]

Participants were characterized as having T2DM using the American Diabetic Association Guidelines [20]. Metabolic syndrome in HAHC-CVD was defined using the criteria proposed by the National Cholesterol Education Program’s Adult Treatment Panel III report (ATP III) [21]. The 14 HIV-uninfected participants were similar in age and gender and underwent similar clinical and immunological assessments as the HIV-infected group.
**Assessment of monocyte intracellular cytokine production**

Monocyte cytokine responses at basal level and following oxLDL-stimulation were evaluated by multi-parametric flow cytometry as previously described [17]. Briefly, whole blood was drawn into EDTA tubes at entry and processed for peripheral blood mononuclear cell (PBMC) isolation within one hour of collection using a standardized ficoll-based protocol. PBMCs were cryopreserved in liquid nitrogen storage until use. Banked PBMCs were thawed in serum-free media containing 10 µg/ml of DNAse and rested overnight at 37°C and 5% CO₂ in a polypropylene 96-well plate. After overnight rest, cells were stimulated with oxidized low-density lipoproteins (oxLDL, 10 µg/ml) or media alone (un-stimulated) for 6 hours in the presence of brefeldin-A (5µg/ml) and monensin (5µg/ml). Cells were then surfaced stained with CD3, CD14, CD16, CD56, CD19, CD20, HLA-DR antibodies, and with Live/Dead fixable yellow dead cell stain. Cells were then fixed, permeabilized, and intracellularly stained with IL-1β, IL-8, IL-6, and TNF-α conjugated antibodies. Data was acquired on a custom 4-laser BD LSRFortessa, and all compensation and gating analyses were performed in FlowJo (TreeStar).

**Statistical analysis**

Comparisons between HIV-infected and uninfected participants were calculated using Mann-Whitney U and Chi-square tests for continuous and categorical variables, respectively. Pearson product-moment correlation and multivariable linear regression were utilized to assess associations. A two-sided probability of p-value <0.05 was
considered statistically significant. Statistical analyses were performed using the SPSS statistical program (SPSS Statistics 22, Armonk, NY).

RESULTS

Patient characteristics

The characteristics of the 33 HIV-infected and 14 uninfected participants are shown in Table 1. Of the 33 HIV-infected participants on ART, the majority was virally suppressed. HOMA-IR and the number of participants with T2DM and metabolic syndrome were higher in the HIV-infected group; however no statistical differences were observed when compared to the HIV-uninfected group.
Table 1. Comparison of baseline measures of study participants\(^{(a)}\)

<table>
<thead>
<tr>
<th></th>
<th>HIV-positive, n=33</th>
<th>HIV-negative, n=14</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>53 [49, 56]</td>
<td>51 [46, 60]</td>
<td>0.552</td>
</tr>
<tr>
<td><strong>Male, n (%)</strong></td>
<td>29 (88%)</td>
<td>14 (100%)</td>
<td>0.302</td>
</tr>
<tr>
<td><strong>Caucasian, n (%)</strong></td>
<td>22 (67%)</td>
<td>9 (64%)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Body mass index, kg/m(^2)</strong></td>
<td>26 [23, 27]</td>
<td>24 [23, 27]</td>
<td>0.601</td>
</tr>
<tr>
<td><strong>History of smoking, n (%)</strong></td>
<td>22 (67%)</td>
<td>11 (79%)</td>
<td>0.724</td>
</tr>
<tr>
<td><strong>History of hypertension, n (%)</strong></td>
<td>10 (30%)</td>
<td>4 (29%)</td>
<td>0.516</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>1.46 [0.79, 2.48]</td>
<td>0.85 [0.62, 1.74]</td>
<td>0.129</td>
</tr>
<tr>
<td><strong>Metabolic syndrome, n (%)</strong></td>
<td>7 (21%)</td>
<td>1 (7%)</td>
<td>0.405</td>
</tr>
<tr>
<td><strong>Type 2 Diabetes Mellitus, n (%)</strong></td>
<td>4 (12%)</td>
<td>0</td>
<td>0.302</td>
</tr>
<tr>
<td><strong>Total cholesterol, mg/dL</strong></td>
<td>175 [146, 189]</td>
<td>173 [151, 192]</td>
<td>0.658</td>
</tr>
<tr>
<td><strong>HDL cholesterol, mg/dL</strong></td>
<td>36 [30, 45]</td>
<td>55 [46, 64]</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>LDL cholesterol, mg/dL</strong></td>
<td>101 [81, 122]</td>
<td>107 [86, 114]</td>
<td>0.585</td>
</tr>
<tr>
<td><strong>Triglycerides, mg/dL</strong></td>
<td>125 [83,161]</td>
<td>78 [56,140]</td>
<td>0.076</td>
</tr>
<tr>
<td><strong>Hepatitis C infection, n (%)</strong></td>
<td>5 (15%)</td>
<td>0</td>
<td>0.303</td>
</tr>
<tr>
<td><strong>Nadir CD4(^+) T cells, cells/µL</strong></td>
<td>181 [63, 275]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>CD4(^+) T cells, cells/µL</strong></td>
<td>574 [450, 713]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>CD4(^+) T cells, %</strong></td>
<td>33 [24, 37]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>CD8(^+) T cells, cells/µL</strong></td>
<td>801 [594, 1087]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>CD8(^+) T cells, %</strong></td>
<td>43 [35, 50]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Activated CD8(^+) T cells (CD38(^{HLA-DR}^{+})), cells/µL</strong></td>
<td>83 [56, 161]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Activated CD8(^+) T cells (CD38(^{HLA-DR}^{+})), %</strong></td>
<td>12 [9, 17]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>HIV RNA &lt;50 copies/mL, n (%)</strong></td>
<td>28 (85%)</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Duration since HIV diagnosis, years</strong></td>
<td>16 [8, 23]</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Duration since ART initiation, years</strong></td>
<td>12 [6, 15]</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>History of NRTI use, n (%)</strong></td>
<td>33 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>History of NNRTI use, n (%)</strong></td>
<td>23 (70%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>History of Protease Inhibitor use, n (%)</strong></td>
<td>21 (64%)</td>
<td>-</td>
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</table>

\(^{(a)}\) Values are shown in median [interquartile range] or frequency, n (%)
Elevated monocyte cytokine responses are associated with immune dysregulation as measured by CD4/CD8 T cell count ratio.

We assessed in HIV-infected participants the relationships of CD4 and CD8 T cell parameters to percentages of monocyte cytokine responses at both basal levels and after oxLDL-stimulation. Nadir CD4 T cell counts showed no correlations with percentages of IL-1β⁺, IL-8⁺, IL-6⁺, and TNF-α⁺ monocytes in either condition. While absolute current CD4 T cell counts did not correlate, current CD4 T cell percentages negatively correlated with basal percentages of IL-6⁺ monocytes (r=-0.389, p=0.025) and oxLDL-stimulated percentages of IL-8⁺ and TNF-α⁺ monocytes (IL-8; r=-0.523, p=0.002 and TNF-α; r=-0.410, p=0.018). No correlations were seen with current CD8 T cell percentages; however absolute current CD8 T cell counts correlated positively with basal percentages of IL-6⁺ monocytes (r=0.368, p=0.035) and oxLDL-stimulated percentages of IL-8⁺ monocytes (r=0.460, p=0.007). Activated CD8 T cell counts and percentages showed no correlations with measured monocyte cytokine responses in either condition. While CD4/CD8 T cell count ratios moderately correlated negatively with unstimulated IL-6 monocyte responses, it strongly correlated negatively with IL-8 and TNF-α monocyte responses after oxLDL stimulation (Figure 1a-c).
Figure 1. Correlation plots of percentages of intracellular cytokine-producing monocytes to CD4/CD8 T cell count ratio in HIV-infected participants. Correlation plots of (a) unstimulated IL-6+, (b) oxLDL-stimulated IL-8+ and (c) oxLDL-stimulated TNF-α+ monocyte percentages to CD4/CD8 T cell count ratio. Solid line represents best-fit line for HIV-infected participants (n=33). *p <0.05, **p <0.01
Elevated monocyte cytokine responses are associated with duration since HIV diagnosis and duration since initiation of ART in HIV-infected participants

Duration since HIV diagnosis correlated positively with basal percentages of IL-1β⁺ (r=0.345, p=0.049), IL-6⁺ (r=0.410, p=0.018) and TNF-α⁺ (r=0.403, p=0.020) monocytes. Duration since ART initiation correlated positively with basal percentages of IL-6⁺ (r=0.503, p=0.003) monocytes. None of the monocyte cytokine responses in stimulated conditions correlated with duration since HIV diagnosis or with duration since ART initiation.

Elevated monocyte cytokine responses are higher in HIV-infected participants with metabolic syndrome and are associated with blood triglyceride levels

Basal and oxLDL-stimulated monocyte responses showed no correlation with total cholesterol, HDL and LDL cholesterol levels in either HIV-infected or uninfected groups. Triglyceride levels correlated only with oxLDL-stimulated percentages of IL-1β⁺ and IL-8⁺ monocytes in HIV-infected participants (IL-1β; r=0.489, p=0.004 and IL-8; r=0.414, p=0.017). No correlations among the HIV-uninfected participants were observed between triglyceride levels and monocyte cytokine responses in either basal or stimulated conditions.

HIV-infected participants with metabolic syndrome had higher percentages of IL-1β⁺ monocytes (p=0.018) as compared to HIV-infected participants without metabolic syndrome. No differences in percentages of IL-6⁺, IL-8⁺ or TNF-α⁺ monocytes were observed. No differences were seen in the HIV-uninfected group.
Elevated monocyte cytokine responses are associated with insulin resistance in HIV-infected participants

There were no correlations between HOMA-IR and basal monocyte responses in either the HIV-infected or uninfected groups. However, when stimulated monocyte responses were analyzed, we observed strong positive correlations between HOMA-IR and percentages of IL-1β+ (r=0.513, p=0.002) and IL-8+ (r=0.504, p=0.003) monocytes among the HIV-infected participants (Figures 2a,b). The strong correlations continued to be seen when the 7 participants with T2DM were excluded. Furthermore, the significant correlations remained when the 5 participants with detectable plasma HIV RNA were excluded. In contrast, no associations were seen between HOMA-IR and IL-1β+ (r=0.060, p=0.837) and IL-8+ (r=0.011, p=0.891) monocytes in the HIV-uninfected group. A trend towards a significant correlation was seen between percentages of TNF-α+ monocytes and HOMA-IR only in HIV-infected participants (r=0.329, p=0.074), while no correlations were seen for percentages of IL-6+ monocytes in either group (Figure 2c,d).
Figure 2. Correlation plots of percentages of intracellular cytokine-producing monocytes to HOMA-IR. Correlation plots of (a) IL-1β+, (b) IL-8+, (c) IL-6+, and (d) TNF-α+ monocyte percentages (oxidized LDL stimulated) to HOMA-IR. Solid line represents best-fit line for HIV-infected participants (n=33) and dashed line represents best-fit line for HIV-uninfected participants (n=14). *p <0.05, **p <0.01
We attempted to assess the independent predictive value of percentages of IL-1β+ and IL-8+ monocytes in explaining increased HOMA-IR when controlled for other variables associated with IR in this population. Because of the small number in our HIV-infected group, it was not statistically possible to control for all parameters of interest. In univariable linear regression analyses, a significant predictive value was seen only for BMI ($\beta=2.028$, $p=0.005$, CI=0.642-3.414) with no significant predictive value seen for other parameters associated with the development of IR in HIV: age, gender, ethnicity (Caucasian vs. non-Caucasian), current or nadir CD4 T cell count, HIV RNA, Hepatitis C infection, duration of HIV infection, or history of protease inhibitor or nucleoside reverse transcriptase inhibitor zidovudine or stavudine use. In multivariable linear regression models controlled for BMI, percentages of IL-1β+ and IL-8+ monocytes in HIV-infected participants continued to significantly predict higher HOMA-IR (IL-1β; $\beta=1.875$, $p<0.0005$, CI=0.940-2.810 and IL-8; $\beta=1.915$, $p=0.007$, CI=0.567-3.264). Exploratory linear regression models that included of age, ethnicity and current or nadir CD4 T cell counts gave similar results.

**DISCUSSION**

Our present study suggests a novel association between stimulated IL-1β and IL-8 monocyte responses in peripheral blood and increased IR among HIV-infected individuals on stable ART regimens. These associations were found to be independent of BMI and were not present among the HIV-uninfected group. Interestingly, we also found un-stimulated monocyte responses to be higher in individuals with longer durations since HIV diagnosis or since ART initiation.
IL-1β is one of the major pro-inflammatory cytokines produced by monocytes/macrophages and is shown to be an important mediator in a number of acute and chronic inflammatory diseases including T2DM in the general population [22]. After prolonged exposure to IL-1β in vitro, induced insulin resistance have been reported in murine and human adipocytes through a decrease in the expression of the glucose transporter Glut 4, which ultimately impairs insulin signaling and action [23]. Others report a cytotoxic effect of IL-1β on insulin-producing pancreatic β cells [22]. Overall, our findings implicate a role for monocyte derived IL-1β as a potential mechanism in the increased risk for IR and metabolic disease among our patient population.

As with IL-1β, IL-8 is a pro-inflammatory cytokine produced by a number of cell types, including monocytes/macrophages, and has been reported to inhibit AKT phosphorylation, increasing IR in human adipocytes [24]. While we did not observe a significant relationship between monocyte IL-6 and TNF-α responses with IR, IL-6 and TNF-α have been reported to be involved in the development of IR [25-27] and have a synergistic relationship with IL-1β and IL-8 responses, further driving IR [17, 23-25, 27, 28].

Targeted treatments towards IL-1β in humans have been described for T2DM and other inflammatory disease states in the general population [22]. A neutralizing IL-1β antibody was well tolerated by T2DM participants and showed modest reduction of HbA1c, fasting glucose, and inflammatory markers [29]. Similarly, Gevokizumab, a human-engineered IL-1β monoclonal antibody, showed improvement in glycemia and reduction of inflammation in T2DM patients [30]. Reductions in HbA1c, systemic
inflammatory markers, as well as proinsulin to insulin ratios in T2DM patients, were demonstrated after 13 weeks of Anakinra administration, a recombinant IL-1R antagonist [31]. Given our study findings, the effect of IL-1β targeted therapeutics on monocyte cytokine responses in HIV may be of interest. Our previous report revealed a positive relationship between monocyte IL-1β and IL-8 responses [17], thus targeting IL-8 as a downstream mediator of inflammation [32-35] in conjunction with targeting IL-1β may also be considered.

Our study found an expected correlation between immune dysregulation as measured by CD4/CD8 ratio [36] and monocyte cytokine responses. Unexpectedly, we found strong correlations (explaining 12 to 25% of the variance) between monocyte cytokine responses and either duration since HIV diagnosis or since ART initiation independent of CD4/CD8 ratio. Both duration of disease/ART as well as the extent of HIV immune dysregulation may be important in explaining the degree of monocyte cytokine responses. Longitudinal studies are warranted to examine the validity of these associations. The increase in monocyte cytokine responses with time may be reflective of increased inflammation associated with elevated cellular turnover and senescence of monocytes [37]. We were not able to decouple the duration effects due to the close relationship between the two parameters, both of which were by self-report. We did not detect a direct relationship between both durations to HOMA-IR, but this may be due to the inability of our small sample size to exhibit such a relationship.

This study is limited by its relatively small sample size. However, the strengths of the study are the careful clinical and cardio-metabolic characterizations performed on HIV-infected and uninfected groups, as well as detailed intracellular cytokine
phenotyping of monocytes that reveal discriminating associations in the HIV-infected group.

In conclusion, higher stimulated monocyte cytokine responses, particularly in IL-1β and IL-8, are associated with increases in IR in HIV-infected but not in HIV un-infected individuals. Duration since HIV diagnosis or since ART initiation may contribute to basal monocyte cytokine responses. The role of monocyte cytokine responses in HIV disease progression and in cardio-metabolic complications warrants further study.
ACKNOWLEDGEMENTS

I first would like to thank our study participants for their enthusiasm in the research conducted at the Hawaii Center for AIDS. Without their commitment and support, none of this work would be possible. I would like to extend much appreciation to Emilie Jalbert and Mary Margaret Byron for their expert guidance in multi-parametric flow cytometry, as well as to Dr. Ivo Sah Bandar, Dr. Alikia Maunakea, Dr. Dominic C. Chow, Dr. Louie Mar A. Gangcuangco, and Dr. Jason D. Barbour for their valuable input and feedback in the study design and data analyses. I finally would like to thank Dr. Cecilia Shikuma and Dr. Lishomwa Ndhlouv, of whom provided sagacious guidance and constructive criticism in all aspects of this study.

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REFERENCES


Chapter 4

Elevation of non-classical (CD14^{+/-}CD16^{++}) monocytes is associated with increased albuminuria and urine TGF-β₁ in HIV-infected individuals on stable antiretroviral therapy.
ABSTRACT

High rates of albuminuria are observed among HIV-infected individuals on stable antiretroviral therapy (ART). Though pro-inflammatory and pro-fibrotic responses are described as components of albuminuria in the general population, it is unclear how these responses are associated to albuminuria during ART-treated chronic HIV infection. We investigated the relationship of monocyte subsets and urine inflammatory and fibrotic biomarkers to albuminuria in ART-treated HIV-infected participants. Cross-sectional analyses were performed on Hawaii Aging with HIV-cardiovascular disease study cohort participants who were required at entry to be ≥40 years old and on ART ≥3 months. Monocyte subpopulations were determined in banked peripheral blood mononuclear cells (PBMC) using multi-parametric flow cytometry. Random urine samples collected at entry were assessed for albumin-to-creatinine ratios (UACR). Urine samples were measured for inflammatory (IP-10, MCP-1, and IL-18) and fibrotic (TGF-β1, TGF-β2, TGF-β3, Collagen IV, and TIMP-1) biomarkers using Luminex technology. Among 96 HIV-infected participants with measured UACR (87% male, 59% Caucasian, and 89% undetectable HIV RNA with median CD4 of 495.5 cells/µL), 18 (19%) had albuminuria. Non-classical (CD14\textsuperscript{low/+}CD16\textsuperscript{++}) monocytes were significantly elevated in participants with albuminuria (p=0.034) and were correlated to UACR (r=0.238, p=0.019). Elevated non-classical monocyte counts were significant predictors of worsening albuminuria, independent of traditional- and ART-associated risk factors (β=0.539, p=0.007). Urine TGF-β1 and collagen IV were significantly higher in albuminuric compared to non-albuminuric participants (TGF-β1; p=0.039 and collagen IV; p=0.042). Urine TGF-β1 was significantly correlated with non-classical monocyte
counts ($r=0.464$, $p=0.017$). Alterations in monocyte subpopulations and urine pro-fibrotic factors may play a role in kidney dysfunction during chronic HIV infection and warrants further study.

INTRODUCTION

Albuminuria is a strong and independent risk factor for renal and cardiovascular disease among the general and HIV-infected populations [1-7]. There is a higher prevalence of albuminuria observed among HIV-infected individuals (4-20%) as compared to those who are uninfected (2%), but mechanisms driving this remain poorly understood [5, 6, 8, 9]. Prior to the introduction of antiretroviral therapy (ART), albuminuria and other renal complications that occurred in individuals infected with HIV were commonly caused by HIV-associated nephropathy [10, 11]. With the widespread use of ART, etiologies of albuminuria have shifted to comorbid diseases such as hypertension and diabetes mellitus, as well as side effects of ART including Tenofovir [12-17]. Persistent pro-inflammatory and pro-fibrotic responses and immune dysfunction driven by chronic HIV infection during suppressive ART may also contribute to the prevalence of albuminuria in HIV-infected individuals [18-24].

Recently, monocytes have been implicated to play a role in the development of non-AIDS comorbidities during chronic HIV infection [25-30]. Through the advancement of flow-cytometry, various monocyte subpopulations have been phenotyped and are traditionally characterized into classical (CD14$^{++}$CD16$^{−}$), intermediate (CD14$^{++}$CD16$^{+}$), and non-classical (CD14$^{++}$/lowCD16$^{++}$) subsets [31, 32]. We recently reported a novel CD14$^{++}$/lowCD16$^{−}$ subpopulation termed transitional monocytes and described this subset
to be associated with increased carotid intima media thickness in HIV-infected individuals [33]. Monocytes contribute to both the production of pro-inflammatory and pro-fibrotic cytokines, and may be major mediators of HIV-associated inflammation and fibrosis [34-38].

Utilizing data and banked blood and urine specimens from a natural history study of individuals with chronic HIV on ART, we investigated the relationship of blood monocyte subsets and urine inflammatory and fibrotic biomarkers to albuminuria. We sought to assess the role of monocyte-associated inflammation and fibrosis in the pathogenesis of albuminuria during chronic HIV infection.

**METHODS**

**Study participants**

This retrospective study was conducted utilizing entry data and specimens from the Hawaii Aging with HIV Cohort-Cardiovascular Disease (HAHC-CVD) study, a 5-year natural history study investigating the role of chronic HIV infection in the development of CVD among HIV-infected participants on suppressive ART. Inclusion criteria into the cohort required documented HIV-positive status, age ≥40 years, and use of combination ART ≥3 months. Further details of this cohort and extensive HIV immunologic and cardio-metabolic assessments have been previously described [39]. IRB approval was obtained from the University of Hawaii, and all participants provided informed consent prior to enrollment into the HAHC-CVD study. At the time of entry into the cohort, all participants gave consent to banking of specimens and use of data and specimens for future studies related to HIV and its complications.
Random urine collections were obtained from participants upon entry into the HAHC-CVD study and urine albumin-to-creatinine ratios (UACR) were calculated. UACR was determined by immunoturbidimetric assay using a Roche/Hitachi Modular P analyzer by a commercial College of American Pathologist (CAP)-certified laboratory (Diagnostic Laboratory Services Inc.). Albuminuria was defined as a UACR ≥30mg/g and was used to separate participants into 2 groups, with or without albuminuria.

**PBMC isolation and flow cytometry assay**

PBMC isolation and flow cytometry assay was done as previously reported [30]. In brief, whole blood was drawn into EDTA tubes and cells were processed for peripheral blood mononuclear cell (PBMC) isolation within one hour of collection and cryopreserved. Banked cryopreserved PBMCs were thawed in serum-free media containing 10 µg/ml of DNase and were rested overnight at 37°C and 5% CO₂ in a polypropylene 96-well plate. Cells were then surface-stained with CD3, CD14, CD16, CD56, CD19, CD20, HLA-DR antibodies, and with Live/Dead fixable yellow dead cell stain (YARD). Data was acquired on a custom 4-laser BD LSRFortessa Cell Analyzer and all compensation and gating analyses were performed in FlowJo analytical software. Percentages of classical, intermediate, non-classical, and transitional monocyte subsets were determined based on CD14 and CD16 staining and absolute numbers of each subset were calculated from WBC and monocyte percent obtained from available clinical CBC performed on each participant.
Detection of urine inflammatory and fibrotic biomarkers by Luminex

Random urine samples from participants collected at HAHC-CVD study entry were aliquoted and cryopreserved. Banked cryopreserved urine samples from randomly selected subjects demonstrating a range of UACR <30mg/g were tested along with all patients with a UACR ≥30mg/g for urinary inflammatory and fibrotic biomarkers. Cryopreserved urine aliquots were thawed and prepared following manufacturer’s guidelines for each kit. Each sample was measured in duplicate. Urine MCP-1 and IL-18 were measured using the Bio-Plex Pro™ RBM Human Kidney Toxicity Panel 1 (Bio-Rad). Urine TGF-β₁, TGF-β₂, and TGF-β₃ were measured using the Bio-Plex Pro™ TGF-β Assay (Bio-RAD). Urine IP-10, Collagen IV, and TIMP-1 were measured using the Milliplex® MAP Human Kidney Injury Magnetic Bead Panel 1 (EMD Millipore). Data was acquired on a Luminex 200™ analyzer (Luminex) and data analysis was done using Bio-Plex Manager™ software (Bio-Rad). Net median fluorescent intensity (MFI) was calculated (MFI value minus background value) and average net MFI of duplicate samples was determined.

Statistical analysis

Comparisons of clinical and laboratory characteristics between groups were calculated using Mann-Whitney U and Chi-square tests for continuous and categorical variables, respectively. UACR, absolute counts of cellular immune parameters, and net MFI averages of urine biomarkers were all log-transformed prior to correlation and linear regression analyses to attain normal distribution. Pearson product-moment correlation and multivariable linear regression were utilized to assess associations. In assessing
the relationship between cellular immune parameters and UACR, cellular compartments with a Pearson product-moment correlation significant at p <0.05 were further examined in a separate multivariable linear regression model with UACR as the dependent variable, adjusting for age, hypertension, HOMA-IR, total cholesterol/HDL cholesterol ratio, and current use of Tenofovir and/or Ritonavir. A two-sided probability of p-value <0.05 was considered statistically significant. Statistical analyses were performed using the SPSS statistical program (SPSS Statistics 22, Armonk, NY).

RESULTS

Characteristics of participants

On baseline evaluation, 96 HIV-infected participants on stable ART with UACR and monocyte subset analyses were available. Of these participants, 18 (19%) had albuminuria with the majority [16 participants (89%)] assessed to have moderately increased albuminuria (UACR 30-300mg/g) with only 2 (11%) having severe albuminuria (UACR >300mg/g). Demographics and clinical parameters are summarized in Table 1. Rates of viral suppression and CD4 T cell counts were comparable between the HIV-infected groups with or without albuminuria. HIV-infected participants with albuminuria were older, had higher rates of hypertension and use of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB), had higher blood glucose levels at 120 minutes during oral glucose tolerance test (OGTT), and had higher insulin resistance measured by homeostatic model assessment-insulin resistance (HOMA-IR); but did not differ in rates of type 2 diabetes mellitus or use of Tenofovir or Ritonavir.
Table 1. Comparison of demographic and clinical parameters of HIV-infected participants on ART with and without albuminuria

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients with albuminuria</th>
<th>Patients without albuminuria</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=96</td>
<td>n=18</td>
<td>n=78</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>57 [49, 62.5]</td>
<td>50 [45, 55]</td>
<td>0.013*</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>16 (88.9%)</td>
<td>67 (86.0%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>12 (66.7%)</td>
<td>45 (57.7%)</td>
<td>0.665</td>
</tr>
<tr>
<td>Other races</td>
<td>6 (33.3%)</td>
<td>33 (42.3%)</td>
<td>0.665</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.9 [21.8, 28.1]</td>
<td>25.7 [23.8, 27.9]</td>
<td>0.789</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>124.5 [119.8, 141.3]</td>
<td>120.0 [111.3, 129.0]</td>
<td>0.043*</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>80.5 [75.0, 84.3]</td>
<td>74.0 [68.0, 80.0]</td>
<td>0.012*</td>
</tr>
<tr>
<td>History of smoking, n (%)</td>
<td>12 (66.7%)</td>
<td>49 (63.6%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.9 [21.8, 28.1]</td>
<td>25.7 [23.8, 27.9]</td>
<td>0.789</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>91.5 [80.5, 96.75]</td>
<td>87.0 [81.0, 94.0]</td>
<td>0.366</td>
</tr>
<tr>
<td>Fasting insulin, µU/mL</td>
<td>9.4 [4.7, 13.5]</td>
<td>5.9 [3.65, 10.1]</td>
<td>0.060</td>
</tr>
<tr>
<td>OGTT (c) blood glucose @ 120 min, mg/dL</td>
<td>113.0 [96.0, 138.0]</td>
<td>97.5 [73.3, 115.5]</td>
<td>0.031*</td>
</tr>
<tr>
<td>Metabolic syndrome (d), n (%)</td>
<td>5 (27.8%)</td>
<td>15 (19.2%)</td>
<td>0.629</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus, n (%)</td>
<td>3 (16.7%)</td>
<td>7 (9.0%)</td>
<td>0.593</td>
</tr>
<tr>
<td>HOMA-IR (e)</td>
<td>2.6 [1, 3, 4]</td>
<td>1.2 [0.8, 2.2]</td>
<td>0.017*</td>
</tr>
<tr>
<td>eGFR CKD-EPI (f), mL/min/1.73 m²</td>
<td>72.8 [63.8, 101.1]</td>
<td>86.6 [76.2, 99.3]</td>
<td>0.071</td>
</tr>
<tr>
<td>UACR (g), mg/g</td>
<td>63.35 [44.40, 156.15]</td>
<td>4.95 [3.38, 8.92]</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dL</td>
<td>13.5 [11.0, 18.0]</td>
<td>13.0 [10.0, 16.3]</td>
<td>0.327</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.1 [0.9, 1.3]</td>
<td>1.0 [0.9, 1.1]</td>
<td>0.168</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>180.0 [138.8, 267.0]</td>
<td>174.5 [150.8, 192.3]</td>
<td>0.383</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>119.0 [75.3, 180.5]</td>
<td>104.5 [83.8, 124.3]</td>
<td>0.315</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>36.0 [31.0, 55.3]</td>
<td>41.0 [32.5, 49.0]</td>
<td>0.683</td>
</tr>
<tr>
<td>Total cholesterol/HDL ratio</td>
<td>4.14 [3.48, 6.58]</td>
<td>4.09 [3.46, 5.29]</td>
<td>0.546</td>
</tr>
<tr>
<td>HIV RNA &lt; 50 copies/mL, n (%)</td>
<td>16 (88.9%)</td>
<td>68 (87.2%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Current use of Tenofovir, n (%)</td>
<td>16 (88.9%)</td>
<td>58 (74.3%)</td>
<td>0.312</td>
</tr>
<tr>
<td>Current use of Ritonavir, n (%)</td>
<td>7 (36.9%)</td>
<td>29 (37.2%)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

a. Median values shown with [median Q1, median Q3] or frequency, n with (%). *p < 0.05, **p < 0.01
b. Angiotensin-Converting Enzyme Inhibitor and Angiotensin II Receptor Blocker
c. Oral Glucose Tolerance Test
d. Metabolic syndrome was defined as having 3 or more of the following: Abnormal obesity, high triglycerides, low HDL cholesterol, high blood pressure, or high fasting glucose.
e. Homeostatic Model Assessment of Insulin Resistance was calculated by: HOMA-IR = [(fasting glucose/18) × fasting insulin]/22.5
f. Estimated Glomerular Filtration Rate was calculated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula: eGFR (if serum creatinine ≤ 0.9 mg/dL) = 141 x (Serum Creatinine (mg/dL)/0.9)^1.209 x 0.993^a; eGFR (if serum creatinine > 0.9 mg/dL) = 141 x (Serum Creatinine (mg/dL)/0.9)^-0.411 x 0.993^a.
g. Urine Albumin/Creatinine Ratio was calculated by: UACR (mg/g) = Urine Albumin (mg/dL)/Urine Creatinine (g/dL) ≈ Albumin excretion in mg/day.
Elevated non-classical (CD14^{+/low}CD16^{++}) monocytes are associated with albuminuria in treated HIV-infected participants

T cell and monocyte subset counts were compared between participants with and without albuminuria. Non-classical monocytes were significantly elevated in HIV-infected participants with albuminuria as compared to HIV-infected participants without albuminuria (Table 2). No significant differences were seen in the T cell subsets counts or in the other monocyte subsets counts. Of the cellular parameters assessed, UACR significantly correlated with non-classical monocyte counts (r=0.238, p <0.05), but not with CD4 T cells (r=0.070), CD8 T cells (r=0.150), activated CD8 T cells (r=-0.039), or with classical (r=0.112), intermediate (r=0.067), or transitional monocytes (r=0.127).

Table 2. Comparison of immunological parameters of HIV-infected participants on ART with and without albuminuria

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients with albuminuria</th>
<th>Patients without albuminuria</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=96</td>
<td>n=18</td>
<td>n=78</td>
<td></td>
</tr>
<tr>
<td>Nadir CD4^+ T cells, cells/µL</td>
<td>92.0 [33.0, 198.25]</td>
<td>170.0 [39.5, 249.5]</td>
<td>0.277</td>
</tr>
<tr>
<td>CD4^+ T cells, cells/µL</td>
<td>496.50 [374.5, 554.5]</td>
<td>495.50 [329.75, 635.75]</td>
<td>0.767</td>
</tr>
<tr>
<td>CD8^+ T cells, cells/µL</td>
<td>885.5 [533.0, 1160.0]</td>
<td>693.0 [552.0, 946.5]</td>
<td>0.177</td>
</tr>
<tr>
<td>Activated CD8^+ T cells (CD38^+), cells/µL</td>
<td>73.2 [53.7, 127.7]</td>
<td>77.8 [46.6, 123.8]</td>
<td>0.962</td>
</tr>
<tr>
<td>Total monocytes, cells/L</td>
<td>4.5x10^6 [3.7x10^6, 6.1x10^6]</td>
<td>3.9x10^6 [3.2x10^6, 5.0x10^6]</td>
<td>0.070</td>
</tr>
<tr>
<td>Classical (CD14^{++}CD16^-) monocytes, cells/L</td>
<td>3.4x10^6 [2.6x10^6, 4.8x10^6]</td>
<td>3.0x10^6 [2.2x10^6, 4.0x10^6]</td>
<td>0.110</td>
</tr>
<tr>
<td>Intermediate (CD14^{++}CD16^+) monocytes, cells/L</td>
<td>8.2x10^6 [2.3x10^6, 1.5x10^7]</td>
<td>5.0x10^6 [2.3x10^6, 1.2x10^7]</td>
<td>0.344</td>
</tr>
<tr>
<td>Non-classical (CD14^{+}CD16^{++}) monocytes, cells/L</td>
<td>2.7x10^7 [2.0x10^7, 5.5x10^7]</td>
<td>2.0x10^7 [1.3x10^7, 3.0x10^7]</td>
<td>0.033*</td>
</tr>
<tr>
<td>Transitional (CD14^{+}CD16^-) monocytes, cells/L</td>
<td>6.8x10^7 [5.2x10^7, 1.1x10^8]</td>
<td>5.8x10^7 [4.5x10^7, 8.2x10^7]</td>
<td>0.116</td>
</tr>
</tbody>
</table>

a. Median values shown with [median Q1, median Q3] or frequency, n with (%). *p <0.05, **p <0.01
We assessed the predictive value of non-classical monocytes in a multivariable linear regression model, adjusting for traditional risk factors of age, hypertension, HOMA-IR, total cholesterol/HDL cholesterol ratio, and ART-associated risk factors of current use of Tenofovir and/or Ritonavir (Table 3). Univariable linear regression analyses of risk factors predicting UACR are as follows: Age, B=0.017, p=0.035, C.I.=0.001-0.033; Hypertension, B=0.463, p<0.001, C.I.=0.235-0.691; HOMA-IR, B=0.077, p=0.010, C.I.=0.019-0.136; Total cholesterol/HDL cholesterol ratio, B=0.067, p=0.046, C.I.=0.001-0.132; Current use of Tenofovir, B=0.215, p=0.131, C.I.=-0.066-0.496; Current use of Ritonavir, B=-0.027, p=0.827, C.I.=-0.274-0.220. The results of Table 3 show that elevation of non-classical (CD14^{low/+}CD16^{++}) monocytes significantly predict worsening albuminuria in HIV-infected participants on stable ART, independent of traditional- and ART-associated risk factors. Inclusion of eGFR CKD-EPI or current use of angiotensin-converting enzyme (ACE) inhibitors and/or angiotensin receptor II blockers (ARB) into the model gave similar results.
Table 3. Multivariable linear regression analysis\(^{(a)}\) of non-classical (CD14\(^{+/low}\)CD16\(^{++}\)) monocyte counts as a predictor of albuminuria in HIV-infected participants on ART while adjusting for risk factors (n=96)

<table>
<thead>
<tr>
<th></th>
<th>Unstandardized Coefficients</th>
<th>Standardized (\beta)-value</th>
<th>(p)-value</th>
<th>95% C.I. for (B)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B-value</td>
<td>Std. Error</td>
<td>(\beta)-value</td>
<td></td>
</tr>
<tr>
<td>Non-classical Monocytes</td>
<td>0.539</td>
<td>0.197</td>
<td>0.259</td>
<td>0.007**</td>
</tr>
<tr>
<td>Age</td>
<td>0.017</td>
<td>0.008</td>
<td>0.212</td>
<td>0.038*</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.260</td>
<td>0.123</td>
<td>0.213</td>
<td>0.037*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.026</td>
<td>0.028</td>
<td>0.234</td>
<td>0.017*</td>
</tr>
<tr>
<td>Total Cholesterol/HDL ratio</td>
<td>0.027</td>
<td>0.030</td>
<td>0.084</td>
<td>0.366</td>
</tr>
<tr>
<td>Current use of Tenofovir</td>
<td>0.357</td>
<td>0.132</td>
<td>0.259</td>
<td>0.008**</td>
</tr>
<tr>
<td>Current use of Ritonavir</td>
<td>0.024</td>
<td>0.013</td>
<td>0.020</td>
<td>0.834</td>
</tr>
</tbody>
</table>

\(a\). The dependent variable for the performed multivariable linear regression model was urine albumin/creatinine ratio (UACR) and was log-transformed prior to analysis. The non-classical monocyte subset count was log-transformed prior to analysis and was inputted as a predictor variable for albuminuria to assess its significance in a multivariable model. *\(p<0.05\), **\(p<0.01\)

**Elevated non-classical (CD14\(^{+/low}\)CD16\(^{++}\)) monocytes are associated with elevated urine TGF-\(\beta\)\(_{1}\) in ART-treated HIV-infected participants**

Urinary inflammatory and fibrotic biomarkers were assessed in all 18 participants with albuminuria and in 19 randomly selected participants out of the total of 78 participants without albuminuria. Between the 19 participants selected for urine biomarker analyses and the 59 participants that were not selected, there were no statistical differences observed in demographic, clinical, or immunological parameters measured including age, history of hypertension, HOMA-IR, and UACR. HIV-infected participants with albuminuria had significantly higher urinary levels of fibrotic markers.
TGF-β1 and collagen IV (Table 4). There were no significant differences observed in measured urine inflammatory biomarkers between groups.

Table 4. Comparison of measured urine biomarkers of HIV-infected participants on ART with and without albuminuria (a)

<table>
<thead>
<tr>
<th>Biomarker (a)</th>
<th>Patients with albuminuria</th>
<th>Patients without albuminuria</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=37</td>
<td>n=18</td>
<td>n=19</td>
<td></td>
</tr>
<tr>
<td>MCP-1(b)</td>
<td>80.0 [22.3, 178.9]</td>
<td>132.0 [7.3, 214.8]</td>
<td>0.663</td>
</tr>
<tr>
<td>IP-10(c)</td>
<td>5.3 [0.4, 17.3]</td>
<td>3.0 [0.4, 14.8]</td>
<td>0.274</td>
</tr>
<tr>
<td>IL-18(d)</td>
<td>4.8 [0.9, 17.4]</td>
<td>8.5 [0.9, 19.0]</td>
<td>0.604</td>
</tr>
<tr>
<td>TGF-β1(e)</td>
<td>14.4 [5.8, 33.8]</td>
<td>3.5 [0.5, 11.5]</td>
<td>0.039*</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>12.8 [5.1, 20.5]</td>
<td>7.5 [5.0, 21.3]</td>
<td>0.503</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>8.1 [6.9, 22.1]</td>
<td>7.3 [3.3, 17.3]</td>
<td>0.152</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>1160.9 [565.9, 1470.1]</td>
<td>702.0 [344.5, 888.5]</td>
<td>0.042*</td>
</tr>
<tr>
<td>TIMP-1(f)</td>
<td>678.8 [235.3, 1420.2]</td>
<td>516.5 [171.5, 751.3]</td>
<td>0.178</td>
</tr>
</tbody>
</table>

a. Medians of net median fluorescence intensity (MFI) averages are shown with [median Q1, median Q3].

* p < 0.05

b. Monocyte Chemotactic Protein-1 (CCL2)
c. IFN-γ-Inducible Protein 10 (CXCL10)
d. Interleukin-18
e. Tumor Growth Factor-β
f. Tissue Inhibitor of Metalloproteinase-1

We assessed the correlation between the measured urine inflammatory and fibrotic biomarkers with non-classical monocytes. Urine TGF-β1 was significantly correlated with non-classical monocytes (r=0.464, p <0.05), while IP-10 (r=0.008), MCP-1 (r=-0.141), IL-18 (r=-0.037), TGF-β2 (r=0.063), TGF-β3 (r=-0.189), collagen IV (r=0.032), and TIMP-1 (r=0.029) did not show significant correlations. No correlations...
were seen between other monocyte subsets or T cell subpopulations and urine inflammatory or fibrotic biomarkers.

We also assessed the correlations among the urine inflammatory and fibrotic biomarkers. TGF-β₁ strongly correlated with TGF-β₂ ($r=0.752$) and TIMP-1 ($r=0.424$). Furthermore, TGF-β₂ correlated with TGF-β₃ ($r=0.389$), collagen IV ($r=0.617$), and TIMP-1 ($r=0.730$). Of the urine pro-inflammatory biomarkers, only urine MCP-1 correlated with TGF-β₂ ($r=0.520$), TGF-β₃ ($r=0.362$), collagen IV ($r=0.558$), and TIMP-1 ($r=0.592$).

**DISCUSSION**

Our study is the first report showing that albuminuria in HIV-infected individuals on stable ART is associated with increased levels of non-classical (CD14⁺⁻/CD16💩) monocytes, independent of traditional and ART-associated risk factors. Treated HIV-infected individuals with albuminuria excrete higher levels of urine TGF-β₁ and collagen IV as compared to those without albuminuria, and increased non-classical monocytes are associated with increased urine TGF-β₁. Furthermore, urinary levels of TGF-β₁ are strongly associated with other urine fibrotic markers.

Despite viral suppression, HIV-infected individuals on stable ART show higher rates of albuminuria, as compared to the general population. Chronic low-grade inflammation may contribute to the development of albuminuria and renal dysfunction, which both pro-inflammatory and pro-fibrotic responses are important components [18, 20, 40-46]. However, mechanisms of albuminuria that occurs during ART-treated chronic HIV infection have been primarily focused on the pro-inflammatory arm. Results
of our present study suggest a dynamic interplay between pro-inflammatory and pro-fibrotic responses.

Human non-classical (CD14$^{\text{low}^+}\text{CD16}^{++}$) monocytes along with the intermediate (CD14$^{++}\text{CD16}^+$) subset, are often grouped as CD16$^+$ monocytes and characterized as a pro-inflammatory cellular compartment, being potent producers of TNF-$\alpha$, IL-6, IL-1$\beta$ and IL-12, and poor producers of IL-10 [47-51]. Several studies have shown that the recruitment of non-classical monocytes in human inflammatory disease states may mediate further pro-inflammatory responses. Individuals with rheumatoid arthritis have been shown to have significantly higher non-classical monocytes as compared to healthy controls [52, 53]. Elevation of this monocyte subpopulation is associated with worsening disease state, higher erythrocyte sedimentation rates, and increased C-reactive protein and rheumatoid factor levels. In respect to kidneys, non-classical monocytes have been shown to accumulate in the glomerular vessels and play a role in the development of lupus-associated glomerulonephritis [34, 54]. Non-classical monocytes were observed to preferentially produce TNF and CCL3 in serum from individuals with lupus.

Elevated levels of peripheral non-classical monocytes have also been described in HIV-infected individuals as compared to HIV-uninfected controls [26, 55, 56]. In addition, we have previously reported increased percentages of total monocytes producing pro-inflammatory cytokines IL-1$\beta$ and IL-8 in ART-treated HIV-infected individuals as compared to HIV-uninfected individuals at basal levels and after stimulation with oxidized low-density lipoproteins and lipopolysaccharides [35]. With these findings, we suspect that the low-level chronic inflammatory environment that has
been characterized in chronic HIV infection may be contributing to elevated levels of non-classical monocytes in peripheral blood and an active inflammatory phenotype that may contribute to the development of albuminuria [22].

Contrasting studies in the general population have shown non-classical monocytes in humans to play a role in the resolution of inflammation and the differentiation into M2 macrophages that aide in anti-inflammatory and wound healing responses [57]. In myocardial infarctions, non-classical monocytes have been shown to demonstrate a beneficial effect in mediating vascular repair and organ function [58-60]. Similarly, recruitment of non-classical monocytes into the brain and spinal cord have shown to be associated with beneficial effects, which include active removal of amyloid-β peptides in neuronal tissue and maintenance of the blood-brain barrier by differentiated perivascular macrophages [61-63].

During the resolution of inflammation, an important component of the response is TGF-β, a multi-functional cytokine that regulates many cellular functions, including cellular growth and differentiation [64]. In the context of HIV infection, TGF-β is observed to be elevated in infected individuals and has been suggested to contribute to the pathogenesis of HIV-associated nephropathy [23, 24, 65]. Mesangial cells have been reported to contribute to the elevated production of TGF-β in the kidneys of HIV-infected individuals [23, 24]. Peripheral blood mononuclear cells have also been found to produce and secrete TGF-β, which associated with increased TGF-β levels in the plasma and tissues [37, 38, 66-69]. Thus, monocytes/macrophages infected with HIV may also contribute to the local production of TGF-β in the kidneys. As our results show, non-classical monocytes are associated with increase intra-renal production of
TGF-β as measured in urine. This suggests that in chronic HIV infection, elevated pro-inflammatory responses may trigger an increase in TGF-β in the kidneys. Moreover, monocyte-derived TGF-β may be increased and contribute to the elevated intra-renal levels, further driving albuminuria. Additional studies are warranted to assess these mechanisms.

This study is limited by its relatively small sample size and the lack of HIV-uninfected controls with measured UACR and phenotyped immunologic cellular subpopulations. However, the strengths of the study are the careful clinical and cardio-metabolic characterizations performed on HIV-infected groups, as well as detailed phenotypes of T cell and monocyte subpopulations and quantification of urine biomarkers that reveal discriminating associations in the HIV-infected groups.

In conclusion, elevation of non-classical (CD14<sup>low/+</sup>CD16<sup>++</sup>) monocytes is associated with worsening albuminuria in HIV-infected individuals on ART. This association is independent of traditional- and ART-associated risk factors of albuminuria. Furthermore, HIV-infected individuals with albuminuria excrete higher levels of urine TGF-β1 and collagen IV as compared to those without albuminuria, and increased non-classical monocytes are associated with increased urine TGF-β1. The role of non-classical monocytes in pro-inflammatory and/or pro-fibrotic responses in the kidney of HIV-infected individuals on ART warrants further study.
ACKNOWLEDGEMENTS

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REFERENCES


Chapter 5

Summary
With successful treatment of ART, individuals who are infected with HIV effectively suppress HIV viremia and preserve immune function as measured by increased CD4 T cell counts. As a result of these beneficial effects, incidences of opportunistic infections and mortality are drastically decreased, as long as compliance to regimens and access to antiretroviral drugs are maintained. However, HIV-infected individuals do not regain full health despite successfully suppressing the virus with ART. It is observed that HIV-infected individuals on ART have higher risk of developing non-AIDS comorbidities including metabolic and renal dysfunctions as compared to the general population. Factors that may be contributing to the comorbidities in the HIV-infected population include traditional risk factors, drug toxicities from both HIV and non-HIV treatments, and inflammation that has been observed to persist despite HIV suppression. While CD8 T cells have been the focus in HIV-immune activation, monocytes have recently been implicated to be involved in HIV-associated inflammation and possibly play a role in the development of non-AIDS comorbidities. However, the link between monocytes and metabolic and renal dysfunction in chronic HIV infection has yet to be understood.

In the first study, we evaluated the relationship between monocyte cytokine responses and insulin resistance. We found a novel association between increased IL-1β and IL-8 monocyte responses as measured by flow cytometry and increased IR as measured by HOMA-IR in HIV-infected individuals on ART. This relationship was not seen in the HIV-uninfected individuals similar in age, gender, and cardiovascular risk as measured by Framingham risk score. Through the use of multivariable linear regression, we were able to further evaluate these relationships while adjusting for BMI,
an important risk factor for IR. IL-1β and IL-8 monocyte responses continued to have a significant relationship with IR, while adjusting for BMI. These finding implicated a potential mechanism of monocyte-derived cytokines in the increased risk of IR in HIV-infected individuals on ART.

Interestingly, we observed significant relationships between increased monocyte cytokine responses at basal levels and longer durations since HIV diagnosis (IL-1β, IL-6, and TNF-α) and since ART initiation (IL-6). These associations remained significant after controlling for CD4/CD8 T cell count ratio, a measure of immune function. Both durations of disease/ART as well as the extent of HIV immune dysregulation may be important to explaining the elevated pro-inflammatory nature of monocyte in the context of chronic HIV infection. Due to the cross-sectional nature of the study, we were not able to differentiate the individual effects of the durations. Longitudinal studies are warranted to further analyze the relationship of duration of infection/ART to monocyte pro-inflammatory responses and its link to the development of non-AIDS comorbidities.

In looking at the relationship between monocytes and albuminuria, we found that non-classical (CD14<sup>low</sup>+CD16<sup>+</sup>) monocytes are associated with worsening albuminuria as measured by urine albumin-to-creatinine ratio. This association was independent of traditional- and ART-associated risk factors for albuminuria. In analyzing urine for intra-renal cytokine profiles, we found that HIV-infected individuals with albuminuria had higher levels of TGF-β<sub>1</sub> and collagen IV as compared to HIV-infected individuals without albuminuria. In assessing the relationship between immune cell populations and urine biomarkers, we observed that non-classical monocytes were strongly associated with increased urine TGF-β<sub>1</sub> levels. These data suggest a possible dynamic role for non-
classical monocytes in renal dysfunction during chronic HIV infection. Having been characterized in previous reports as a pro-inflammatory subpopulation, non-classical monocytes may induce a heightened pro-inflammatory response in the kidneys and as a counteractive measure to resolve the inflammation, resident cells of the kidney such as mesangial cells may produce elevated levels of TGF-β1, which may account for the increase of this cytokine. Another possibility is that non-classical monocytes may be contributing to the intra-renal level of TGF-β1 in the kidney, since it has been previously shown that monocytes have the capacity to produce this cytokine. A major limitation to this study is the lack of HIV-uninfected controls. In future studies, we plan to compare HIV-infected individuals with albuminuria to HIV-uninfected individuals with albuminuria to see if there is a heightened level of non-classical monocytes as well fibrotic markers in the infected group.

Overall, the results show a significant association between activated/inflammatory monocytes and abnormalities of glucose metabolism and kidney function. Monocyte are shown to be possibly involved in pro-inflammatory responses as in the case of insulin resistance, as well as pro-fibrotic responses as in the case of albuminuria. Further studies are needed to investigate these possible mechanisms in the context of chronic HIV. The significance of these results is that it may direct therapeutic/preventative interventions for these comorbidities in HIV-infected individuals on ART. In addition, monocyte phenotype and function may be utilized as important biomarkers of inflammation/fibrosis and may be a means of measuring efficacy of new interventions.