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Endothelial Colony Forming Cells and Inflammatory Monocytes in HIV

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Abstract

The relationships between HIV infection, monocyte activation, and endothelial colony forming cells (ECFCs) are unknown. We compared ECFC, intermediate monocytes (CD14⁺CD16⁺), and non-classical monocytes (CD14^{dim}CD16⁺⁺) levels in HIV-infected participants virologically-suppressed on antiretroviral therapy, HIV-infected treatment-naïve participants, and HIV-uninfected healthy controls. ECFC levels were significantly higher in the HIV-infected, virologically-suppressed group compared to the uninfected controls. CD14⁺CD16⁺ percentages (but not CD14^{dim}CD16⁺⁺ cells) were significantly higher in both HIV-infected groups vs uninfected controls. In the HIV-infected groups, ECFCs and CD14⁺CD16⁺ intermediate monocytes were significantly and inversely correlated. Lower availability of ECFCs may partly explain the relationship between greater intermediate monocytes and atherosclerosis in HIV.

Keywords

endothelial progenitor cell; endothelial colony forming cells; non-classical monocyte; intermediate monocyte; HIV; flow-mediated dilation

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INTRODUCTION

Cardiovascular disease (CVD) is more common in HIV-infected vs. uninfected populations.^{1,2} Greater systemic inflammation, and more specifically, monocyte activation, may contribute to this increased risk of CVD in HIV.^{3,4} In addition, untreated HIV may be associated with impaired endothelial function, perhaps due to HIV-associated inflammation, but antiretroviral (ART)-induced virologic suppression does not fully reverse either systemic inflammation or endothelial dysfunction.⁵⁻⁷

Endothelial progenitor cells (EPCs) can be found in the peripheral blood, are of bone marrow origin, and possess the ability to maintain vascular homeostasis.⁸ EPCs play a role in maintaining a healthy endothelium by homing to denuded or dysfunctional areas of endothelium and assisting in vascular repair either by direct integration or via paracrine mechanisms.⁹ Reduced circulating numbers of EPCs have been found in those who have suffered CVD events, and may portend a poor prognosis even after adjustment for traditional risk factors.⁹ These studies have traditionally used the original definition of an EPC by Asahara et al.⁸ as detected by flow cytometry using the cell surface markers CD34 and KDR (i.e. VEGFR-2) but have not necessarily confirmed that these cells have clonal capabilities or have endothelial cell phenotypic characteristics. This confusion and lack of consensus definition of purported EPCs has perhaps led to conflicting results regarding the effects of HIV infection on EPC circulating levels.¹⁰

Recently Mund et al., have proposed a new “gold standard” method for enumeration of true, bona fide EPCs, which are termed endothelial colony forming cells (ECFCs). By using multi-parametric flow cytometry acquisition and analysis, and restricting cell populations that yield false positive events in previously used methods (CD14, glyA, and LIVE/DEAD), a new optimum technique has emerged.¹¹ Therefore, we sought to evaluate the effects of HIV infection, antiretroviral therapy, and monocyte activation on circulating levels of ECFCs as a potentially novel mechanism underlying the increased risk of CVD in HIV. We also examined the relationships between ECFCs and monocyte cell subsets with *in vivo* endothelial function.

METHODS

Study Design and Population

We performed a prospective, cross sectional pilot study of 20 HIV-infected patients (10 ART-naïve and 10 virologically suppressed). After an 8 hour fast, whole blood samples for flow cytometry measurement of ECFCs and activated monocyte subset populations and measurement of soluble monocyte activation markers were obtained followed by flow-mediated dilation (FMD) of the brachial artery to measure *in vivo* endothelial function. The same flow-cytometry and FMD measurements (but not soluble markers) from a separate study of 17 HIV-uninfected healthy controls drawn from the Indiana University campus were also incorporated into this analysis as an additional control group.

The HIV-infected participants were eligible if they were ≥ 18 years of age; non-smoking; not pregnant or breastfeeding, without known CVD, diabetes, uncontrolled hypertension

(>160/100 mm Hg), or other pro-inflammatory condition; and with creatinine clearance 60 mL/min. To be included in the virologically suppressed group, the participants needed to have an HIV-1 RNA level < 50 copies/mL while receiving co-formulated emtricitabine/tenofovir/efavirenz for at least six months as their initial therapy. All participants provided written informed consent, and all protocol versions and consents were reviewed and approved by the Indiana University Institutional Review Board.

Procedures

FMD was measured in all participants as per established guidelines¹² by a single technician and with all vascular parameters measured by a blinded, single investigator (SKG). In our laboratory's most recent assessment of reproducibility, we performed paired FMD studies over 1-4 weeks in 11 healthy volunteers; the intraclass correlations for baseline brachial artery diameter, reactive hyperemia, and FMD were 0.98, 0.98, and 0.70, respectively.

Whole blood was collected in EDTA tubes with subsequent plasma isolation and enumeration of ECFCs and both non-classical monocytes (CD14^{dim}CD16⁺⁺) and intermediate monocytes (CD14⁺CD16⁺) using multi-parametric flow cytometry (Supplemental Digital Content 1, which describes flow cytometry methods in more detail).^{11, 13, 14} Levels of soluble CD14 and soluble CD163 were measured in batch from plasma samples which had been frozen at -80C after collection using Quantikind enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, Minnesota).

Statistical Methods

As ECFC measurements have not previously been performed in HIV-infected patients, we could not justify the sample sizes in this initial study an *a priori* power calculation; instead, we chose to include 10 participants in each of the HIV-infected groups based on resource availability.

Categorical variables were summarized by frequencies and percentages and were compared between the groups using Fisher's exact test. Normality held for all continuous variables, so these variables were summarized by mean (standard deviation) and compared using Student's t-tests and analysis of variance as appropriate with adjustment for multiple comparisons using Tukey's method. Pearson's correlations without adjustment for multiple comparisons were calculated to assess the relationships between FMD and all the circulating markers and between ECFCs and the monocyte subsets.

RESULTS

Study Population

The characteristics and laboratory data of each study group are listed in Table 1. Due to delays in whole blood processing for a few participants on the day of the study visits, ECFC and monocyte subset enumeration were performed in only 8 of the HIV-infected, virologically-suppressed group and in 9 of the HIV-infected, ART-naive group. Mean age was 35 years overall but varied among the groups, with mean age of 28 years in the uninfected control group compared to 43 years in the virologically-suppressed group

($p=0.01$). The groups were similar in regards to systolic blood pressure and body mass index. The HIV-infected groups had similar CD4 counts with the mean HIV-1 RNA level in the untreated group being 30,100 copies/mL.

Endothelial colony forming cells

As shown in Table 1, mean ECFC levels were significantly higher in the virologically suppressed group compared to the uninfected, control group (0.1073 vs. 0.0198, $p=0.03$). These results remained significant after adjustment for age ($p = 0.04$). ECFC levels were not significantly higher in the ART-naive HIV-infected group compared to the uninfected controls.

Monocyte Subsets

There were no significant differences amongst the groups in regards to the non-classical monocyte subset ($CD14^{\text{dim}}CD16^{++}$). However, levels of intermediate monocytes ($CD14^{+}CD16^{+}$) were significantly higher in each of the HIV-infected groups compared to the uninfected controls (ART-naive 9.16% vs. controls 3.05%, $p=0.01$; virologically suppressed 8.68% vs. 3.05%, $p=0.01$), although there was no statistically significant difference between the two HIV-infected groups. As shown in Figure 1, among all 17 HIV-infected participants with available data, we found a strong negative correlation between ECFCs and intermediate monocytes ($r = -0.65$, $p=0.005$). The magnitudes of the correlations within the suppressed group ($r = -0.68$, $p=0.06$) and the ART-naïve group ($r = -0.69$, $p=0.04$) were similar to the overall group. No significant correlations were found between ECFCs and activated monocytes in the overall HIV-infected group or the two HIV-infected subgroups.

Soluble Monocyte Activation Markers

sCD163 levels did not differ between the HIV-infected groups (698 ng/mL in the virologically suppressed group vs. 734 ng/mL in the ART-naïve group, $p = 0.77$). There was a trend towards higher levels of sCD14 in the virologically suppressed group compared to ART-naïve group (2523 ng/mL vs. 2094 ng/mL, $p = 0.09$). Among the HIV-infected participants, the $CD14^{+}CD16^{+}$ levels (but not the $CD14^{\text{dim}}CD16^{++}$ levels) significantly correlated with both sCD14 ($r = 0.49$, $p=0.048$) and sCD163 ($r = 0.53$, $p=0.03$).

Flow mediated dilation

There were no significant differences in FMD amongst the three groups and no significant correlations were found between FMD and any of the circulating markers assessed.

DISCUSSION

The relationships between immune activation and EPCs (or ECFCs), and how these cell subsets affect vascular homeostasis, in HIV-infected persons are yet to be understood. The literature on EPCs in HIV has been mixed, likely due to the variable definitions of EPCs and the mixed methods of enumeration. Using the new gold standard technique in ECFC enumeration, we report the novel finding of elevated ECFCs among our virologically suppressed HIV-infected participants compared to the uninfected control group.

Furthermore, we were able to show a negative correlation among the inflammatory subset of monocytes and ECFCs among our HIV-infected participants.

One explanation for the higher ECFCs in those virologically suppressed compared to the uninfected control group may be that ART in combination with HIV may have adverse effects on the endothelium, which then consequently signal the release of ECFCs to repair the vascular damage. Given the small numbers in our study, which may have precluded finding differences in ECFC levels between the two HIV-infected groups and between the ART-naïve group and the uninfected controls, we cannot state that ART in general has a direct effect on ECFC levels. Of note, in order to limit variability in the results that could potentially have been introduced by different ART regimens, we required all virologically suppressed participants to receive the same ART combination, namely co-formulated emtricitabine/tenofovir/efavirenz. We chose this regimen due to its common use. As efavirenz may have negative effects on the endothelium,^{15, 16} it is plausible that the higher levels of ECFCs found in this group were due to a direct toxic effect of this drug.

Although most data regarding circulating EPCs in non-HIV infected individuals show an inverse correlation in CVD, others have found the opposite.¹⁷ For example, Guven et al. reported in an HIV-uninfected cohort that the highest EPC levels were found among those with the greatest angiographic stenosis severity.¹⁷ Notably this study used EPC culture techniques that correlate most closely to the flow cytometry enumeration procedure we performed in our study. Vecchiet et al. also employed EPC culture methods and reported higher ECFC levels in HIV-infected, ART-naïve patients compared to uninfected controls; ECFC levels were lower in those on ART, correlated with HIV viral load, and inversely correlated with CD4 cell counts.¹⁸ Overall, the differences in EPC results amongst earlier non-HIV and HIV studies are most likely due to the differences in technique as well as nomenclature. It is now well known that two clonogenic progenitors are involved in endothelial maintenance. Colony-forming unit-endothelial cells (CFU-ECs) are hematopoietic in origin and do not possess true post-natal vasculogenic properties; however, they may contribute to neoangiogenesis by secretion of local paracrine factors. Bone fide EPCs (i.e. ECFCs) possess true clonal proliferation capabilities, lack myeloid markers, and can create functional blood vessels.^{19, 20} The two previously mentioned studies, one in HIV-uninfected individuals and the other in HIV-infected patients, both implemented cell culture techniques and found higher ECFCs in those with CVD and in those free of CVD but HIV-infected.

Our data regarding the different monocyte subsets are consistent with other recently published studies in HIV.^{21, 22} We found a significant trend from lowest to highest frequencies of inflammatory monocytes from uninfected controls to virologically suppressed HIV-infected participants to ART-naïve HIV-infected group. A recent large study by Baker et al. showed that among HIV-infected patients with immune restoration, higher circulating CD16⁺ monocytes predicted coronary artery calcium progression.²¹ Our present study adds to these findings by demonstrating a negative correlation between the ECFCs and the intermediate monocytes in the HIV-infected participants. One possible explanation for this finding is that the elevated intermediate monocyte fraction in HIV could be causing arterial

inflammation and endothelial damage, and consequently, either due to HIV and/or ART effects, there is deregulation in biofeedback or bone marrow release of ECFCs.

One limitation to our study was the relatively small number of participants and the subsequently reduced power to find differences in FMD and ECFC amongst all study groups or correlations between FMD and the circulating cell fractions and soluble markers studied here. The cross-sectional design only allowed us to assess association but not causality. In addition, the uninfected group was significantly younger than the HIV-infected groups, although our findings remained significant after adjustment for age. We also did not measure fasting lipids or glucose in this study and cannot determine if imbalances in these parameters potentially affected the results.

To our knowledge, this is the first study of its kind using the new gold standard methodology for ECFC enumeration in an HIV-infected cohort. We found higher ECFC levels in HIV-infected patients receiving ART compared to uninfected controls and that these ECFC levels are negatively correlated with CD14⁺CD16⁺ monocyte levels in our overall HIV-infected cohort. As such, it is reasonable to speculate that the association between higher pro-inflammatory monocytes and development of atherosclerosis in HIV may be in part due to reduced numbers of ECFCs. Larger, longitudinal studies are needed to assess if ECFC levels vary by ART regimen, change with alterations in levels of monocyte subsets, and, most importantly, predict development of atherosclerosis and future CVD events in HIV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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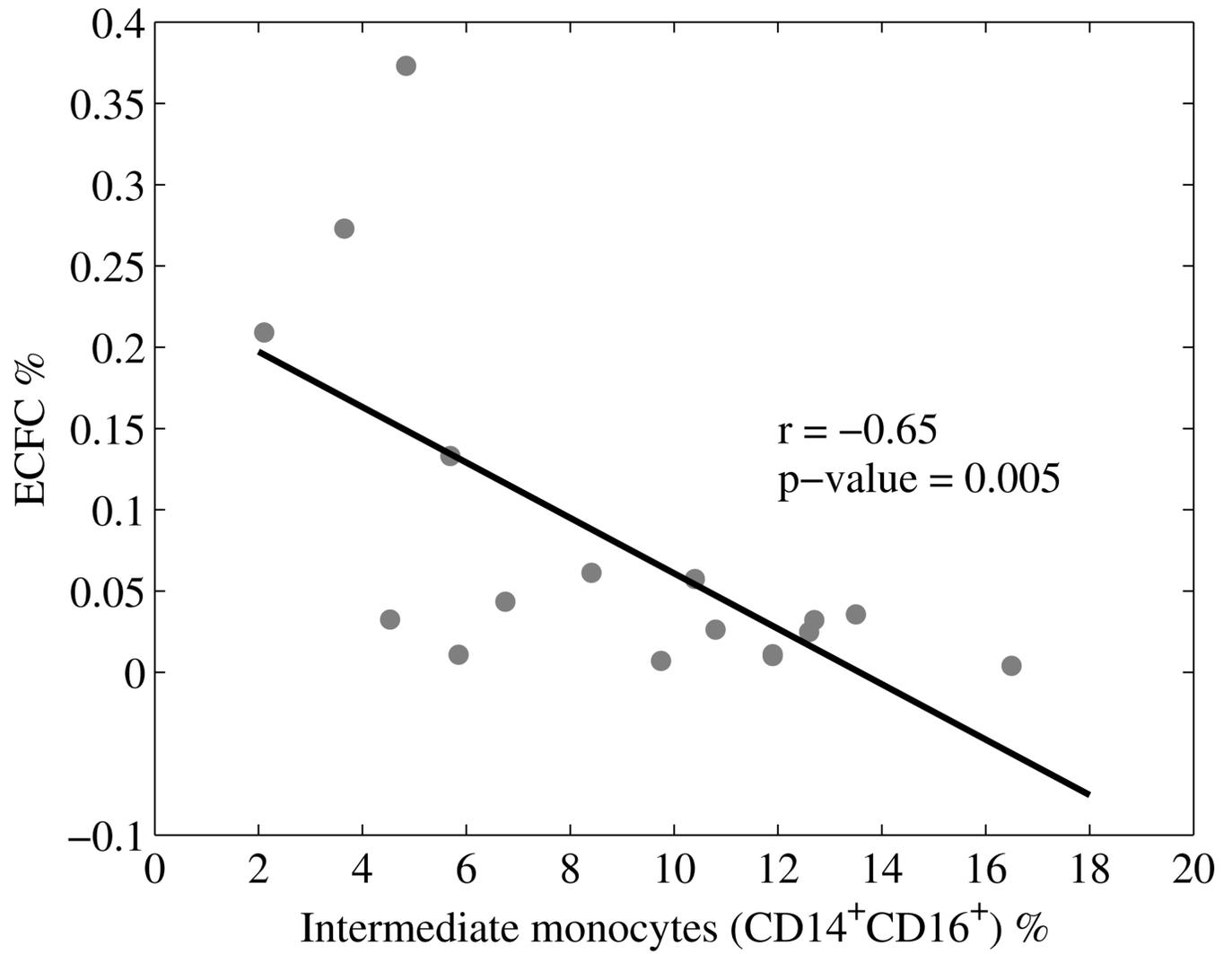


Figure 1. Correlation between percentages of intermediate monocytes (CD14⁺CD16⁺) and endothelial colony forming cells (ECFC) in 17 HIV-infected study participants (8 virologically suppressed and 9 antiretroviral-naïve).

Table 1

Characteristics of the study groups.

Characteristic	HIV-infected		HIV-uninfected (n=17)
	Virologically-suppressed ¹ (n=10)	ART-naïve (n=10)	
Age, y	43 (11.6)	37 (13.3)	28 (8.1)
Male Sex, n (%)	8 (80)	5 (50)	7 (41)
Black race, n (%)	5 (50)	6 (60)	2 (12)
BMI, kg/m ²	28 (6.84)	29 (5.42)	25 (2.79)
SBP, mm Hg	123 (12)	122 (14)	125 (15)
CD4, cells/ μ L	649 (311)	678 (204)	n/a
HIV-1 RNA level, copies/mL	<50	30,100 (34,007)	n/a
² ECFC, %	0.1073 (0.142)	0.054 (0.0608)	0.0198 (0.0324)
² CD14 ⁺ /CD16 ⁺ , %	8.68 (4.69)	9.16 (3.71)	3.05 (2.21)
² CD14 ^{dim} /CD16 ⁺ , %	4.62 (6.74)	6.26 (11.43)	3.47 (4.27)
sCD14, ng/mL	2523 (360.96)	2094 (656.14)	n/a
sCD163, ng/mL	698 (263.58)	734 (277.54)	n/a
FMD, %	4.22 (2.98)	4.73 (3.86)	3.25 (2.89)

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; SBP, systolic blood pressure; ECFC, endothelial colony forming cells; FMD, flow mediated dilation; n/a, not available or not applicable.

All data presented as n (%) or mean (standard deviation).

¹ All were treated with co-formulated emtricitabine, tenofovir, and efavirenz.

² Flow cytometry measurements only performed in 8 of the virologically-suppressed group and in 9 of the antiretroviral-naïve group.