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Endothelial Colony-Forming Cell Function Is Reduced During HIV Infection

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Background. Human immunodeficiency virus (HIV) may be related to cardiovascular disease through monocyte activation–associated endothelial dysfunction.

Methods. Blood samples from 15 HIV-negative participants (the uninfected group), 8 HIV-positive participants who were not receiving antiretroviral therapy (ART) (the infected, untreated group), and 15 HIV-positive participants who were receiving ART (the infected, treated group) underwent flow cytometry of endothelial colony-forming cells (ECFCs) and monocyte proportions. IncuCyte live cell imaging of 8 capillary proliferative capacity parameters were obtained from cord blood ECFCs treated with participant plasma.

Results. The ECFC percentage determined by flow cytometry was not different between the study groups; however, values of the majority of capillary proliferative capacity parameters (ie, cell area, network length, network branch points, number of networks, and average tube width uniformity) were significantly lower in infected, untreated participants as compared to values for uninfected participants or infected, treated participants ($P < .00625$ for all comparisons). $CD14^+CD16^+$ intermediate monocytes and soluble CD163 were significantly and negatively correlated with several plasma-treated, cord blood ECFC proliferative capacity parameters in the combined HIV-positive groups but not in the uninfected group.

Conclusions. Cord blood ECFC proliferative capacity was significantly impaired by plasma from infected, untreated patients, compared with plasma from uninfected participants and from infected, treated participants. Several ECFC functional parameters were adversely associated with monocyte activation in the HIV-positive groups, thereby suggesting a mechanism by which HIV-related inflammation may impair vascular reparative potential and consequently increase the risk of cardiovascular disease during HIV infection.

Keywords. HIV-1; endothelium; ECFC; monocytes; cardiovascular.

Cardiovascular disease (CVD) has emerged as a leading cause of death in the human immunodeficiency virus (HIV)–infected population [1]. Systemic inflammation, particularly monocyte activation, is heightened during HIV infection and has been implicated in the pathogenesis of cardiovascular disease in HIV-infected individuals [2, 3]. How monocyte activation leads to CVD in HIV-infected patients, even in those receiving virologically suppressive antiretroviral therapy (ART), remains unclear.

There has been great interest in the role of endothelial progenitor cells in the development and treatment of CVD. Although most data regarding circulating endothelial progenitor cells in non–HIV-infected individuals show an inverse correlation of the proangiogenic endothelial progenitor cell in CVD [4–6], others have found the opposite [7–9]. These studies have traditionally used the classical definition of an endothelial progenitor cell by Asahara et al [10], as detected by flow cytometry using the cell surface markers CD34 and KDR (ie, VEGFR-2). However, these studies have not confirmed that these endothelial progenitor cells have vasculogenic capabilities or display true endothelial cell phenotypic characteristics.

It is now established that there are 2 clonogenic progenitor classes involved in endothelial maintenance. The cells identified by Asahara et al are now termed circulating angiogenic cells, myeloid angiogenic cells, or colony-forming unit–endothelial cells (CFU-ECs; also called CFU-Hill cells), which are hematopoietic in origin and do not possess postnatal vasculogenic properties [11]. These CFU-EC/Hill cells may contribute to neo-angiogenesis by secretion of local paracrine factors but do not actually undergo a lineage fate switch to become endothelial cells or to engraft into endothelium. In contrast, our group has identified "true" endothelial progenitor cells, which we refer to as "endothelial colony-forming cells" (ECFCs) and which possess clonal proliferation capabilities, lack myeloid markers, and can create functional blood vessels upon implantation to restore or regenerate an injured vascular bed [12–18]. By using multiparametric flow cytometry acquisition and analysis and by restricting cell populations (CD14, CD235a, and LIVE/DEAD)

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that yield false-positive events in previously used methods, we have developed a widely accepted gold standard flow cytometry technique to identify the viable circulating endothelial cells containing true ECFC activity [19].

Using this new flow cytometry signature, we found in a preliminary study that circulating ECFC numbers were higher in HIV-positive patients receiving efavirenz-based treatment, compared with HIV-negative controls [20]. We also found in this previous study that $CD14^{+}CD16^{+}$ intermediate monocyte proportions, found to be linked with atherosclerosis progression in HIV-infected patients [21], were inversely correlated with ECFC numbers, thus suggesting a potentially detrimental link between activated monocytes and endothelial regenerative capacity. Because efavirenz has been linked to endothelial dysfunction [22–24], we performed this current study to assess ECFC numbers in patients receiving modern integrase inhibitor–based regimens, which have not previously been linked to endothelial dysfunction or CVD in HIV. We also studied ECFC functional parameters, namely the ability of cord blood ECFCs, to proliferate new capillary networks when exposed to HIV-positive and HIV-negative plasma samples from these same study participants.

METHODS

Study Design and Participants

We performed a prospective comparative study of HIV-negative healthy volunteers (hereafter, the uninfected group), HIV-positive participants not receiving ART (hereafter, the infected, untreated group), and HIV-positive participants receiving ART (hereafter, the infected, treated group). After an 8-hour fast, a blood specimen was collected for enumerating ECFCs and proportions of $CD14^{++}CD16^{-}$ cells (classical monocytes), $CD14^{+}CD16^{+}$ cells (intermediate monocytes), and $CD14^{dim}$CD16+ cells (nonclassical monocytes) by flow cytometry. Each participant in the uninfected group and the infected, treated group attended 2 study visits within 14 days for collection of blood specimens, to provide a more stable, average estimate of ECFC counts and monocyte proportions. The infected, untreated participants also were invited to attend 2 visits, but they uniformly chose to forgo the second study visit because of their wish to initiate ART as soon as possible per current treatment guidelines.

There were no exclusions based on ethnicity, race, or sex. For all 3 groups, participants were ≥18 years of age, had no known CVD, did not have other proinflammatory conditions (although hepatitis B virus and HIV coinfection was allowed), had not undergone treatment for malignancy within 6 months of the study visits, were not diabetic, were not pregnant or breastfeeding, did not have fever at the time of the blood specimen collection, did not have an acute infection within 7 days of the study visits, and did not have uncontrolled blood pressure (defined as >160/>110 mm Hg). For the infected, untreated participants, there was neither a $CD4^{+}$ T-cell count nor an HIV-1 RNA load restriction. For the infected, treated group, they must have had an HIV-1 RNA level of <200 copies/mL (with no $CD4^{+}$ T-cell count restrictions) while currently receiving as their initial ART regimen of an integrase inhibitor (dolutegravir, raltegravir, or elvitegravir/cobicistat), combined with a backbone of tenofovir plus emtricitabine, for at least 6 months prior to enrollment.

The Indiana University Institutional Review Board approved this study. All participants provided written, informed consent.

Study Measurements

Peripheral blood samples were collected in vessels containing ethylenediaminetetraacetic acid. Plasma was isolated and then frozen at −80C for eventual batch analysis of soluble proteins.

Plasma Biomarkers

Levels of plasma soluble CD14 and soluble CD163 were measured using Quantikind enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). We then measured a panel of soluble proteins associated with angiogenesis (high-sensitivity C-reactive protein [hsCRP], C-C motif ligand 5 [CCL5], soluble vascular cell adhesion molecule 1 (sVCAM-1), angiostatin, E-selectin, vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor receptor 1 [VEGFR1] and 2VEGFR2, interleukin 6 [IL-6], interleukin 8 [IL-8], CCL2, soluble tumor necrosis factor receptor 1 [sTNFR1] and sTNFR2, angiopoietin 2 [Ang-2], endoglin, and fibroblast growth factor 2 [FGF-2]), using enzyme-linked immunosorbent assay (ELISA) multiplex kits (EMD Millipore, Billerica, MA) [25]. See Supplementary Methods for the intraassay coefficients of variation of these biomarkers.

Flow Cytometry for Enumerating ECFCs and Monocyte Subsets

Populations of ECFC and monocytes were enumerated using multiparameter flow cytometry as previously described [26–28]. The ECFCs were expressed as percentage of the total circulating mononuclear cell population. Multiparameter flow cytometry was performed in real time within 24 hours after blood specimen collection. See Supplementary Methods for additional details.

ECFC Proliferative Capacity Measurements

The ECFCs used in the functional capacity experiments were isolated from human umbilical cord blood specimens and cultured as previously described [29]. These cord blood ECFCs were passaged on collagen-coated dishes in complete Endothelial Cell Growth Medium 2 (EBM-2; Lonza, Walkersville, MD). The cells were allowed to adhere to the dish for a minimum of 6 hours. The puc2CL6IEGwo viral supernatant (Supplementary Methods) was then used to transduce the ECFCs with green fluorescent protein for visualization using IncuCyte software; this viral supernatant was added directly

to the culture medium and incubated overnight at 37°C in 5% CO_2 as previously described [29]. Medium was aspirated the next morning, and the cells were washed with phosphate-buffered saline, and fresh EGM-2 was added. The green fluorescent protein–expressing ECFCs were documented using flow cytometry as previously described [29].

Ninety-six–well plates were coated with Matrigel basement membrane matrix (Corning Discovery Labware, Bedford, MA) at 37° C for 30 minutes as previously described [30]. A total of 1×10^4 ECFCs in 100 μL of EBM-2, supplemented with either 10% defined fetal bovine serum (HyClone, Logan, UT) for control or 2.5% of each participant plasma sample, were placed onto the matrix and incubated at 37° C for 12 hours in the IncuCyte ZOOM (Sartorius, Ann Arbor, MI). Eight cord blood ECFC proliferation measurements were analyzed with IncuCyte ZOOM angiogenesis software (version 2016A). These included network branch points (the sum of the branch point counts of all of the capillary networks in the image divided by the image area, $1/mm^2$), cell area (the sum of the areas of all the cells in the image divided by the image area, mm^2/mm^2), the average tube width uniformity (the average tube width uniformity of all of the networks in the image, or a measure of how parallel the edges of the tube are to the skeleton mask that is generated after processing), networks (the number of total networks within an image divided by the image area, in 1/square millimeters), the network area (the sum of the areas of all of the networks in the image divided by the image area, in square millimeters/square millimeters); network length (the sum of the lengths of all of the networks in the image divided by the image area, in millimeters/square millimeters), the average network length (the average length of all the networks in the image divided by the image area, in millimeters/square millimeters), and the average tube width (the average of the tube widths of all the networks in the image, in micrometers). All IncuCyte data were obtained from 2 independent experiments with triplicate samples in each experiment.

Statistical Analysis

Continuous variables were summarized by means and standard deviations (SDs) and were compared across the 3 study groups, using 1-way analysis of variance (ANOVA). Categorical variables were summarized by counts and percentages and were compared using the Fisher exact test.

Bonferroni correction was adopted for the 8 ECFC functional parameters, the primary study outcomes, at a type I error of 0.05; hence, a P value of <.00625 was considered statistically significant. If one of these parameters was significantly different across the 3 groups, pairwise comparisons were then conducted with further Bonferroni correction with a multiplicity of 3; hence, a P value of <.00208 was considered statistically significant.

ECFCs and their functional parameters were further examined with respect to their Pearson correlations with immunologic markers. For these correlations, the linear step-up method was adopted to adjust for multiple testing at a false-discovery rate of 20%.

Soluble angiogenesis biomarkers were compared across groups by using ANOVA. Their correlations with ECFCs and their functional parameters were calculated as exploratory analyses, so no corrections for multiple testing were performed for these analyses. All analyses were performed using SAS (SAS Institute, Cary, NC).

RESULTS

The characteristics of the study groups are shown in Table 1. The 3 groups were similar in terms of age, sex, current smoking, body mass index, systolic blood pressure, lipid levels, and 10-year Framingham risk scores. The uninfected group had fewer black participants than the 2 HIV-positive groups. The 2 HIV-positive study groups had similar $CD4^+$ T-cell counts, with mean values of >500 cells/μL. All of the infected, untreated participants were ART naive.

ECFC Parameters

There were no differences ($P = .67$) in ECFC percentages, based on flow cytometry findings, among the study groups (Table 1). Adjustments for demographic characteristics or smoking status did not change this result (data not shown). However, there were differences when examining patient-derived, plasma-treated cord blood ECFC proliferative capacity parameters. As shown in Table 2, there were significant differences among the groups (all $P < .00625$) in cell area, network length, network branch points, networks, and average tube width uniformity. Adjustments for demographic characteristics and smoking did not change these results (data not shown). There were significantly lower cell areas, network lengths, and network branch points in the infected, untreated group as compared to the uninfected group ($P < .00208$ for all comparisons). When comparing the 2 infected groups, significantly lower cell areas, network lengths, network branch points, and average tube width uniformities were found in the infected, untreated group, compared with the infected, treated group ($P < .00208$ for all comparisons). Trends ($.00208 < P < .05$) were found for lower network areas, network lengths, network branch points, average tube widths, and networks in the infected, treated group as compared to the uninfected group. Figure 1 shows representative IncuCyte images from the 3 study groups, demonstrating the reduced capillary network formation in the uninfected group as compared to the 2 infected groups.

Monocyte Parameters

There were no differences ($P > .10$ for all comparisons) among the study groups in any of the 3 monocyte subsets or in sCD14

Table 1. Clinical Characteristics, Endothelial Colony-Forming Cell (ECFC) Percentage, and Monocyte Parameters of the 3 Study Groups

Characteristic	Uninfected (n = 15)	Infected, Untreated (n = 8)	Infected, Treated (n = 15)	P[a]
Age, y	37.45 ± 12.06	34.46 ± 8.03	43.90 ± 9.59	.09
Male sex	9 (60)	5 (62.50)	13 (86.67)	.26
Black race	1 (6.67)	5 (62.50)	9 (60.00)	.003
$CD4^+$ T-cell count, cells/μL	NA	525.75 ± 220.27	584.47 ± 323.73	.65
HIV-1 RNA load, copies/mL	NA	56 889 ± 89 227	<100[b]	.001
Current smoker	0 (0)	1 (12.50)	4 (26.67)	.08
BMI[c]	31.23 ± 8.80	30.22 ± 7.61	30.97 ± 7.16	.96
Systolic blood pressure, mm Hg	115.07 ± 11.21	118.75 ± 13.98	121.40 ± 14.32	.42
Cholesterol level, mg/dL				
Total	162.71 ± 40.17	158.00 ± 37.91	177.08 ± 40.50	.53
LDL	89.64 ± 27.62	92.33 ± 32.67	101.21 ± 36.15	.62
HDL	48.00 ± 17.65	43.67 ± 11.06	53.23 ± 13.75	.42
Triglycerides level, mg/dL	125.57 ± 70.03	110.33 ± 41.74	140.85 ± 82.72	.68
Framingham 10-y risk score, %	0.6 ± 7.2	1.3 ± 4.7	6.2 ± 6.2	.076
ECFCs, %	0.05726 ± 0.1677	0.02226 ± 0.0150	0.07358 ± 0.1380	.67
$CD14^{++}CD16^-$ cells, %	67.26 ± 18.41	63.28 ± 12.39	63.68 ± 17.36	.75
$CD14^+CD16^+$ cells, %	1.84 ± 1.36	2.48 ± 2.32	1.37 ± 1.35	.22
$CD14^{dim}CD16^+$ cells, %	0.72 ± 0.94	3.52 ± 4.42	4.25 ± 7.77	.11
sCD14 level, ng/mL	1485 ± 242	1395 ± 284	1565 ± 407	.49
sCD163 level, ng/mL	547 ± 215	1006 ± 252	469 ± 187	<.0001

Data are mean ± SD or no. (%) of participants. Participants were HIV negative (uninfected group), HIV positive and not receiving antiretroviral therapy (ART; infected, untreated group), or HIV positive and receiving ART (infected, treated group).

Abbreviations: HDL, high-density lipoprotein; HIV-1, human immunodeficiency virus type 1; LDL, low-density lipoprotein; NA, not applicable; sCD14, soluble CD14; sCD163, soluble CD163.

[a]By analysis of variance, with values of <.05 considered statistically significant.

[b]All values were <20 copies/mL except for those for 2 participants, who had levels of 80 and 75 copies/mL.

[c]Body mass index (BMI) is calculated as the weight in kilograms divided by the height in meters squared.

levels (Table 1), even when adjusted for demographic characteristics and smoking status (data not shown). But we did detect differences in sCD163 levels ($P < .0001$), with the highest levels in the infected, untreated group.

Correlations Between ECFC Parameters and Monocyte Parameters

We then correlated ECFC percentages enumerated by flow cytometry and each of the 8 participant-derived, plasma-treated cord blood ECFC proliferative capacity measures with the 3 monocyte subsets, 2 monocyte activation markers, the $CD4^+$ T-cell count, and the HIV-1 RNA level (Supplementary Table 3). At an FDR of 0.20, we found that $CD14^{++}CD16^-$ monocytes were significantly negatively correlated with both network area and network length in the infected, treated group. $CD14^+CD16^+$ monocytes were significantly positively correlated with the ECFC percentage in the uninfected group;

Table 2. Plasma-Treated Cord Blood Endothelial Colony-Forming Cell (ECFC) Proliferative Capacity Functional Parameters of the 3 Study Groups

Characteristic	Uninfected (n = 15)	Infected, Untreated (n = 8)	Infected, Treated (n = 15)	P[a]
Network area, mm/mm^2	0.11 ± 0.037	0.076 ± 0.031	0.10 ± 0.046	.018
Cell area, mm^2/mm^2	0.19 ± 0.034	0.14 ± 0.022[b,c]	0.18 ± 0.051	.0008
Network length, mm/mm^2	2.62 ± 0.48	1.73 ± 0.43[b,c]	2.38 ± 0.56	<.0001
Network branch points, $1/mm^2$	7.27 ± 2.25	3.29 ± 1.54[b]	5.71 ± 2.86	<.0001
Average tube width, μm	42.39 ± 0.85	43.14 ± 0.60	42.88 ± 0.33	.023
Networks, $1/mm^2$	4.07 ± 0.36	3.80 ± 0.25[c]	4.42 ± 0.53	.0053
Average network length, mm/mm^2	1.14 ± 0.33	0.94 ± 0.54	0.89 ± 0.18	.13
Average tube width uniformity	0.68 ± 0.0064	0.67 ± 0.0114[b]	0.68 ± 0.0060	.0028

Participants were human immunodeficiency virus (HIV) negative (uninfected group), HIV positive and not receiving antiretroviral therapy (ART; infected, untreated group), or HIV positive and receiving ART (infected, treated group).

[a]Values of <.00625 were considered statistically significant for group differences across all 8 parameters. Values of <.00208 were considered statistically significant for pairwise comparisons for each individual parameter.

[b]Statistically significant pairwise comparison for the infected, untreated group versus the uninfected group.

[c]Statistically significant pairwise comparison for infected, untreated group versus the infected, treated group.

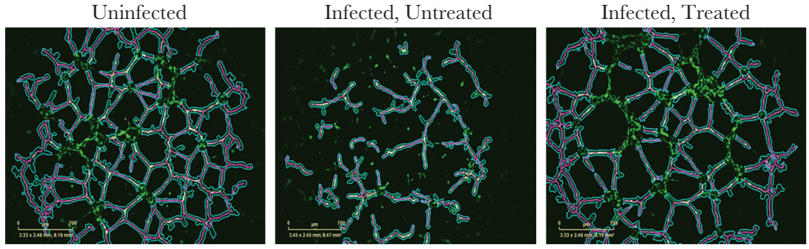

Figure 1. Phase contrast images, with both network and skeletal masks, of representative capillary networks from individuals who were human immunodeficiency virus (HIV) negative (uninfected), HIV positive and not receiving antiretroviral therapy (ART; infected, untreated), or HIV positive and receiving ART (infected, treated), using the IncuCyte live image analysis platform. The skeletal mask is set to identify intact vascular cords with single endothelial cells lining the walls of the capillary-like structures. Cord blood endothelial colony-forming cells were treated with plasma from the study participants. The capillary network from the infected, untreated participant and, to a lesser extent, from the infected, treated participant, was appreciably reduced as compared to that from the uninfected control participant.

negatively correlated with the network area in the infected, treated group and the overall group; negatively correlated with the cell area in the infected, treated group, both infected groups combined, and the overall group; negatively correlated with network length in both infected groups combined and the overall group; negatively correlated with network branch points in the infected groups combined; and positively correlated with average network length in the infected, treated group. sCD14 was positively correlated with the average network length in the uninfected group, whereas sCD163 was negatively correlated with both cell area and network branch points in the infected groups combined. ECFC percentage, $CD14^{dim}CD16+$ monocytes, $CD4^{+}$ T-cell counts, and HIV-1 RNA levels were not correlated with any plasma-treated cord blood ECFC proliferative capacity variables.

Correlations Between ECFC Proliferative Capacity and Soluble Angiogenesis Markers

As we found that plasma from the infected study participants had a negative effect on several proliferative capacity measures, we then explored whether soluble angiogenesis factors may explain these findings. Table 3 shows the comparative values from the panel of circulating angiogenesis biomarkers assessed in this study. CCL5, sVCAM-1, VEGFR1, and VEGFR2 were the only biomarkers for which statistically significant differences (all $P < .05$) were found among the 3 study groups; the levels of these 4 markers were higher in the infected study groups as compared to the uninfected control group.

We then correlated these angiogenesis biomarkers with the 8 plasma-treated cord blood ECFC proliferative capacity measures to determine if these factors were negatively associated

Table 3. Soluble Angiogenesis Biomarker Levels Among the 3 Study Groups

Biomarker	Uninfected (n = 15)	Infected, Untreated (n = 8)	Infected, Treated (n = 15)	P[a]
hsCRP level, ng/mL	8806.40 ± 9709.69	8392.34 ± 10601.47	6822.13 ± 7213.54	.82
CCL5 level, pg/mL	26887 ± 17138	55074 ± 28515	45153 ± 31821	.04
sVCAM-1 level, ng/mL	781.01 ± 179.64	991.61 ± 301.43	687.78 ± 116.82	.01
Angiostatin level, pg/mL	82619 ± 42952	124059 ± 88659	131358 ± 62571	.09
E-selectin level, pg/mL	98239 ± 49949	100662 ± 47471	95222 ± 40799	.96
VEGFR1 level, pg/mL	686.71 ± 417.08	825.35 ± 448.70	1269.13 ± 603.11	.01
VEGFR2 level, pg/mL	13126 ± 3426	13522 ± 4180	20005 ± 7055	.01
VEGF-A level, pg/mL	60.60 ± 59.62	93.90 ± 132.40	167.17 ± 152.40	.06
IL-6 level, pg/mL	4.65 ± 3.19	3.54 ± 2.71	4.85 ± 3.50	.63
IL-8 level, pg/mL	3.46 ± 2.11	3.01 ± 1.39	5.74 ± 4.64	.09
CCL2 level, pg/mL	292.20 ± 90.18	298.65 ± 119.06	422.97 ± 254.76	.11
sTNFR1 level, pg/mL	1311.94 ± 348.59	1127.93 ± 449.59	1339.55 ± 385.15	.43
sTNFR2 level, pg/mL	6481.50 ± 1898.91	8313.06 ± 2483.12	6869.28 ± 1927.46	.13
Angiopoietin level, pg/mL	1163.76 ± 370.45	1217.78 ± 604.05	1788.01 ± 1287.52	.14
Endoglin level, pg/mL	1121.46 ± 448.55	1573.02 ± 558.73	1434.22 ± 606.09	.12
FGF-2 level, pg/mL	77.76 ± 36.21	74.79 ± 48.63	134.64 ± 138.65	.19

Data are mean ± SD. Participants were human immunodeficiency virus (HIV) negative (uninfected group), HIV positive and not receiving antiretroviral therapy (ART; infected, untreated group), or HIV positive and receiving ART (infected, treated group).

Abbreviations: FGF-2, fibroblast growth factor 2; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; IL-8, interleukin 8; sTNFR1, soluble tumor necrosis factor receptor 1; sTNFR2, soluble tumor necrosis factor receptor 2; sVCAM-1, soluble vascular cell adhesion molecule 1; VEGF-A, vascular endothelial growth factor A; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2.

[a]By analysis of variance, with values of <.05 considered statistically significant.

with ECFC function (Supplemental Table 5). We did not find significant correlations between CCL5, sVCAM-1, VEGFR1, and VEGR2 and any proliferative capacity measure in the infected, untreated group. In the infected, treated group, VEGFR1 was significantly correlated with network area (r = 0.65; P = .0074) and network length (r = 0.56; P = .029); no significant correlations were found with CCL5, sVCAM-1, or VEGFR2 in this group. Of note, we found hsCRP to be negatively correlated with network area, network length, and network branch points in the infected, untreated group (−0.78 < rho < −0.73; all P < .04).

DISCUSSION

To our knowledge, this is the first study to comprehensively assess the effects of HIV infection and ART on ECFC function. We found that several plasma-treated cord blood ECFC proliferative capacity measures are reduced in infected, untreated patients, compared with uninfected controls and infected, treated patients. In addition, intermediate monocytes may be specifically associated with this ECFC dysfunctional regenerative potential in HIV infection, as we did not find similar correlations in the uninfected control group. These findings thereby suggest a possibly unique connection underlying the relationship between monocyte inflammation and vascular dysfunction in HIV-positive patients.

We could not replicate our initial study, which found the ECFC percentage to be significantly increased in HIV-positive patients treated with the regimen of efavirenz/tenofovir disoproxil fumarate/emtricitabine [20]. In the current study, infected, treated patients receiving regimens comprised of tenofovir/emtricitabine and an integrase inhibitor did not have a higher ECFC percentage than uninfected controls or infected, untreated patients, thus suggesting a specific effect of efavirenz on the ECFC percentage. Given that we and others have previously found deleterious effects of efavirenz on the endothelium [22–24], it is plausible that the increased ECFC percentage with efavirenz may reflect a reflexive increase in ECFC numbers to repair drug-induced endothelial damage, which is not found with more-modern integrase inhibitor therapies.

We then extended our study by assessing ECFC function with regard to proliferation of cord blood ECFCs into new capillary networks when treated with participant plasma. We have previously reported that patients with peripheral arterial disease leading to amputation possess circulating ECFCs and resident arterial ECFCs that have reduced ECFC function characterized by limited proliferative potential and lack in vivo capillary forming potential [18]. Of interest, both human embryonic stem cells and induced pluripotent stem cells give rise to ECFCs that display properties similar to umbilical cord blood ECFCs when differentiated along a specific differentiation protocol [18]. These human embryonic stem cell–derived ECFCs and human induced pluripotent stem cell–derived ECFC can rescue the avascular regions in the eyes of newborn mice exposed to high oxygen tension. These ECFCs also could regenerate the vascular endothelium in mice that had undergone femoral artery excision and to restore blood flow significantly better than control treated mice [18]. Thus, ECFC function may have both physiological and clinically relevant implications. The IncuCyte system has been used to date to primarily assess tumor proliferation characteristics [31–33] and only recently has been used to assess endothelial cell properties [34]. To our knowledge, the IncuCyte system for assessing cord blood ECFC proliferative capacity has never been used in the context of assessing potential vascular reparative parameters in patients with HIV infection. The IncuCyte system assesses cell proliferation through live imaging analysis and can provide a series of angiogenesis readouts. Our investigation using 8 specific capillary formation readouts suggests that plasma from the infected, untreated participants significantly reduced 3 angiogenesis capacity parameters and nonsignificantly reduced 4 others, compared with uninfected controls, thereby suggesting a global effect of plasma from infected, untreated individuals on ECFC proliferation. Of note, several proliferative capacity parameters were lower in the infected, treated group as compared to uninfected controls but did not achieve statistical significance, based on our stringent Bonferroni-corrected P values; future larger-scale studies are needed to determine whether virologic suppression truly does not fully normalize plasma-induced cord blood ECFC proliferation. It should be noted that we assessed each IncuCyte proliferative capacity readout individually in this study because it is yet unknown which of these is clinically relevant in HIV. Additional research should be performed to determine the relationships between these 8 parameters and physiologic endothelial function, atherosclerosis development, and actual CVD events.

Although the 3 monocyte subsets assessed by flow cytometry were not significantly different among the 3 study groups, we found that $CD14^{+}16^{+}$ intermediate monocytes (and, to a lesser degree, $CD14^{++}CD16^{-}$ monocytes) were negatively correlated with several plasma-treated cord blood ECFC proliferative capacity parameters in both the infected, treated group and the combined infected study groups. These results suggest that these activated, intermediate monocytes play an adverse role in impairing HIV-related vascular regenerative potential and that virologic suppression with ART does not abrogate this impairment. This finding is in line with other recent publications suggesting that activated monocytes are linked to atherosclerosis progression during HIV infection [21]. In addition, we found that the monocyte activation marker sCD163 was negatively correlated with several ECFC functional parameters, which is line with other studies demonstrating that sCD163 is associated with increased noncalcified coronary plaque and arterial inflammation during HIV infection [2, 35, 36].

We then examined in an exploratory analysis if soluble angiogenesis factors may explain how plasma from infected

patients led to impaired cord blood ECFC proliferative capacity. Although CCL5, sVCAM-1, VEGFR1, and VEGFR2 levels were significantly elevated in the infected groups as compared to the uninfected controls, we did not find evidence of negative relationships between these markers and plasma-treated cord blood ECFC proliferative capacity measures in either infected study group. In fact, there was evidence of positive relationships between VEGFR1 and both network area and network length, perhaps suggesting that VEGFR1, a marker of proangiogenesis activity, was reflexively increased in response to reduced ECFC functional capacity during HIV infection. We did not find that any of the angiogenesis biomarkers measured was negatively correlated with plasma-treated cord blood ECFCs in the infected, treated group. Thus, additional research will be needed to identify the soluble factors that negatively affect cord blood ECFC function in HIV-positive patients. Of interest, hsCRP levels were negatively correlated with several ECFC proliferative capacity measures in the infected, untreated group, which supports other studies demonstrating hsCRP as a predictor of CVD events during untreated HIV infection [37].

There are several limitations to our study. First, we could not assess causal relationships due to the cross-sectional nature of the analysis. Although samples sizes in the infected groups were larger than in our previous study, we acknowledge that the modest sizes of the study groups in the current study, especially in the infected, untreated group, may not have been adequate power to detect differences in the ECFC percentage or to detect significant correlations between the ECFC and monocyte parameters, especially given our conservative approach to correction for multiple testing. We cannot extend our findings to HIV-positive patients receiving different ART regimens, although the regimens chosen in this study are not known to cause endothelial damage, thereby limiting confounding effects by treatment.

In summary, although we did not find that the numbers of ECFCs as determined by flow cytometry differed between those with and those without HIV infection, regardless of ART use, plasma-treated cord blood ECFC function was impaired in the infected, untreated group (and may be modestly impaired in the infected, treated group), compared with uninfected controls. Intermediate $CD14^{+}CD16^{+}$ monocyte proportions were negatively associated with several proliferative capacity parameters in the infected groups combined. Future research should be directed at understanding causal mechanisms by which monocyte inflammation may impair endothelial regenerative potential and how this consequently could lead to the higher risk of CVD observed in HIV-positive patients.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interests. S. K. G reports having received consultancy/advisory fees from Gilead Sciences, GSK-ViiV, and BMS and travel support to present study results at conferences from Gilead Sciences. All other authors report no potential conflicts.

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