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Dysfunctional HDL from HIV+ individuals promotes monocyte-derived foam cell formation *in vitro*.

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Running title: HDL function and atherogenesis in HIV

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ABSTRACT

Objective: The role of HDL function in HIV-related atherosclerotic cardiovascular disease (CVD) is unclear. HDLs isolated from HIV+ [HIV(+)HDL] and HIV-uninfected individuals (HDL) were assessed for HDL function and ability to promote monocyte-derived foam cell formation (MDFCF) (a key event in HIV-related CVD) *ex vivo*.

Design/Methods: Using an established *in vitro* model of atherogenesis and plasma samples from an established cross-sectional study of virologically-suppressed HIV+ males on stable effective antiretroviral therapy (ART) and with low CVD risk (median age: 42 years; n=10) , we explored the impact of native HDL [HIV(+)HDL] on MDFCF. In this exploratory study we selected HIV-HDL known to be dysfunctional based on two independent measures of impaired HDL function: a) antioxidant (high HDL_{ox}) b) ability of HDL to release apoA-I [low HDL-apoA-I exchange (HAE %)]. Five healthy males matched by age and race to the HIV+ group were included. Given that oxidation of HDL leads to abnormal HDL function, we also compared proatherogenic effects of HIV-HDL versus chemically-derived HDL_{ox}. The *ex vivo* atherogenesis assay was performed using lipoproteins (purchased or isolated from plasma using ultracentrifugation) and monocytes purified via negative selection from healthy donors.

Results: HIV(+)HDL known to have reduced antioxidant function and rate of HDL/ApoAI exchange promoted MDFCF to a greater extent than HDL (33.0% vs 26.2% foam cells; p=0.015). HDL oxidized *in vitro* also enhanced foam cell formation as compared to non-oxidized HDL (p<0.01).

Conclusion: Dysfunctional HDL in virologically suppressed HIV+ individuals may potentiate atherosclerosis in HIV infection by promoting monocyte-derived foam cell formation.

Subject codes: Inflammation, Lipids and Cholesterol, Vascular Biology, Oxidant Stress, Atherosclerosis

Key Words: HDL function, Human Immunodeficiency Virus, cardiovascular disease, atherosclerosis, monocyte-derived foam cells

ACCEPTED

BACKGROUND

Despite potent antiretroviral therapy (ART) HIV-1-infected (HIV+) individuals have an approximately 2-fold increased risk of atherosclerotic cardiovascular disease (CVD), but the mechanisms are unclear^[1]. High-density lipoprotein cholesterol (HDL-C) levels are a powerful independent negative predictor of CVD, however, HDL structure and function rather than absolute level may more accurately predict atherosclerosis^[1]. HIV+ ART-treated individuals have a higher prevalence of dyslipidemia, low HDL levels^[1], impaired lipoprotein metabolism and increased HDL lipid hydroperoxide content and redox activity (HDL_{ox})^[2] that are associated with CVD in some, but not all studies^[3-5]. In HIV-uninfected (HIV-) persons, abnormal HDL function strongly correlates with CVD^[6], but there is limited evidence in HIV infection.

Cell-based assays such as cholesterol efflux assays^[7] have been used to determine HDL function, but have numerous drawbacks including heterogeneity with regards to cells used, reported readout, and standardization^[3, 4]. Cell-free assays may give more robust measures of HDL function compared with cell-based assays^[4]. We have developed a novel cell-free fluorometric method that measures HDL-associated lipid peroxidation (HDL_{ox}) and hence reduction of HDL antioxidant function^[3, 4]. In HIV+ ART-treated participants with low CVD risk, the readout from our assay correlated with coronary artery calcium score, carotid intima media thickness (cIMT; surrogate measures of subclinical atherosclerosis) and macrophage activation^[3-5]. Furthermore, impaired HDL antioxidant function (as measured by higher HDL_{ox}) was associated with impaired HDL remodeling^[8] (a CVD-relevant measure of HDL function^[9]) using a cell free assay that measures the ability of HDL to release apoA-I (HDL-apoA-I

exchange (HAE))^[9, 10]. Thus, use of two independent cell-free assays of HDL activity may more accurately reflect HDL dysfunction in treated HIV+ persons.

Monocytes play a central role in the development of atherosclerosis, and HIV-related chronic monocyte activation may contribute to increased CVD, but the mechanisms are unknown. Using an established *in vitro* model of early atherosclerotic events, we have shown that monocytes from HIV+ individuals have a greater potential to develop into lipid-laden foam cells (which are associated with plaque progression) following transendothelial migration than monocytes from age-matched HIV- controls^[11]. Furthermore, we found that serum factors from ART-treated HIV+ individuals further promote monocyte-derived foam cell formation (MDFCF)^[11]. We have shown that dysfunctional HDL persists in virologically suppressed HIV+ individuals^[3, 4] on potent ART and may be the circulating factor that directly promotes MDFCF, a key event in HIV-related CVD. This research question can be reliably studied in a large prospective study of HIV-1 infected persons on chronic ART and well matched uninfected controls, where complex effects of confounders on MDFCF need to be dissected. Given the above complexities, known limitations of all HDL function assays, and lack of any preliminary data that support the role of dysfunctional HDL in promoting MDFCF, it would be difficult to design such as prospective study. Thus, to address this question, we conducted an exploratory study using a subset of samples from an established cohort of HIV+ individuals on potent ART with low CVD risk specifically selected to be matched for relevant factors. We isolated native HDL from a homogeneous group of HIV+ persons and incubated it directly with monocytes from healthy participants (to dissect effects of HIV(+)HDL, independent to the documented effect of HIV on MDFCF^[11] in an established *ex vivo* model of early atherosclerotic events.

METHODS

Detailed methods are described in the Supplemental Material, <http://links.lww.com/QAD/B159>.

Study Design and Participants

This work utilized samples from the Center for Clinical AIDS Research and Education (CARE) HDL function study; a cross-sectional study investigating impaired HDL function among viremic and virologically-suppressed HIV+ males on stable ART^[8]. Sociodemographic and clinical characteristics, and measures of HDL function (HDL_{ox}, HAE %) have been described^[8]. To avoid confounding effects from gender^[12], inflammation (other than HIV), traditional CVD risk factors and ART on atherogenesis, we selected HIV+ persons with the following inclusion criteria: i) males, ii) no known inflammatory comorbidities other than HIV, iii) no known CVD or risk factors for CVD (e.g. metabolic syndrome, diabetes, dyslipidemia, use of lipid lowering medication, hypertension, family history of CVD, Framingham Risk Score $\geq 6\%$), iv) on stable and identical ART > 6 months prior to visit and v) with dysfunctional HDL as defined by two independent cell-free assays of HDL function. In the setting of our exploratory study, we selected 10 HIV-1 infected persons from this group who had the highest HDL_{ox} and lowest HAE to explore the question whether dysfunctional HDL in the setting of low CVD risk directly upregulates MDFCF. Five healthy males matched by age and race to the HIV+ group were also included. All individuals enrolled in the study provided written informed consent and the study was approved by the local Institutional Review Boards.

Lipoprotein preparation, oxidation and determination of HDL function

HDL was purchased (EMD Millipore, Billerica, MA) or isolated from plasma using ultracentrifugation and lipoproteins were oxidized *in vitro* as previously [9, 10]. HDL_{ox} was quantified using a validated fluorometric assay that measures HDL lipid peroxidation based on the oxidation of the fluorochrome Amplex Red [9, 10]. HAE assays were performed as previously described [8]. Different aliquots of identical HDL were used for the HDL_{ox}, HAE and the foam cell assays.

In vitro model of monocyte migration and foam cell formation

The *in vitro* MDFCF assay was performed using monocytes purified via negative selection (Miltenyi Biotec, Cologne, Germany) from freshly-isolated peripheral blood mononuclear cells from healthy donors [11]. Monocytes were pre-incubated with 20 µg/mL donor or commercial HDL (unmodified or oxidized with copper(II) sulfate (CuSO₄) or 13(S)-hydroperoxy-9Z, 11E-octadecadienoic acid (13(S)-HPODE)) in serum-free media for 1 hr. Monocytes were then added to Tumor necrosis factor (TNF)-activated human umbilical vein endothelial cells (HUVEC) monolayers on type I fibrous collagen gels to transmigrate and form foam cells as previously described (Supplemental Figure 1, <http://links.lww.com/QAD/B159>). Lipids were maintained in the media throughout the experiment.

RESULTS

Baseline characteristics

Baseline characteristics of the study participants are shown in Table 1. HIV+ participants (median age: 42 years; n=10) had suppressed viremia (<50 copies/mL) and the group overall had a low CVD risk (Framingham Risk Score < 6%). All HIV+ persons were on stable ART > 6 months prior to visit with efavirenz/emtricitabine/tenofovir disoproxil fumarate (EFV/TDF/FTC). HIV+ and control participants had comparable lipid profiles, Framingham Risk Score and rates of CMV seropositivity.

Dysfunctional HDL present in virologically suppressed, ART-treated HIV-1 infection directly increases MDFCF

To investigate whether HDL dysfunction (as indicated by cell-free assays) was associated with proatherogenic effects, we used isolated HDL, assessed to be dysfunctional, from ART-treated HIV+ individuals [(HIV(+))HDL, n=10)] and compared their influence on monocyte-derived foam cell formation *in vitro* to HDL isolated from healthy controls (HIV(-) HDL, n=5). The decreased function of the isolated HDL assessed using 2 independent assays is presented in Figure 1. Median HDL_{ox} of HIV+ persons was ~50% higher compared with uninfected participants (Figure 1A, p<0.001). ART-treated HIV+ subjects had ~35% lower median %HAE compared with HIV- participants (p<0.001, Figure 1B, Table 1).

Next, isolated donor HDL were pre-incubated with monocytes from HIV- individuals prior to (and maintained in culture throughout) analysis in our *in vitro* foam cell model (Supplemental Figure 1 , <http://links.lww.com/QAD/B159>). When media containing

dysfunctional HIV(+)HDL was added to TNF-activated HUVEC , a significantly increased proportion of monocytes differentiated into foam cells as compared with the same monocytes exposed to media containing HDL from HIV- persons (median foam cells 33% vs 26.2%, respectively, $p=0.015$, Figure 1C; from $n=3$ replicate experiments using monocytes from 3 individual HIV- donors).

***In vitro* oxidized HDL directly increases MDFCF**

Given that oxidation of HDL leads to abnormal HDL function, we next compared the magnitude of the proatherogenic effects of HIV(+)HDL with chemically-derived HDL_{ox}. *In vitro* oxidation of lipoproteins using CuSO₄, although widely used, has uncertain physiological relevance since this type of oxidation does not occur *in vivo*. Therefore, we also used 13(S)-HPODE, a potent *in vivo*-generated lipid that renders HDL dysfunctional^[8] as a comparator. Both forms of HDL_{ox} were associated with a significant increase in foam cell formation by healthy monocytes following transendothelial migration as compared with monocytes incubated with unmodified HDL ($p=0.004$ and 0.0006 for CuSO₄ and 13(S)-HPODE HDL_{ox} vs unmodified HDL, respectively; Figure 1D). Indeed, the direct stimulation of foam cell formation with HDL_{ox} generated with the more physiological reagent 13(S)-HPODE (Figure 1) was comparable to the effects of HIV(+)HDL (Figure 1C).

DISCUSSION

In this exploratory study, we found that HIV(+)HDL from HIV+ individuals on effective ART had abnormal HDL function based on two independent cell-free assays and increased MDFCF in an established *in vitro* model of atherogenesis. Notably, the selected HIV+ participants were all receiving a Nucleoside reverse transcriptase inhibitors (NRTI)/ Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) regimen that is associated with a more favorable lipid profile compared with Protease inhibitor (PI) -containing regimens, and had low overall CVD risk. Given our previous finding that monocytes isolated from HIV+ donors have heightened MDFCF potential ^[11], it is possible that dysfunctional HDL from HIV+ persons may synergize with these proatherogenic monocytes, resulting in even more prominent effects on foam cell formation. We also showed that treatment of monocytes with HDL oxidized *in vitro* by two different mechanisms enhanced MDFCF. Given our prior data that dysfunctional HDL in chronic treated HIV is oxidized, this suggests that oxidation underlies the atherogenic properties of HIV(+)HDL. Our study is among the first that provides important mechanistic insight into the role of dysfunctional HDL as a possible driver of CVD in chronic, treated HIV infection. Our hypothesis is shown in Supplemental Figure 2 , <http://links.lww.com/QAD/B159>. Elucidating the mechanisms driving HDL-induced foam cells may identify oxidized HDL as a potential therapeutic target to reduce HIV-related CVD risk.

In vitro studies indicate that oxidative modification of HDL may impair cholesterol efflux activity and inhibit HDL remodeling/exchange of apoA-I ^[8], a CVD-relevant measure of HDL function ^[11]. We previously showed that dysfunctional HDL with impaired antioxidant HDL function (as measured by higher HDL_{ox}) in HIV+ individuals on long-term ART and without

clinical CVD i) was associated with *in vivo* progression of CVD^[4], ii) may stimulate endothelial cells to induce monocyte/macrophage chemotaxis^[3,4], iii) was positively correlated with non-calcified coronary atherosclerotic plaque^[5], iv) independently correlated with several markers of inflammation and immune activation^[9] and v) was associated with impaired HDL remodeling^[8]. Here we show that foam cell formation is enhanced when monocytes were exposed to chemically-oxidized HDL thus linking HDL oxidation and impaired HDL function to processes which have a central role in HIV-related CVD.

The strengths of our study are the careful covariate phenotyping of our selected study population, including novel measures of HDL function and established physiologically relevant model of atherogenesis. Of note, MDFCF is not considered a function of HDL and thus the hypothesis that 2 abnormal HDL functions lead to a third impaired HDL function cannot explain our finding that dysfunctional HDL directly promotes MDFCF. However, all assays of HDL function have limitations^[10] and HDL proteomics and cell-based cholesterol efflux assays were not performed. Other important limitations of our study include the small sample size and the inclusion of men only. Larger studies are needed to elucidate the differential effects of dysfunctional HDL on MDCF given the complex and unclear underlying physiological modulators of lipid metabolism responsible for the differences between men and women^[12]. For our *in vitro* assay we used monocytes from HIV(-) donors only and, as mentioned above, it is possible that foam cell formation in response to HIV(+) HDL might be further enhanced in monocytes from ART-treated and/or HIV-infected individuals. Here, we preselected HIV+ persons with evidence of dysfunctional HDL from a retrospective study, to specifically assess whether this dysfunction was associated with altered MDFCF. Such an approach is reasonable in the setting of a hypothesis-generating study, and we were able to demonstrate direct *ex vivo*

proatherogenic effects of dysfunctional HDL. These findings will inform larger studies to elucidate the mechanisms responsible for heightened MDFCF, and determine whether these effects may be more prominent in older HIV-1 infected persons and/or those with higher CVD risk.

In conclusion, our data provide important mechanistic insights into the role of dysfunctional HDL as possible driver of increased atherosclerotic risk in this population which warrants further investigation in larger prospective studies of subclinical atherosclerosis and CVD clinical events.

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Conflicts of Interest:

M.N.O. is a founder of and owns a significant stake in Seer Biologics, Inc. but the content of this manuscript provides no benefit to Seer BioLogics, Inc. Potential benefit in no way influenced the thoroughness, stringency, interpretation and presentation of this manuscripts content. All other authors have no conflicts of interest to disclose.

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Table 1: Characteristics by group.

	HIV+ ON ART (n=10)	HIV (-) (n=5)	P value
Demographics			
Age (years)	42 (10.5)	38.5 (15.0)	0.832
Race/Ethnicity (Non-Hispanic White)	10 (100%)	5 (100%)	(-)
Body Mass Index (BMI) (kg/m²)	23.8 (4.3)	22.7 (3.6)	0.524
HIV related parameters and lipids			
Current CD4 T cell count (cells/m³)	550 (325)	ND	(-)
Viral load (copies/ml)	<50	NA	(-)
CMV seropositive (IgG) (%)	9 (90%)	4 (80%)	0.915
ApoA-I (mg/dL)	121.4 (26.4)	166.6 (63.0)	<0.01
Total cholesterol [mg/dL]	130 (45.0)	118.0 (42.0)	0.884
Triglycerides [mg/dL]	114.0 (54.0)	97 (27.0)	0.859
HDL cholesterol [mg/dL]	36.0 (14.0)	41.0 (17.0)	0.874
LDL cholesterol [mg/dL]	84.5 (31.6)	74.2 (34.1)	0.905
Non-HDL cholesterol [mg/dL]	115.0 (31.0)	105.5 (42.3)	0.903
LDL-cholesterol/HDL-cholesterol ratio	2.3 (0.4)	1.8 (0.4)	0.234
HDL function measurements			
HDL_{ox} (normalized ratio to pooled control)	1.416 (0.7)	0.879 (0.1)	<0.001
%HAE	35.9 (12.6)	53.6 (7.1)	<0.001

Notes: Median (first – third quartiles) or number (%). ND: not determined; NA: not applicable

FIGURE LEGENDS

Figure 1: HDL from virologically suppressed HIV+ individuals has impaired function and directly promote foam cell formation in an *in vitro* model of foam cell formation. Normalized (by HDL_C amount and pooled control) HDL lipid peroxide content (A, HDL_{ox}; no units) and % HDL-apoA-I exchange (B, HAE) of HDL from HIV- (n=5) and HIV+ (n=10) individuals are shown. HDL lipid peroxide content (HDL_{ox}) was measured by Amplex Red assay of lipid peroxidation and was normalised by total HDL levels and readout of pooled HDL control as described in methods and previously. HAE is expressed as a percentage of maximum response as measured by EPR spectroscopy and as described in methods and previously. Monocyte-derived foam cell formation following incubation with HDL isolated from the above individuals (C, each HDL sample tested separately) or commercially produced HDL oxidized with CuSO₄ or 13(S)-HPODE (D, HDL_{ox}) was measured using monocytes from 3 (C) or ≥ 4 individual HIV-donors an *in vitro* model of transendothelial migration and foam cell formation. Foam cell formation is expressed as % of total migrated cells (calculated as the median of n=12 technical replicates per donor), and the % foam cells averaged for all 3 (C) or ≥ 4 (D) donors for each HDL tested. Box and whisker plots show median, IQR and 1.5 standard deviations (Tukey plot). All comparisons made using Mann-Whitney U test; *p<0.05, **p<0.01, ***p<0.001.

