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Subclinical carotid artery atherosclerosis is associated with increased expression of peripheral blood IL-32 isoforms among women living with HIV

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Competing financial interests

Dr. Jorge R. Kizer has stock ownership in Bristol-Myers Squibb, Johnson & Johnson, Medtronic, Merck and Pfizer. All other co-authors have no conflict of interest to declare.

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Abstract

Background.—Persistent inflammation in HIV infection is associated with elevated cardiovascular disease risk, even with viral suppression. Identification of novel surrogate biomarkers can enhance cardiovascular disease risk stratification and suggest novel therapies. We investigated the potential of IL-32, a proinflammatory multi-isoform cytokine, as a biomarker for subclinical carotid artery atherosclerosis in virologically-suppressed women living with HIV (WLWH).

Methods and Results.—Nested within the Women's Interagency HIV Study (WIHS), we conducted a cross-sectional comparison of IL-32 between 399 WLWH and 100 women without HIV, followed by a case-control study of 72 WLWH (36 carotid artery plaque cases *vs.* 36 age-matched controls without plaque). Plasma IL-32 protein was measured by ELISA, and mRNA of IL-32 isoforms (IL-32α, β, γ, D, ε, θ) was quantified by RT-PCR from peripheral blood mononuclear cells (PBMCs). Plasma IL-32 protein levels were higher in WLWH compared to women without HIV (p=0.02). Among WLWH, while plasma IL-32 levels did not differ significantly between plaque cases and controls, expression of IL-32 isoforms α, β and ε mRNA was significantly higher in PBMCs from cases (p=0.01, p=0.005, and p=0.018, respectively). Upregulation of IL-32β and IL-32ε among WLWH with carotid artery plaque persisted after adjustment for age, race/ethnicity, smoking, systolic blood pressure, body mass index, and history of hepatitis C virus (p=0.04 and p=0.045); the adjusted association for IL-32α was marginally significant (p=0.07).

Conclusion.—IL-32 isoforms should be studied further as potential cardiovascular disease biomarkers. This is of particular interest in WLWH by virtue of altered IL-32 levels in this population.

Keywords

HIV; cardiovascular disease; atherosclerosis; carotid artery; IL-32

Introduction

Unresolved low-grade inflammation in HIV infection, even with antiretroviral therapy (ART), contributes to cardiovascular disease (CVD) [1]. Multiple factors are believed to contribute to this chronic inflammation, including persistent immune stimulation fuelled by residual HIV viremia and possibly antigens from other co-infections such as cytomegalovirus (CMV) or hepatitis C virus (HCV), imbalance of intestinal microbiota composition towards pathogenic bacteria (i.e., gut dysbiosis), and bacterial and fungal translocation as a result of compromised gut mucosal barrier integrity [2–10]. These mediators may sustain overt inflammation by upregulating a large number of inflammatory factors, including TNF-α, IL-1β, VCAM-1, hsCRP, D-dimer, sCD14, sCD163, and IL-6 [11–15]. Some of these factors, such as hsCRP, are used as biomarkers to enhance risk stratification of

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CVD and associated mortality [16]. However, the role of hsCRP as an inflammatory marker is limited to prognosis and risk prediction since it does not seem to represent a causal factor for CVD [17], and moreover the association of hsCRP with atherosclerosis is blunted in the setting of HIV infection [18]. Other factors, including IL-6, have the potential to be used as both biomarkers and therapeutic targets [19]. However, clinical trials with tocilizumab, a monoclonal antibody targeting the IL-6 receptor, have reported considerable side effects such as increased total and low-density cholesterol levels [20]. Thus, the identification of novel inflammatory factors, especially candidates upstream of IL-6 signaling, may provide better alternatives for CVD risk stratification as well as lead to potential treatment targets. In this regard, we have recently reported that IL-32, a proinflammatory cytokine that is expressed in multiple isoforms (α, β, γ, D, ε, θ, ζ, η, and small/sm) [21–23], is upregulated in HIV infection and its expression is not normalized with ART [24]. We have also demonstrated that IL-32 induces a strong inflammatory response in T-cells by enhancing the production of IL-6, TNF-α and IFNγ, and further induces HIV transcription from latently infected cells [24,25]. These observations suggest that IL-32 may contribute to the persistent immune activation and inflammation that are the major etiologic mediators of atherosclerosis [26] and may represent a biomarker of future CVD. Here, we investigated this hypothesis by studying expression of IL-32 and its isoforms in a case-control study of ART-treated, virally suppressed women living with HIV (WLWH), comparing those with and without subclinical carotid artery atherosclerosis.

Methods

Study design and population

We conducted a study nested within the Women's Interagency HIV Study (WIHS), a long-standing prospective multi-center cohort of WLWH and women at risk for HIV infection from the same communities [27,28]. The WIHS is now part of the MACS/WIHS Combined Cohort Study[29]. To study expression of total IL-32 protein by HIV serostatus, we randomly selected 499 participants (399 WLWH and 100 without HIV) with stored plasma and peripheral blood mononuclear cells (PBMCs) collected during WIHS Visit 34 (April to September 2011). WLWH selected for this study were required to be taking ART and virally suppressed with <100 HIV RNA copies/mL at the time of the visit (COBAS TaqMan v2.0 HIV-1, Roche). Despite good adherence to ART, viral blips <100 copies/mL are relatively common among people with HIV without being associated with clinical variables [30].

Next, we conducted a case-control study among WLWH examining expression of IL-32 isoforms based on presence or absence of subclinical carotid artery atherosclerosis. This study was nested within a vascular substudy of the WIHS. Briefly, starting in 2004, WIHS participants were invited to undergo high-resolution B-mode ultrasound every two to three years to image the carotid artery [31,32]. Among participants in the fourth wave (2010–2012) of the vascular substudy, we selected 36 WLWH with subclinical atherosclerosis (cases), and matched them by age with 36 WLWH without subclinical atherosclerosis (controls). WLWH were also required to be taking ART and virologically suppressed (<100 copies/mL) at the time of the most recent scan, as well as free of coronary heart disease (self-report of angina, myocardial infarction, or coronary revascularization) at the baseline vascular substudy visit.

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Case definition

As part of the vascular substudy, six locations in the right carotid artery were imaged: the near and far walls of the common carotid artery, carotid bifurcation, and internal carotid artery [31,32]. A standardized protocol was used at all centers, [33] and measurements were obtained at a centralized reading center (University of Southern California). Cases of subclinical atherosclerosis had plaque, defined as a focal wall protuberance into the lumen of the artery with a minimal diameter of 1.5 mm at its maximum point, measured in at least one of the six aforementioned artery locations. Controls were found to not have plaque at any of the imaged locations. While mean intima-media thickness (IMT) was also assessed from standardized ultrasound images by automated computerized edge detection at the far walls of the common carotid artery and the carotid bifurcation, our previous studies have not found these measurements to be positively associated with HIV serostatus [31], and therefore we did not design our study to examine IL-32 isoforms in relation to mean IMT.

IL-32 measures

Total IL-32 protein was quantified from stored plasma using the human IL-32 ELISA kit (R&D System, Cat #DY3040–05). Total RNA was isolated from cryopreserved human PBMCs using the RNeasy plus mini kit from Qiagen as *per* the manufacturer's protocol (Catalog #74134). Quantification of IL-32 isoforms (α, β, γ, D, ε and θ) was performed using One-step SYBR Green reverse transcription quantitative PCR (RT-qPCR) performed on LightCycler 480II machine (Roche) with QIAGEN QuantiTect (Catalog #204243). Relative expression of IL-32 RNA was normalized to the housekeeping gene β-glucuronidase. Primer sets for the different IL-32 isoforms and β-glucuronidase, conditions for the quantitative PCR and analysis were done as recently reported[24].

Potential confounders

All comparisons in the case-control study accounted for age as part of the matched design. We considered the following variables as additional potential confounders: race/ethnicity; education; current crack/cocaine or alcohol use; history of injection drug use; smoking history; body mass index (BMI); systolic blood pressure; total, LDL, and HDL-cholesterol levels (Quest standard lipid panel); history of diabetes mellitus; menopause status; and current use of medications for hypertension, hyperlipidemia, or diabetes. History of hepatitis C virus (HCV) infection was defined by presence of antibody to HCV by second-generation or third-generation ELISA (Ortho-Diagnostic Systems) or presence of HCV-RNA by HCV-branched DNA (Quantiplex 2.0, Bayer-Versant Diagnostics) and RT-PCR (COBAS Amplicor HCV detection kit, Roche). We also considered current and nadir CD4+ T-cell count, history of clinical AIDS, CD4:CD8 ratio, and hsCRP levels.

Statistical analysis

Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA) and SAS 9.4 (SAS Institute, Cary, NC). Differences by HIV serostatus or case status were assessed by the Mann-Whitney U test or chi-square test, as appropriate. Correlations were assessed with the non-parametric Spearman test. Multivariable logistic regression analyses controlled for potential confounders, including those significantly associated with carotid artery plaque in

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bivariate analyses (P<0.10), as well as age and smoking status based on a priori knowledge [34]. Levels of IL-32 isoforms were log-transformed when not normally distributed, and scaled to Z-score prior to model fitting. We used alpha<0.05 to determine statistical significance.

Ethical considerations

Participants provided written informed consent, and all analyses were performed in accordance with the guidelines and regulations approved by the Institutional Review Boards (IRBs) of the "Centre Hospitalier de l'Université de Montréal" Research Center (approval #CE.11.063) and each participating WIHS center.

Results

Total IL-32 protein was quantified in plasma from n=399 ART-treated, virologically suppressed WLWH and n=100 women without HIV. While characteristics between WLWH and women without HIV were generally similar, WLWH were slightly older (median age 50 *vs.* 47.5), had higher total cholesterol levels (median 185.5 *vs.* 177.5 mg/dL), lower systolic blood pressure (median 119 *vs.* 124 mm Hg), lower CD4 counts (median 598 *vs.* 989 cells/mm^3), and had fewer behavioral risk factors like smoking history (66% *vs.* 80%), current crack/cocaine use (4% *vs.* 10%), and current alcohol use (33% *vs.* 59%). Plasma samples were blinded to presence of subclinical atherosclerosis to confirm the upregulation of IL-32 in HIV infection as we previously reported [24], replicated here in an independent sample.

As shown in Figure 1A, plasma IL-32 protein levels were significantly higher in WLWH compared to women without HIV (p=0.02). Among WLWH, IL-32 showed a positive correlation with participant age (r=0.13, p=0.0084, Figure 1B) and, similar to our previous findings [25], were negatively correlated with CD4 count (r=−0.1, p=0.04, Figure 1C), albeit weakly. After restricting the analysis to the 36 atherosclerosis cases and 36 age-matched controls (Table 1), total IL-32 plasma protein did not differ significantly between the two groups (p=0.35) (Figure 1D).

Given the multitude of IL-32 isoforms and their differential functions as we and others have previously shown [22,24], we aimed to investigate whether these isoforms are differentially expressed based on atherosclerosis status. As shown in Figure 1E, levels of the IL-32α, β and ε isoforms were significantly higher in PBMCs isolated from WLWH with subclinical atherosclerosis (p=0.01, p=0.005, and p=0.018, respectively) compared to age-matched controls without atherosclerosis. Upregulation of the IL-32β and ε isoforms persisted after additional adjustment for age, race/ethnicity, smoking status, systolic blood pressure, BMI, and history of hepatitis C virus (p=0.04 and p=0.045, respectively). After adjustment, every Z-score increase in IL-32β was associated with a 96% higher odds of plaque (adjusted odds ratio [aOR] 1.96, 95% confidence interval [CI] 1.05–3.68) Similarly, every Z-score increase in IL-32ε was associated with a 92% higher odds of plaque (aOR 1.92, 95% CI 1.01–3.63). The adjusted association for IL-32α was marginally significant (aOR 1.80, 95% CI 0.96–3.37, p=0.07). Levels of IL-32γ and IL-32D were higher in plaque cases compared with controls, but these differences were not statistically significant.

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Discussion

Our study provides evidence for a potential role of the proinflammatory cytokine IL-32 as a biomarker for subclinical atherosclerosis in well-controlled WLWH. This role was restricted to cell-associated IL-32 mRNA that distinguished differentially expressed isoforms, rather than circulating total IL-32 proteins detected by a common set of antibodies. Of note, we previously showed in a separate sample that mRNA levels of IL-32 isoforms positively correlate with cell-associated IL-32 proteins but not with plasma IL-32 [24]. These data suggest that differences in mRNA expression observed in the current study between WLWH with or without subclinical atherosclerosis are likely to be translated into functional proteins at the cellular level.

We showed here that the IL-32α, β and ε isoforms were highly expressed (and IL-32β and ε significantly so) in WLWH with subclinical atherosclerosis, independent of age, smoking status, and other CVD risk factors including BMI. Of note, higher BMI is known to be associated with low-grade inflammation, due in part to the production and secretion of proinflammatory cytokines in adipose tissue [35]. However, in the current study, average BMI was lower among cases with subclinical atherosclerosis compared with controls (Table 1). Despite this, expression of IL-32 isoforms was significantly higher among cases, bolstering the link between IL-32 and subclinical atherosclerosis and suggesting the potential for use of these isoforms as biomarkers of CVD. However, we acknowledge the limitation of the relatively small sample size used in the current study. Thus, replication of these observations in larger cohorts of both men and women living with HIV is warranted.

Moreover, the functional consequence for the simultaneous co-expression of the three IL-32 isoforms (IL-32α, β and ε) remains to be determined. While IL-32β plays a proinflammatory role by inducing IL-6 and IFNγ in activated T cells [24] (likely inducing a Th1 phenotype) and similarly IL-32ε induces a distinct form of caspase-independent apoptosis [36], IL-32α shows anti-inflammatory potential since it induces IL-10 expression but not IL-6, as we have previously shown [24]. However, IL-32β and IL-32ε are expressed at a relatively higher ratio compared to IL-32α (100 and 10 fold more, respectively[24]) and therefore the overall dominant function of IL-32 expression is likely to be inflammatory, which favors atherogenesis with plaque development and growth.

In conclusion, our observations align with mounting evidence for a potential role of IL-32 as a key player in vascular inflammation and CVD [37–39] and warrant further investigations to build the case for this novel proinflammatory cytokine as a CVD biomarker and therapeutic target.

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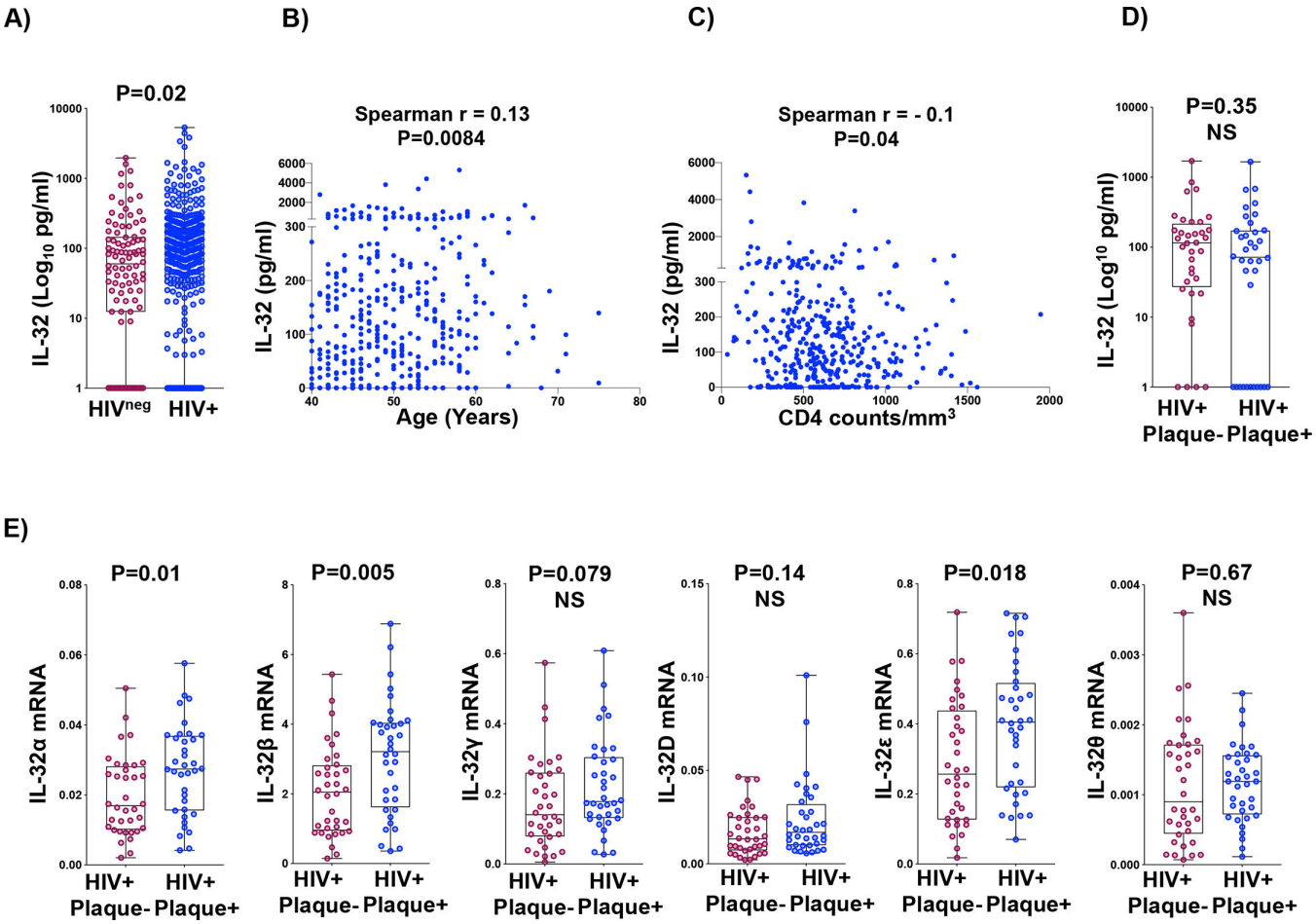

Figure 1: Total IL-32 protein levels and isoforms mRNA expression in PBMCs of WLWH with or without subclinical atherosclerosis.

A) Total IL-32 protein measured by ELISA in plasma from n=100 women without HIV and n=399 WLWH. B) Correlations of IL-32 protein in WLWH (n=399) with participant age. C) Correlations of IL-32 protein in WLWH (n=399) with participant CD4 T-cell count. D) IL-32 protein measured by ELISA in plasma from WLWH with (n=36) or without (n=36) subclinical atherosclerosis (HIV+plaque^{neg} and HIV+plaque+, respectively). **E)** RT-qPCR data for IL-32 isoforms (α, β, γ, D, ε and θ) amplified from total PBMCs of HIV+plaque^{neg} (n=36) compared to HIV+plaque+ (n=36). IL-32 mRNA levels were normalized to the housekeeping gene β-glucuronidase. *P* values are calculated with the two-tailed non-parametric Mann-Whitney test in A, D and E and Spearman correlations in B&C. NS: Non-Significant.

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Table 1.

Demographic and clinical parameters of ART-treated virally suppressed case-control study participants.

	Controls (no plaque, N=36) N (%) or median (IQR)	Cases (plaque, N=36) N (%) or median (IQR)	*P*-value
Demographic characteristics			
Age, years	55 (51–58.5)	55 (51–58.5)	0.92
Race/ethnicity			
Black, non Hispanic	15 (42)	21 (58)	0.08
Hispanic	19 (53)	10 (28)	
White, non Hispanic or other	2 (6)	5 (14)	
Education			
Did not complete high school	17 (47)	18 (50)	0.99
Completed high school	11 (31)	8 (22)	
At least some college	8 (22)	10 (28)	
Behaviour-related characteristics			
Current crack/cocaine use	0 (0)	3 (8)	0.24
Current alcohol use	8 (22)	8 (22)	0.99
History of injection drug use	12 (33)	18 (50)	0.23
History of HCV infection	12 (33)	20 (56)	0.10
History of smoking	21 (58)	27 (75)	0.21
Cardiometabolic risk factors			
Body mass index, kg/m^2	30.1 (26.6–38.9)	26.9 (24.3–31.0)	0.01
Systolic blood pressure, mm Hg	116 (108–125.5)	123.5 (113.5–137)	0.049
Current use of anti-hypertensive medications	16 (44)	22 (61)	0.24
Total cholesterol, mg/dL	184.5 (164.5–224)	185 (150–221)	0.59
LDL cholesterol, mg/dL	103 (77–125)	96.5 (78–124)	0.79
HDL cholesterol, mg/dL	59 (47–70.5)	53 (42–68)	0.30
Current use of lipid-lowering medications	12 (33)	13 (36)	0.99
History of diabetes mellitus	9 (25)	12 (33)	0.60
Current use of diabetes medications	7 (19)	5 (14)	0.75
Menopausal (includes surgical)	25 (69)	27 (75)	0.79
Current use of anti-inflammatory medications	7 (19)	7 (19)	0.99
IMT*, common carotid artery (mm)	0.733 (0.662–0.803)	0.830 (0.738–0.954)	0.0003
IMT, bifurcation (mm)	0.816 (0.739–0.888)	0.884 (0.788–1.003)	0.02
HIV-specific characteristics and biomarkers			
CD4+ count, cells/uL	627 (520.5–779)	600.5 (452.5–796)	0.74
Current ART regimen			0.67
Integrase inhibitor-based	6 (17)	7 (19)	
NNRTI-based**	15 (42)	11 (31)	
PI-based***	13 (36)	17 (47)	
Other	2 (6)	1 (3)	
History of clinical AIDS	20 (56)	17 (47)	0.64
Nadir CD4+ count, cells/uL	194.5 (131.5–353)	241.5 (105–348.5)	0.82
CD4:CD8 ratio	0.82 (0.49–1.03)	0.67 (0.49–0.91)	0.32

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	Controls (no plaque, N=36) N (%) or median (IQR)	Cases (plaque, N=36) N (%) or median (IQR)	*P*-value
hsCRP, ug/mL	1.9 (1.0–4.2)	1.9 (1.0–6.8)	0.82

*IMT: Intima-media thickness

**NNRTI: Non-nucleoside reverse transcriptase inhibitor

***PI: Protease inhibitor.