Relationship between Circulating Inflammatory Monocytes and Cardiovascular Disease Measures of Carotid Intimal Thickness

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Aims: Cardiovascular disease (CVD) remains the leading cause of death worldwide despite improvements in the treatment of atherosclerosis, an inflammatory disease and major underlying cause of CVD. Monocytes, an innate immune cell type, are linked to CVD progression; however, given their heterogeneity, the association between distinct monocyte subsets and increased risk of CVD remains unclear. This study investigated the association between peripheral monocyte subpopulation numbers and carotid intima-media thickness (cIMT), a sensitive measure of CVD risk, in a cohort of adults recruited from the general population.

Methods: We used clinical data and peripheral blood mononuclear cell (PBMC) specimens from 67 individuals. cIMT was measured by high-resolution, B-mode, ultrasound images of the right carotid artery. PBMCs were stained with conjugated monoclonal antibodies to define monocyte subpopulations based on CD14 and CD16 co-expressions into classical (CD14^{++}CD16$^-$), intermediate/inflammatory (CD14^{++}CD16$^+$), and non-classical/patrolling (CD14$^{low/+}$CD16^{++}) monocytes.

Results: We found a higher intermediate monocyte count was significantly correlated with increased right common carotid artery (RCCA) and right carotid bifurcation (RBIF) intima-media thickness (IMT) ($p=0.004$ and 0.006, respectively), even after adjusting for CVD-associated clinical data ($p=0.006$ and 0.004, respectively).

Conclusion: Our study demonstrated a strong correlation between inflammatory monocyte counts and cIMT. These results suggest that, in the general population, there is a relationship between intermediate monocyte expansion and elevated predictors for CVD risk, and intermediate monocytes may be involved in the development of atherosclerosis and metabolic diseases. Strategies targeting inflammatory monocytes may be needed to slow CVD progression.

Key words: Intermediate monocyte, cIMT, Atherosclerosis, Inflammation

Introduction

Atherosclerosis is an inflammatory disease characterized by accumulating plaque and associated with cardiovascular disease (CVD) development[1]. Despite improvements in interventional therapies and treatments for atherosclerosis, CVD remains the leading cause of death in developed countries[2]. Peripheral monocytes, an innate circulating cell population with inflammatory properties, were suggested to play a key role in atherosclerotic plaque formation[1, 3]. Based on CD14 and CD16 surface co-expression, monocytes are classified into three different subsets: classical (CD14^{++}CD16$^-$), intermediate or inflammatory (CD14^{++}CD16$^+$), and non-classical or patrolling (CD14$^{low/+}$CD16^{++})[4]. Existing studies have demonstrated monocytes' roles in various disorders, including those that are associated with CVD. Higher levels of classical monocytes have been correlated with insulin resistance, a metabolic disorder closely related to

CVD[5]. In patients with ongoing systemic inflammation, such as HIV infection, an increase in non-classical monocytes was suggested to contribute to CVD progression, even when these individuals were on stable antiretroviral therapy[6]. Intermediate monocytes were also associated with CVD progression. Increased intermediate monocyte count was independently associated with CVD incidence in non-dialysis, chronic kidney disease (CKD) patients. Non-CKD patients, referred for elective coronary angiography, had higher intermediate monocyte counts as independent predictors of CVD risk[7, 8].

However, despite compelling data, another study did not associate monocyte subsets with increased risk of atherosclerosis rupture, rendering their specific role in atherosclerosis and CVD inconclusive[9]. This contradicting result may be explained by differences in the study population and CVD severity. Our study focused on a relatively healthy population, with no known CKD, and explored the correlation between monocyte subsets, particularly intermediate monocytes, and measurements that predict future CVD risk in healthy individuals. We used carotid intima-media thickness (cIMT), a demonstrated non-invasive method of measuring carotid plaque and predicting future CVD risk[10]. This study investigated the association between the number of monocyte subpopulations and cIMT indices as a reliable predictive measure of CVD risk among adults recruited within the general population.

Materials and Methods

Study Participants

This is a cross-sectional analysis of data on HIV-negative adult participants who were enrolled as the HIV-uninfected control comparison group in the Hawaii Aging with HIV Cardiovascular (HAHC-CVD) study. The HAHC-CVD cohort was a five-year longitudinal study, which assessed the role of oxidative stress and inflammation in HIV cardiovascular risk. The cohort details have been published previously[11]. The parent study was approved by the University of Hawaii Institutional Review Board and conducted in accordance with the Declaration of Helsinki.

Clinical Assessment

General medical history, with special emphasis on CVD, was obtained. Clinical parameters, including blood pressure (BP), height, weight, body mass index (BMI), and waist-to-hip ratio, were measured. Blood tests, including fasting for 12 hours, estimated glomerular filtration rate (GFR), high-sensitivity C-reactive protein (hsCRP), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, and glucose, were performed. HIV-seronegative status was confirmed by enzyme-linked immunosorbent assay (ELISA).

Hypertension was defined as systolic BP $\geq$ 140 mmHg, or diastolic BP $\geq$ 90 mmHg on entry visit, self-reported history of hypertension, or use of antihypertensive medications. Diabetes mellitus was defined by a fasting blood sugar (FBS) $\geq$ 126 mg/dL, two-hour oral glucose tolerance test (OGTT) $\geq$ 200 mg/dL, or self-reported history of diabetes mellitus. Ten-year coronary heart disease (CHD) risk was calculated by Framingham risk score (FRS), using the National Cholesterol Education Program website (http://hp2010.nhlbihin.net/atpiii/calculator.asp)[12]. Participants with diabetes (as a CVD equivalent) or clinical CVD (history of myocardial infarction, angina, coronary disease-related cardiac surgery, or ischemic stroke) were automatically classified as having ten-year CHD risk by a FRS of 20%. Clinical CVD events were adjudicated by two physician-researchers (CMS and DCC).

Carotid Artery Intima-Media Thickness

A single, high-resolution, B-mode ultrasound image of the right carotid artery was obtained to measure the cIMT of the far wall of the distal common carotid artery (CCA) and bifurcation (BIF) with automated edge detection. Ultrasound images were acquired at the Queen's Medical Center in Honolulu and analyzed at the University of Southern California Atherosclerosis Research Unit Core Imaging and Reading Center.

Cell Staining and Flow Cytometric Analysis

In brief, cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed and surface-stained with a conjugated antibody panel designed to selectively define and quantify peripheral monocyte subpopulations, as previously described[5]: V500-conjugated anti-CD3, Qdot605-conjugated anti-CD14, Alexa700-conjugated anti-CD16, PE-Cy7-conjugated anti-CD56, PE-Cy7-conjugated anti-CD19, PE-Cy7-conjugated anti-CD20, and APC-H7-conjugated HLA-DR monoclonal antibodies (mAbs). Monocytes were identified in the HLA-DR positive gate and further into monocyte subpopulations based on CD14 and CD16 expression (**Fig. 1**). All antibodies were from BD Biosciences, except for Q605-conjugated anti-CD14 and yellow Live/Dead (Life Technologies). Stained PBMCs were acquired by flow cytometry using a 4-laser custom BD-Fortessa instrument (Becton Dickinson) and analyzed with FlowJo software (Treestar Inc Ashland, OR) as previously described[5].
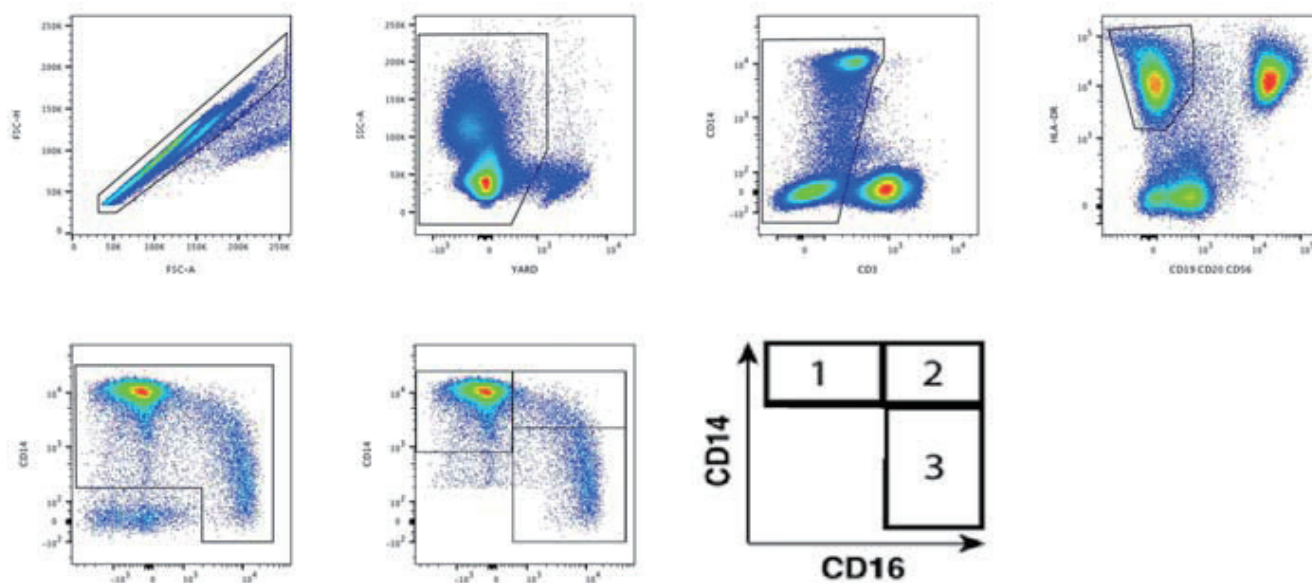
Fig. 1. Multiparametric flow cytometry phenotype gating strategy for identification of monocyte subsets

To selectively define and quantify peripheral monocyte subpopulations, a conjugated monoclonal antibody panel was designed to exclude doublets (FSC-H against FSC-A), dead cells (YARD against SSC-A), CD3 positive T-cells, B-cells, and NK-cells (CD19/20 and CD56). Monocytes were identified in the HLA-DR positive gate and further classified into monocyte subpopulations based on CD14 and CD16 expression: (1) classical monocytes (CD14^{++}CD16$^-$), (2) intermediate monocytes (CD14^{++}CD16$^+$), and (3) non-classical monocytes (CD14$^{low/+}$CD16^{++})

The total monocyte count was calculated from white blood cell (WBC) count, and percent monocyte values on the complete blood count, conducted as part of entry evaluations on the same blood specimen used for flow cytometry in line with our previous reports[5].

Statistical Analyses

Demographic and CVD risk characteristics were described using the median, first quartile (Q1), and third quartile (Q3) for continuous variables as well as frequency and percent for categorical variables. Pearson correlation was conducted to correlate demographic and CVD risk characteristics with monocyte subset count. Multivariable linear regression was conducted on each of the intima-media thickness outcomes to assess their association with intermediate monocytes with adjustments for 1) Age, 2) Gender, 3) BMI, 4) LDL cholesterol, 5) HDL cholesterol, 6) Diabetes mellitus, 7) Hypertension, and 8) Current cigarette smoking. Due to the small sample size, manual backward selection was performed on each regression model to determine the most important covariates. A two-sided p-value of less than 0.05 was considered statistically significant. Analyses were conducted using SAS (Version 9.4, 2002-2012 SAS Institute Inc., Cary, NC) and SPSS (IBM, Version 24, Armonk, NY).

Results

There were 67 patients who had available monocyte and cIMT data. The participants' demographic and CVD risk characteristics are presented in **Table 1**. The participants were primarily male (80%) and Caucasian (65.7%), with a median age of 54 years. The cohort had a median BMI of 26.8 kg/m^2, systolic BP of 121 mmHg, diastolic BP of 73 mmHg, and FRS of 4%. The median fasting plasma glucose was 78 mg/dL, GFR was 98.8 mL/min, LDL cholesterol was 115 mg/dL, and HDL cholesterol was 55 mg/dL. Of the 67 participants, 18 (27%) had hypertension, and 4 (6%) were diabetic. Ten patients (15%) were smoking at the time of entry, 39 (58%) had a past history of smoking, and 28 (42%) had never smoked. The median total WBC count was 5230 cells/µL, hsCRP was 1.0 mg/dL, RCCA cIMT was 0.75 mm, and RBIF cIMT was 0.84 mm.

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Table 1. Baseline characteristics of study participants

N	67
Age, years [median (Q1, Q3)]	54.4 (48.0, 59.8)
Male gender, *n* (%)	54 (80)
Ethnicity	
Caucasian, *n* (%)	44 (65.7)
African American, *n* (%)	1 (1.5)
Native Hawaiian/Pacific Islander, *n* (%)	0 (0)
Asian, *n* (%)	6 (8.9)
More than one race, *n* (%)	16 (23.8)
BMI, kg/m^2 [median (Q1, Q3)]	26.8 (23.5, 29.4)
Hypertension, *n* (%)	18 (27)
Blood pressure	
Systolic blood pressure, mmHg [median (Q1, Q3)]	121 (114, 131)
Diastolic blood pressure, mmHg [median (Q1, Q3)]	73 (68, 79)
Fasting plasma glucose, mg/dL [median (Q1, Q3)]	78 (73, 85)
GFR, mL/min [median (Q1, Q3)]	98.8 (87.5, 121.7)
Diabetes mellitus, *n* (%)	4 (6)
LDL, mg/dL [median (Q1, Q3)]	115 (94, 139)
HDL, mg/dL [median (Q1, Q3)]	55 (46, 67)
Smoking history	
Current, *n* (%)	10 (14.9)
Past, *n* (%)	39 (58.2)
Never smoked, *n* (%)	28 (41.7)
10-year CHD risk estimated by FRS, % [median (Q1, Q3)]	4 (2, 7)
Total WBC count [median (Q1, Q3)]	5230 (4460, 5950)
hsCRP [median (Q1, Q3)]	1 (1, 2)
RCCA cIMT [median (Q1, Q3)]	0.75 (0.69, 0.84)
RBIF cIMT [median (Q1, Q3)]	0.84 (0.76, 0.93)

BMI, body mass index; GFR, glomerular filtration rate; LDL, low-density lipoprotein; HDL, high-density lipoprotein; CHD, coronary heart disease; FRS, Framingham risk score; WBC, white blood cell; hsCRP, high-sensitivity C-reactive protein; RCCA, right common carotid artery; RBIF, right carotid bifurcation; cIMT, carotid intima-media thickness

tions based on CD14 and CD16 expression: classical (CD14^{++}CD16$^-$), intermediate (CD14^{++}CD16$^+$), and non-classical (CD14$^+$CD16$^+$) (**Fig. 1**). The participants' monocyte characteristics are presented in **Table 2**. The cohort had a median total monocyte count of 450 cells/µL, a classical monocyte count of 295 cells/µL (69%), an intermediate monocyte count of 21 cells/µL (5%), and a non-classical monocyte count of 36 cells/µL (8%).

In unadjusted Pearson correlation, intermediate monocytes were positively correlated with age (*r*= 0.24, *p*=0.05) and FRS (*r*=0.37, *p*=0.001) and negatively correlated with LDL cholesterol (*r*=-0.21, *p*= 0.08) and total cholesterol (*r*=-0.21, *p*=0.08). Similarly, intermediate monocytes were correlated with right common carotid artery intima-media thickness (RCCA; *r*=0.34, *p*=0.004) and bifurcation intima-media thickness (RBIF; *r*=0.33, *p*=0.006). By backward selection, age, BMI, and LDL cholesterol were

found to be the most important for both models, as well as HDL cholesterol and diabetes for RCCA and RBIF, respectively. In the adjusted models, shown in **Table 3**, intermediate monocyte count was significantly associated with both RCCA (standardized *β* = 0.31, *p*=0.006) and RBIF (standardized *β*=0.33, *p*= 0.004).

No associations were observed with the classical (RCCA *p*=0.066, RBIF *p*=0.194) or non-classical (RCCA *p*=0.182, RBIF *p*=0.076) monocyte subsets.

Discussion

In this study, we investigated the association between intermediate monocytes and a marker for atherosclerosis, cIMT, in a group of healthy individuals. As part of the innate immune system, monocytes play a critical role in many inflammatory diseases, represent an important source of cytokines and chemo-

Table 2. Monocyte characteristics of study participants

Total monocyte count [median, (Q1, Q3)]	450 (325, 544)
Classical monocyte count [median, (%), (Q1, Q3)]	295 (69) (232, 379)
Intermediate monocyte count [median, (%), (Q1, Q3)]	21 (5) (13, 32)
Non-classical monocyte count [median, (%), (Q1, Q3)]	36 (8) (20, 52)

Table 3. Multivariable linear regression predicting RCCA IMT and RBIF IMT from intermediate monocytes

RCCA*				RBIF*			
Parameter	Unstandardized Beta	Standardized Beta	p	Parameter	Unstandardized Beta	Standardized Beta	p
IM	0.001	0.31	0.006	IM	0.001	0.33	0.004
Age	0.002	0.22	0.043	Age	0.001	0.15	0.156
BMI	0.002	0.17	0.118	BMI	0.003	0.27	0.023
LDL	0.000	0.18	0.095	LDL	0.000	0.14	0.191
HDL	-0.001	-0.24	0.033	Diabetes	0.05	0.16	0.170

N=67
*RCCA: right common carotid artery; RBIF: right carotid bifurcation; Log-10 transformed to correct for normality.
IM, intermediate monocyte; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

kines, and may be major contributors to CVD[1, 13]. Monocyte subsets may also be a useful tool to help identify individuals at risk of future CVD[3, 7, 8]. However, previous data regarding peripheral monocytes' prognostic value remains controversial. Several studies have demonstrated a significant association between monocyte subsets and atherosclerosis plaque severity, vulnerability, and progression of CVD[7, 8, 14-17]. Additionally, CD14^{+}CD16^{+} monocytes were associated with restenosis, after percutaneous transluminal angioplasty, in individuals with peripheral artery disease[18].

In support of the aforementioned data, our study showed that higher intermediate monocyte subset count with older age was significantly associated with increased cIMT at the CCA region. Similarly, the correlation between intermediate monocyte count and cIMT at the BIF region was also significant. The correlation between intermediate monocytes and cIMT at both the CCA and BIF regions persisted even after adjusting for age, gender, BMI, LDL cholesterol, HDL cholesterol, diabetes mellitus, hypertension, and current cigarette smoking. In summary, this study reported the association between intermediate monocyte numbers with cIMT as predictors of CVD risk in a relatively healthy adult population.

Our study contradicted the results from a previous report that did not associate monocytes and their subsets with the risk of atherosclerosis rupture in patients with severe atherosclerosis[9]. Additionally, our results contradicted another study, which stated that, unlike non-classical monocytes, there is no significant association between intermediate monocytes and cIMT changes during a two-year period[6]. These contradicting results may be explained partially by the different study populations. Most of the previous studies involved participants with severe and clinically symptomatic atherosclerosis, whereas our study participants were relatively healthy individuals with subclinical atherosclerosis. Monocyte influx is paramount in the atherogenesis process in mouse studies[19]. However, monocyte recruitment may be less crucial in more severe and advanced atherosclerotic plaque[20]. Monocytes' diverse roles in different stages of atherosclerosis development may account for the contradicting results of our study and the study by Meeuwsen *et al.*[9]. HIV infection may also contribute to the different results presented by our study and the study by Chow *et al.*[6]. Previous reports demonstrated that coronary atherosclerosis was more common in HIV-infected individuals, and that coronary plaque was larger compared to those of HIV-uninfected individuals[21]. Additionally, immunosuppression and HIV viremia were also associated with more atherogenic lipid profiles in aging individuals with HIV[22]. Our study focused mainly on exploring monocyte subsets as predictive markers for future atherosclerosis in healthy individuals. Our data showed that intermediate monocytes may be used as potential markers to predict future atherosclerosis establishment in individuals without any current atherosclerotic clinical symptoms.

Monocytes expressing varying degrees of CD16 are important in several diseases, as the subsets' differences in frequency suggest that they may modulate a disease course[4, 23]. Intermediate monocytes represent

only a fraction of the total monocyte population. However, they have been identified as a translational population, sharing phenotypic and functional characteristics as well as expressing surface markers at levels between the classical and non-classical subsets[4]. Intermediate monocytes also have significant inflammatory properties, increase oxidative stress, and expand in population during chronic inflammation[24]. A study by Justo-Junior *et al.* demonstrated that individuals with unstable angina had elevated counts of intermediate monocytes that expressed the highest concentrations of the chemokine receptors CCR2, CCR5, and CX3CR1 as well as the pattern recognition receptors TLR2 and TLR4 compared to the other monocyte subsets[25]. These receptors are involved in recognizing damage-associated molecular patterns and recruiting immune cells to inflammatory sites; thus, they may contribute to inflammation and the progression of atherosclerosis[25]. Additionally, this study showed that HSP60, a molecule produced in the presence of oxidized lipids and during hypertension periods, induced the production of IL-12p70 in intermediate monocytes[25]. The cytokine IL-12p70 promotes Th1 T-lymphocyte differentiation, proinflammatory cytokine release, and macrophage activation, which may thereby increase inflammation and atherosclerotic plaque development[26]. In individuals with stable atherosclerotic disease, an increased proportion of inflammatory monocytes is associated with elevated levels of lipoprotein a, which is a proatherogenic plasma lipoprotein[27]. Furthermore, lower HLA-DR expression in intermediate monocytes was associated with metabolic disorders[14]. These properties, along with their association with increased cIMT in our study, suggested that intermediate monocytes may be involved in the pathogenesis of atherosclerosis.

Heine *et al.* associated elevated intermediate monocyte count with increased CVD risk in dialysis patients[28]. Another study, by Rogacev *et al*, independently associated increased intermediate monocyte count with CVD incidence in non-dialysis patients with CKD[7]. Both studies could provide a mechanistic model on how CKD, monocyte dysfunction, and atherosclerosis are interrelated. Uremia-related immune dysfunction in dialysis patients leads to increased pro-inflammatory cytokine production by monocytes[28]. Additionally, reduced renal cytokine clearance may contribute to elevated cytokine levels in the peripheral blood[26]. These data put dialysis patients at a higher atherosclerotic risk than the general population. CKD also accelerates atherosclerosis progression and increases CD16-positive monocyte count, compared to healthy controls, which puts them at a higher risk of CVD compared to the general population[7]. Our

study focused on a healthier population, and none of our study participants had CKD based on their estimated GFR. We also did not find any correlation between intermediate monocytes and GFR. Our data suggested that intermediate monocytes may act as independent predictors of future CVD risk.

This study was limited because it was a cross-sectional observation that was restricted by the sample size and demonstrated correlation, not causality. Despite these limitations, our study demonstrated a strong relationship between intermediate monocyte count and cIMT, an atherosclerosis marker, in a well-characterized population of adults with extensive investigation of arterial injury markers. Further investigation involving longitudinal study into the mechanism of monocytes in atherosclerosis development as well as the qualitative cut off and clinical outcomes of cIMT is warranted.

Our study demonstrated a strong correlation between inflammatory monocyte counts and cIMT. These results suggest that the relationship between intermediate monocyte expansion and elevated predictors for CVD risk occurs in the general population, and intermediate monocytes may be involved in the development of atherosclerosis and metabolic diseases.

Conflict of Interest

None of the authors have any relevant conflicts of interest to disclose.

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