LDL associates with pro-inflammatory monocyte subset differentiation and increases in chemokine receptor profile expression in African Americans

Cristhian A. Gutierrez-Huerta[1], **Briana S. Turner**[1], **Sophie E. Claudel**[1], **Nicole Farmer**[2], **Rafique Islam**[1], **Valerie M. Mitchell**[1], **Billy S. Collins**[1], **Yvonne Baumer**[1], **Alan T. Remaley**[3], **Tiffany M. Powell-Wiley**[1,4,#]

[1]Social Determinants of Obesity and Cardiovascular Risk Laboratory, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA.

[2]Clinical Center, National Institutes of Health, Bethesda, MD 20892, USA.

[3]Lipoprotein Metabolism Section, Translational Vascular Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA.

[4]Intramural Research Program, National Institute on Minority Health and Health Disparities, National Institutes of Health, Bethesda, MD, USA.

Abstract

Background: In the United States, African Americans (AAs) have greater risk for Class III obesity and cardiovascular disease (CVD). Previous reports suggest that AAs have a different immune cell profile when compared to Caucasians.

Methods: The immune cell profile of AAs was characterized by flow cytometry using two experimental setups: *ex vivo* (N=40) and *in vitro* (N=10). For *ex vivo* experiments, PBMC were treated with participant serum to understand how lipid contents may contribute to monocyte phenotypic differences. For *in vitro* experiments, monocytes were low-density lipoprotein (LDL)- or vehicle-treated for four hours and subsequently analyzed by flow cytometry and RT-qPCR.

Results: When PBMCs were treated with participant sera, subsequent multivariable regression analysis revealed that serum triglycerides and LDL levels were associated with monocyte subset

differences. *In vitro* LDL treatment of monocytes induced a phenotypic switch in monocytes away from classical monocytes accompanied by subset-specific chemokine receptor CCR2 and CCR5 expression changes. These observed changes are partially translation-dependent as determined by co-incubation with cycloheximide.

Conclusions: LDL treatment of monocytes induces a change in monocyte subsets and increases CCR2/CCR5 expression in a subset-specific manner. Understanding the molecular mechanisms could prove to have CVD-related therapeutic benefits, especially in high-risk populations with hyperlipidemia and increased risk for CVD.

Graphical Abstract:
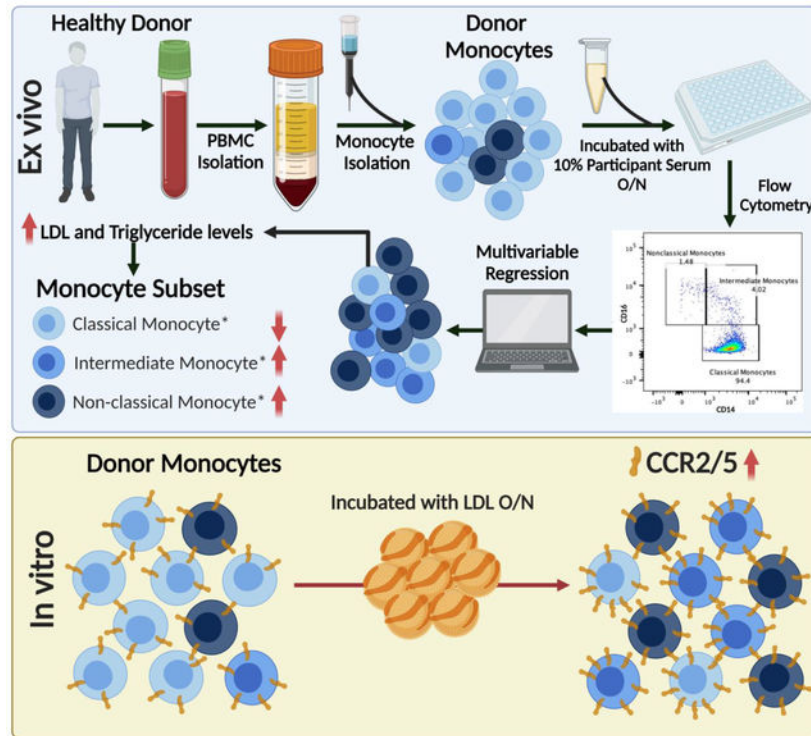
LDL associated changes on monocyte subsets and CCR2/5 expression.

Keywords

health disparities; monocyte subset phenotype; cardiovascular disease; LDL

Introduction:

Obesity remains a major contributor to cardiovascular disease (CVD) risk and mortality worldwide [1] and is described as a chronic, low-grade inflammatory disease that is often associated with hyperlipidemia and hyperglycemia [2]. Atherosclerosis, the main underlying cause of CVD, is driven by the infiltration of immune cells into the vessel wall, with monocytes being a significant contributor [3]. Monocytes, amongst other cell types, are recruited to the vascular wall by chemokine receptors, notably the C-C

Motif chemokine receptors 2 and 5 (CCR2 [4] and CCR5 [5], respectively). Peripheral blood monocytes are heterogenous and possess either a classical (CM; $CD14^{++}CD16^{-}$), intermediate (IM; $CD14^{++}CD16^{+}$), or nonclassical (NCM; $CD14^{+}CD16^{++}$) phenotype [6]. Specifically, elevated IM population frequencies have been traced to adverse cardiac events in patients with coronary artery disease [7], end-stage renal disease [8], psoriasis [9], and rheumatoid arthritis [10]. Increased IM and NCM populations have also been observed in individuals with obesity [11–13]. Additionally, notable monocyte subset sex differences have been reported with women having a less favorable monocyte subset phenotype[11]. In addition, our group has recently shown in a smaller exploratory study that African Americans (AAs) have higher IM and NCM populations when compared to Caucasians, even in the absence of known CVD and seemingly independent of age and sex [14]. Remarkably, hypercholesteremia has been shown to directly impact hematopoiesis by specifically inducing monocytosis [15]. Clinical correlations have also assessed a direct link between lipid profile and monocyte subsets in humans[16]. However, these studies have not been performed in diverse study cohorts nor have they assessed the causal effects of these lipids *in vitro*. Therefore, studies are needed to investigate the association between cholesterol content and its direct contribution to pro-inflammatory monocyte lineages in a population at high risk for obesity, hyperlipidemia, and CVD, like AAs.

In this study we aim to: 1) identify whether lipid profile is associated with a shift in monocyte lineage towards NCM; among a community-based cohort of African Americans; 2) determine if LDL cholesterol is directly associated with monocyte subset differentiation and chemokine receptor expression using *ex vivo* and *in vitro* approaches; and 3) elucidate whether surface marker expression of CD14, CD16, CCR2, and CCR5 is a result of translation-dependent protein synthesis. We hypothesize that individual LDL content will be associated with increased populations of NCM in *ex vivo* experiments and changes in chemokine receptor expression *in vitro* experiments. Also, we hypothesize that chemokine receptor expression differences are related to internalization mechanisms rather than de novo synthesis.

Methods:

A cohort of African Americans residing in Washington, D.C. were included in this study (Table 1). Study approval was obtained from the Institutional Review Board at the NIH in accordance with the Principles of Declaration of Helsinki. All guidelines for good clinical practice and those set forth in the Belmont Report were followed. Data for all study participants were obtained under the clinical protocols NCT01143454 and NCT00001846. All study participants provided written informed consent. Detailed information for all experiments can be found in the Supplementary Methods.

Results:

LDL and TG levels in serum is associated with monocyte subset shifts.

Freshly isolated PBMCs from a healthy individual were treated with bio-banked serum from 40 individuals in an *ex vivo* experimental setup (Patient characteristics summarized in Table 1). LDL and TG were found to be significantly associated with the various monocyte

subsets (Table 2). A negative association was observed between serum LDL levels and CM (ß=−0.32; p=0.046) which became non-significant after adjusting for FRS score, BMI, and clinical use of statin treatment. A positive association was observed between LDL levels and NCM proportions (ß=0.33, p=0.036) that was sustained after adjusting for FRS and BMI (ß=0.36, p=0.028). Interestingly, additional adjustment for clinical use of statin therapy resulted in a loss of significance of this association (ß= 0.30, p=0.084), reemphasizing that LDL and LDL-related pathways may be involved in the observed changes. A positive trend was also observed between LDL and IM. TG levels in the serum were negatively associated with CM (ß=−0.41, p=0.009) and positively associated with IM (ß=0.42, p=0.006). Both associations between TG with CM and IM were sustained after adjustment for FRS and BMI (ß=−0.36, p=0.02 and ß=0.37, p=0.006, respectively). Associations remained significant even after an additional adjustment for clinical use of statin treatment (ß =−0.39, p=0.02 and ß=0.41, p=0.02). Neither BMI nor lipid levels were associated with surface expression in CCR2 and CCR5 of serum-treated monocytes in the *ex vivo* experiments (Supplementary Table 3). No relationships were found between estradiol levels and monocyte phenotype (Supplementary Table 4).

In vitro application of LDL contributes to monocyte phenotype observed differences.

Aiming to establish the potential for a direct relationship between LDL and monocyte subset differentiation, we treated isolated monocytes with or without 50 mg/dL of LDL and determined that neither the total amount of monocytes (Figure 1A) nor overall CCR2/5 expression (Figure 1B) changed with LDL treatment. However, LDL-treated monocytes showed a decrease in the proportion of CM (92.67% to 89.52%, p=0.007) and an increase in the proportion of IM (3.84% to 4.82%, p=0.022) and NCM (3.27% to 4.95%, p=0.004) when compared to untreated monocytes (Figure 1C). CCR2 expression, as measured by median fluorescence intensity (MFI), was significantly increased in all three subsets in LDL-treated monocytes (Figure 1D; CM CCR2 p=0.037, IM CCR2 p=0.006, and NCM CCR2 p=0.027). CCR5 expression increased in CM (p=0.049) and IM (p=0.027) after LDL treatment (Figure 1E).

Cyclohexamide inhibits monocyte subset shift and NCM CCR2 expression changes induced by LDL.

Cyclohexamide (CHX) is a non-specific, translation elongation inhibitor that can be used to assess whether de novo protein synthesis is responsible for treatment-driven phenotypic changes [17]. As previously reported in this study, LDL induced an increase in CCR2 and CCR5 expression of monocytes in a subset-specific way. When comparing LDL-only treated monocytes with monocytes dually treated with CHX and LDL (Figure 1F), we observed, that the frequency of all monocyte subsets returned to control levels, indicating a normalization of the LDL-induced shift in monocyte subsets (CM LDL: 71.35% to CHX+LDL: 88.35%, p=0.02; IM LDL: 10.24% to CHX+LDL: 3.56%, p=0.01; NCM LDL: 15.97% to CHX+LDL: 7.55%, p=0.03). CCR2 expression between LDL-treated and LDL+CHX-treated monocytes was not altered on CM and IM subsets; however, LDL-only treated NCM had higher surface marker expression of CCR2 when compared to cells that were dually treated with LDL and CHX (521.83 MFI to 415.83 MFI, p=0.02), suggesting a normalization to control levels when translation is inhibited in the presence of LDL (Figure

1G). CCR5 expression in CM was dampened when comparing LDL-treated monocytes with dually treated CM (1312.67 MFI to 1000.5 MFI, p=0.005). No significant CCR5 expression differences were noted in IM/NCM subsets (Figure 1H). These results suggest that this acute LDL-induced CCR expression upregulation is only partially dependent on genetic regulation and occurs in a subset-specific way.

RT-qPCR confirms subset-specific differences in CCR2/5 gene expression

RT-qPCR was performed on isolated monocytes and isolated monocyte subsets. RT-qPCR of all monocytes showed that that there were no overall gene expression changes observed in CD14, CD16, CCR2, nor CCR5 when monocytes were treated with LDL compared to control group (Supplementary Figure 2). To investigate if changes in gene expression were responsible for subset-specific upregulation of CCR2 and CCR5, RT-qPCR was performed after treatment with LDL and subsequent FACS sorting. No statistically significant differences were observed in monocyte subset expression of CCR2 when monocytes were treated with LDL potentially arising from individual donor differences, suggesting that CCR2 regulation is independent of regulation of gene expression (Figure 1I). However, treatment with LDL showed a 54-fold increase in CCR5 gene expression in CM (p-val=0.008) and a 0.84-fold decrease in CCR5 gene expression in IM (p-val=0.056) when compared to untreated control (Figure 1J).

Discussion:

Our *ex vivo* experimental setup showed that LDL and TG were associated with monocyte subset differentiation highlighting their important role as CVD risk factors. Interestingly, BMI did not correlate with monocyte subset phenotypes in our clinical cohort, this may relate to limitations of BMI as a marker of CVD risk, especially in racially/ethnically diverse populations [18]. Other clinical markers of CVD risk such as HOMA-IR, serum insulin levels, CRP, and HDL cholesterol did not associate with monocyte subset phenotype either. The association between monocyte subset phenotypes with LDL [19] and TG [7] have both been explored and verified in prior studies involving patients with a history of coronary artery disease. In contrast, the participants in our study have a variety of CVD risk factors (Table 1) and no clinical diagnosis of known coronary artery disease. The regression analysis performed demonstrated that LDL associates with a monocyte subset shift towards NCM even after adjustment for BMI and FRS (Table 2) suggesting that changes in LDL and TG levels might influence a pro-inflammatory, pro-atherogenic monocyte subset shift. This is particularly interesting, as recent literature has demonstrated that NCM have been associated with carotid-intima-media thickness progression [20], coronary artery calcium progression in HIV-1 infected adults on antiretroviral therapy [21], endothelial dysfunction in patient with CAD [22], and cardiometabolic disorders and CVD.[23]

LDL has long been associated as a critical CVD risk factor [24], but even in the setting of well-controlled LDL levels, studies have shown that elevated triglycerides predict CVD risk [25]. Of note, an interesting paradox has been well-reported that suggests AA present with decreased amounts of TG and increased HDL serum lipid levels when compared to other ethnicities [26]. This data has motivated our group to study the effects of LDL on

monocyte subset phenotype and CCR expression *in vitro* as it appears that LDL might be a major contributor to CVD in AA populations. Our results suggest that LDL may change the monocyte phenotype from a classical phenotype to a CVD-associated phenotype. In addition to phenotype-switching, CCR2 expression on each individual subset and CCR5 expression on CM and IM appears to increase in the presence of LDL. We hypothesize that this LDL-associated subset-specific increase in CCR2 and CCR5 could lead to greater monocyte migration from the circulation to the vascular wall when an individual is in a hyperlipidemic state. Tjaden *et al.* has previously shown that human monocytes engage in more chemokinesis when treated with LDL [27] though surface CCR expression of these monocytes was not determined in their study. Therefore, the findings presented here strengthen the argument that LDL increases monocyte chemokinesis.

The application of CHX, a non-specific translation elongation inhibitor, in addition to LDL-treatment was employed to explore if the observed LDL-induced monocyte subset shifts are dependent on *de novo* protein synthesis. Our findings further suggest that LDL may promote the synthesis of a protein that likely cleaves CD14. Alternatively, LDL may promote the synthesis of CD16, but the full mechanism of this induced effect is not fully understood. In spite of this, it is likely that CD14 is cleaved as soluble CD14 (sCD14) in the plasma as it has been shown to be associated with CVD risk [28]. In fact, recent studies suggest that this sCD14-CVD association may be most prevalent in the AA population. On the other hand, increased populations of CD16+ monocytes have been shown to activate pro-atherosclerotic features in human arterial and venous endothelium [29]. Therefore, future experiments should be undertaken to fully answer this question as the identification of this protein and subsequent inhibition may have CVD-related therapeutic benefits, especially in AA populations. The dual treatment of CHX and LDL suggests that the increased CCR2/5 expression of monocyte subsets is only partially regulated by genetic translation processes and is subset specific. The data shown by RT-qPCR after FACS sorting also confirms that *de novo* synthesis only partially contributes to the increase in chemokine receptor expression and is subset-specific. This suggests the possibility that increased CCR2 expression of CM and IM in response to LDL treatment might be due to the inhibition of CCR2 internalization [30] rather than translation-dependent mechanisms (through sterol regulation [31] or activation of PPARγ [32]), though the mechanism remains to be elucidated. Interestingly, dual treatment of CHX and LDL dampened the LDL-induced CCR5 increase in CM and the LDL-induced CCR2 increase in NCM, highlighting the different mechanisms involved in regulating CCR2 and CCR5 receptor expression within different subsets. These data indicate that the LDL-induced change in monocyte subset and the increase of CCR5 surface expression of CM are at least partially mediated by processes involving genetic regulation likely acting through the upregulation of CREB-1 [33].

Study Limitations:

Comparing a 4-hour acute treatment to a 24-hour serum treatment of PBMC may be seen as a limitation of our study design, but in order to see the impact of cholesterol/lipids in a 10%-study participant serum environment without additional supplements like FBS, we believe prolonged exposure is required. Evaluating the complete effects of LDL on monocyte subset and chemokine receptor expression is limited due to the limited available

clinical data of healthy control blood bank samples provided by the NIH blood bank. The precise biochemical transduction mechanism of LDL-induction on monocyte subset phenotype and chemokine receptor expression has yet to be fully elucidated; yet, we believe that demonstrating monocyte subset-specific differences in chemokine receptor regulation is of extreme importance. Epigenetic modifications, such as DNA methylation, a proxy for premature cellular senescence, has also been associated with body mass index in AA women [34] and we can therefore not exclude any potential contributions made by epigenetic modifications.

Conclusion:

Our data suggest that LDL contributes to monocyte subset differentiation and to chemotactic receptor expression, supporting its role as a crucial CVD risk factor. In our experiments, high concentration LDL decreases the CM and increases the IM/NCM populations. Due to a small, homogeneous cohort, the findings in this report should be replicated utilizing larger, racially/ethnically diverse cohorts in order to assess the intersectionality of hyperlipidemia, and race/ethnicity as a marker of social and environmental exposures on monocyte subset distributions and chemokine receptor expression *in human clinical studies*. Nonetheless, elucidating the underlying mechanisms that explain how LDL may change monocyte phenotype could prove to have therapeutic benefit, especially in populations who are at high-risk for obesity, hyperlipidemia, and CVD. Therefore, the molecular pathway of LDL-induced phenotype-switching of monocytes should be of keen interest of future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

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Highlights

- Little is known about the impact of LDL cholesterol on monocytes in African Americans.

- LDL cholesterol is associated with monocyte phenotypes in African Americans.

- LDL cholesterol promotes monocyte subset distribution and monocyte chemokine receptor expression in a subset-specific way from *in vitro* studies.

- LDL-induced changes are partly mediated by translation in a subset-specific manner.
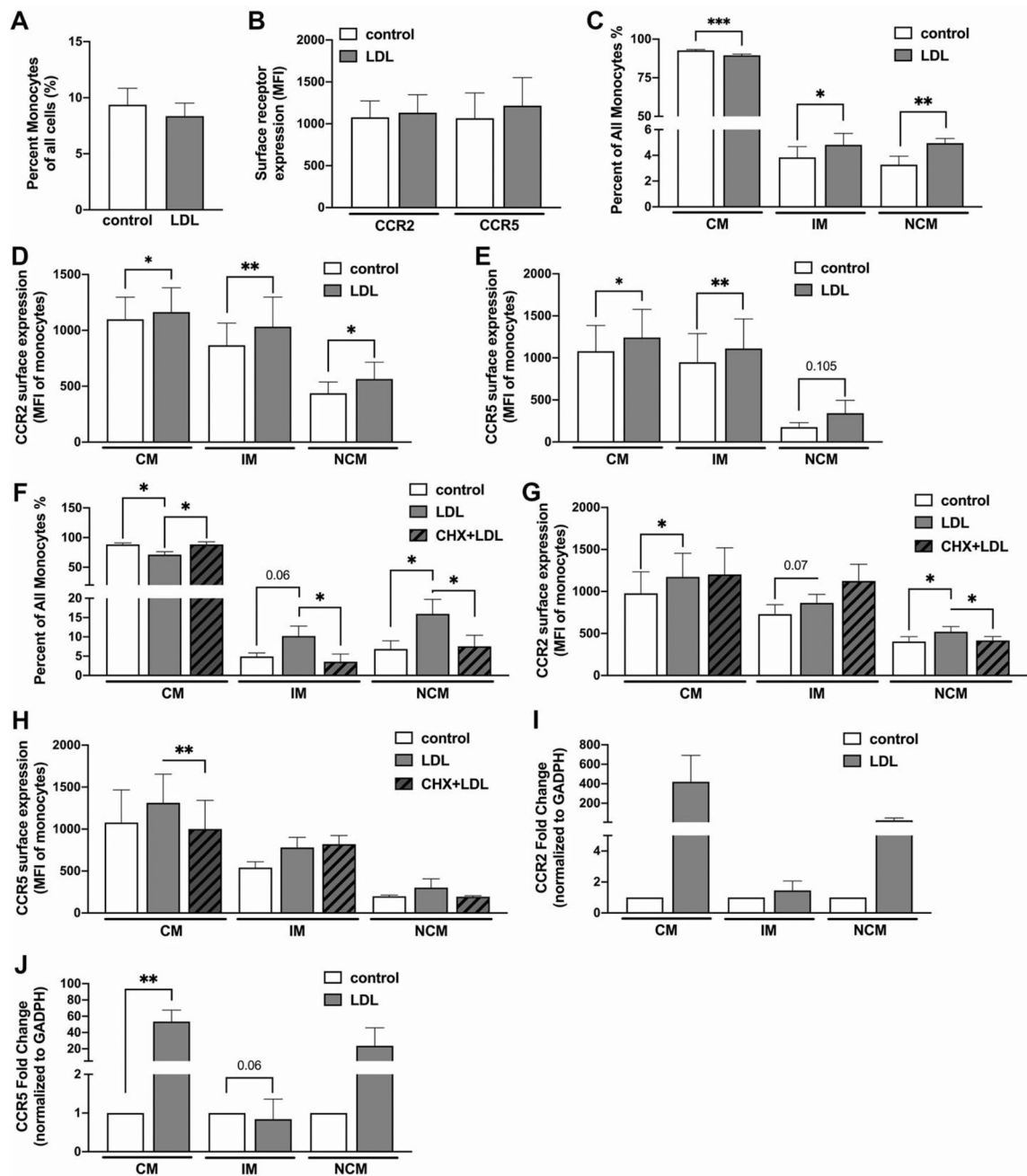
Figure 1: Phenotypic changes on monocytes due to LDL treatment.
Monocytes were isolated from healthy BBD and treated as indicated for 4hours. **(A-H)**
Monocyte subsets according to their CD14/CD16 expression and CCR2/5 surface expression
was measured utilizing flow cytometry. **(A)** Total amount of monocytes with and without
LDL treatment from all PBMC. Displayed as percentage of all cells (n=10, Mann-Whitney
test). **(B)** CCR expression on all monocytes displayed as MFI (n=10, unpaired T-test or
Mann-Whitney depending on Shapiro-Wilk test result per dataset). **(C)** Proportion of CM,
IM, and NCM of all monocytes with and without LDL treatment (n=10, paired T-test for
all datasets). **(D)** CCR2 expression, as determined by MFI, on individual monocyte subsets

(n=10, Wilcoxon test for all datasets). **(E)** CCR5 expression, as determined by MFI, on individual monocyte subsets after being treated with and without LDL (n=10, Wilcoxon test for all datasets). **(F-H)** Monocytes (N=6) were isolated and split into three samples. Sample 3 was pre-treated with 10 μg/μL CHX for 30 minutes. Then, 50 mg/dL LDL was added to Samples 2 and 3. All samples were incubated for four hours at 37°C/5% CO_2. Flow cytometry was used to characterize monocyte subsets **(F)**, CCR2 expression on individual subsets **(G)**, and CCR5 expression on individual subsets **(H) (F-H)** RM One-way ANOVA or Friedman test depending on Shapiro-Wilk test result per dataset with Tukey or Dunn's corrections was utilized as statistical test. **(I/J)** Additionally, flow cytometry was used to sort monocyte subsets by CD14 and CD16 expression profile for subsequent RT-qPCR analysis (unpaired T-test or Mann-Whitney depending on Shapiro-Wilk test result per dataset). (*p-val<0.05; **p-val<0.01 for all graphs)

Table 1:

Patient demographics for Cohort 2. Categorical variables expressed as N (%). Continuous variables expressed as mean ± SD[#] (range).

	Participants (N=40)
Female Sex, N (%)	36 (90%)
African American, N (%)	40 (100%)
Age, years	60.55 ± 10.67
BMI, kg/m2	32.94 ± 7.71 (20.5 to 50.8)
SBP, mmHg	132.33 ± 15. 61 (103 to 176)
DBP, mmHg	73.18 ± 10.94 (52 to 94)
TC, mg/dL	198.46 ± 39.94 (103 to 285)
HDL, mg/dL	58.73 ± 20.10 (18 to 98)
LDL, mg/dL	111.90 ± 34.93 (52 to 200)
TG, mg/dL	82.70 ± 26.41 (39 to 153)
Serum Insulin, mg/dL	16.43 ± 12.43 (5 to 59)
Glucose, mg/dL	105.56 ± 18.31 (84 to 156)
CRP, mg/dL	4.97 ± 9.47 (0.3 to 157)
FRS, percent (%)	11.34 ± 7.63 (0.7 to 36.7)
Estradiol[##], pg/mL	28.68 ± 49.33 (5 to 208)

SD[#] = standard deviation

[##] = N=16 due to samples being under detection limit

BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, TC = total cholesterol, HDL = high-density lipoprotein, LDL = low-density lipoprotein, TG = triglycerides, CRP = high-sensitivity C-reactive protein, FRS = Framingham risk score

Table 2:

Linear regression associations between monocyte subset populations obtained in *ex vivo* experiments and BMI and serum levels of TC, HDL, LDL, and TG. Unadjusted and adjusted models shown. Shown as standardized ß (p-val).

	CM	Adj. for FRS and BMI	IM	Adj. for FRS and BMI	NCM	Adj. for FRS and BMI
BMI	−0.11 (0.50)	−0.12 (0.45)[^]	0.11 (0.49)	0.13 (0.44)[^]	−0.00 (0.99)	−0.01 (0.92)[^]
TC	−0.27 (0.09)	−0.26 (0.11)	0.26 (0.11)	0.25 (0.13)	0.23 (0.15)	0.25 (0.13)
HDL	0.04 (0.83)	−0.02 (0.89)	−0.04 (0.81)	0.021 (0.89)	0.03 (0.86)	0.01 (0.95)
LDL	**−0.32 (0.046)** [*]	−0.31 (0.057)	0.29 (0.06)	0.29 (0.08)	**0.33 (0.036)** [*]	**0.36 (0.028)** [*]
TG	**−0.41 (0.009)** [**]	**−0.36 (0.02)** [*]	**0.42 (0.006)** [**]	**0.37 (0.015)** [*]	0.08 (0.63)	0.11 (0.47)
Serum Insulin	−0.05 (0.76)	0.05 (0.74)	0.05 (0.74)	−0.05 (0.74)	−0.03 (0.86)	−0.00 (0.99)

[*] = p-val <0.05.

[**] = p-val < 0.01.

[^] = Adjusted for FRS only.