RESEARCH ARTICLE

Reduced Population of CD36^{-}/ABCA1^{+} Macrophages is Correlated with An Increase of Coronary Artery Disease Risk Markers in Type 2 Diabetes Mellitus

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Abstract

BACKGROUND: Cluster of differentiation (CD)36 and adenosine triphosphate-binding cassette transporter A1 (ABCA1) are 2 macrophages-expressed receptors that promote cholesterol uptake and efflux, in which their imbalance might be associated with the foam cell formation risk. Type 2 diabetes mellitus (T2DM) has been correlated with the increase of this plaque formation. Therefore, it is necessary to determine whether expression of CD36 and ABCA1 in macrophages are correlated with coronary artery disease (CAD) risk markers in T2DM cases.

METHODS: Peripheral blood mononuclear cells (PBMC) were isolated from 13 diabetic patients and 11 healthy donors. Then, the PBMC-derived macrophages were cultured with supplement of oxidized low-density lipoprotein (ox-LDL) or lipopolysaccharide (LPS). Expression of CD36 and ABCA1 was measured using flowcytometry, meanwhile the supernatant concentration of interleukin (IL)-1β and IL-10 was measured by multiplex immunoassay.

RESULTS: T2DM subjects more likely to have low proportion of CD36^{-}ABCA^{+} macrophages compared to healthy donors (p=0.041) and it had negative correlation with glucose homeostasis and insulin resistance markers, including fasting blood glucose (FBG, r=-0.408, p=0.048), glycated hemoglobin (HbA1c, r=-0.380, p=0.049), triglyceride glucose index (r=-0,518, p=0.009), and high-sensitivity C-reactive protein (hs-CRP, r=-0.556, p=0.005). Moreover, it also had a negative correlation with atherogenic markers such as triglyceride (r=-0.417, p=0.043), triglyceride/HDL, and LDL/HDL, but had positive correlation with HDL (r=0.540, p=0.007). Most of T2DM subjects had high IL-1β/IL-10 ratio after ox-LDL and LPS stimulation (p=0.02 and p=0.05, respectively).

CONCLUSION: Reduced proportion of CD36^{-}ABCA1^{+} macrophages followed with high IL-1β/IL-10 can be a marker of CAD in T2DM.

KEYWORDS: type 2 diabetes mellitus, coronary artery disease, macrophages, ABCA1, CD36

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Introduction

In 2019, International Diabetes Federation estimated that more than 463 million adults are living with diabetes meliitus (DM) (1), and Indonesia is rank as the 5th country with the most DM cases in productive ages (2,3). Among these DM patients, 90% of them were classified as type 2 diabetes mellitus (T2DM).(1,2) DM is associated with an increase of atherosclerosis risk and its complications, including myocardial infarction.(4) Insulin resistance, main feature of T2DM is proposed as a central feature of the metabolic

syndrome that leads to atherosclerosis and coronary artery disease (CAD).(5) Therefore, T2DM is a substantial risk factor for cardiovascular disease and its consequences.

Macrophages are the key to all stages of atherosclerosis and are widely considered as a therapeutic target.(6) Diabetes patients are prone to have more pro-inflammatory type of macrophages and those contributes to various diabetic complications.(7) Components of the diabetic environment, such as oxidized low density lipoprotein (ox-LDL) and lipopolysaccharide (LPS) can stimulate macrophages to release cytokines that produce inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α.(8)

Macrophages internalize native lipoproteins, such as LDL and oxidized lipoproteins in plaques through micropinocytosis, phagocytosis of aggregated LDL and through scavenger receptor-mediated uptake including scavenger receptor type A (SRA), lectin like oxidized LDL (LOX1), scavenger receptor class B type 1 (SRB1) and cluster of differentiation (CD)36.(9) Macrophages also have an active transport system through transporters such as ATP-binding cassette transporters A1 (ABCA1), ATP-binding cassette transporters G1 (ABCG1), and SR-B1 which are responsible for the elimination of most of the cholesterol (cholesterol efflux) from the macrophages to extracellular acceptors.(10) Among those receptors, CD36 and ABCA1 are the primary roles in regulating cholesterol uptake and efflux. An increased of CD36 expression were correlated with foam cell formation and proinflammatory response via c-Jun N-terminal kinase (JNK) and nuclear factor kappa B (NF-kB) pathway.(11,12) In addition, CD36 also mediated ox-LDL induced IL-1β secretion and led to inflammasome formation.(13) On the other hand, ABCA1 can be considered as an atheroprotective receptor because it can inhibit cholesterol uptake and induce cholesterol efflux.(14) ABCA1 receptor also could enhance anti-inflammatory response via Janus kinase 2/STAT3 and induce IL-10 stimulation through protein kinase A.(8,15,16) T2DM is associated with increase of atherosclerotic plaque formation. In addition, the concentration and function of ABCA1 were reduced in T2DM and it was associated with high density lipoprotein (HDL) level.(17)

Peripheral blood mononuclear cells (PBMC) are source of cells with single nucleus, including circulatory monocyte. In circulation, monocyte and macrophage will invade tunica media, then will develop foam cells after certain induction such as ox-LDL.(18) Therefore, it is necessary to analyze the expression of CD36 and ABCA1 in macrophages-derived circulatory monocytes of diabetic patients after ox-LDL stimulation. In this study, we also measured IL-1β and IL-10 level as the representatives of pro/anti-inflammatory response that mediated by CD36 and ABCA1 receptor. This study was conducted to determine whether these receptors can be used as a marker to assess foam cell formation in T2DM patients who had a higher risk of developing atherosclerosis.

Methods

Study Subjects

The *in vitro* study was conducted with 13 T2DM patients and 11 healthy donors that were recruited from Ministry of Energy and Mineral Resources Outpatient Clinic and Prodia Clinical Laboratory (Greater Jakarta Region Office & PHC Kramat). The healthy donors were <18 years old subjects with body mass index (BMI) <25 kg/m^2, fasting blood glucose (FBG) <100 mg/dL and glycated haemoglobin (HbA1c) <5.7%. Meanwhile, T2DM subjects were patients in the same age range who had been diagnosed with T2DM by physician based on American Diabetes Association criteria with BMI >25 kg/m^2, FBG >126 mg/dL and HbA1c >6.4%. Subjects were excluded from the study if they were pregnant, having cancer, using immunosuppressants and steroids (during the sample collection), or having blood clotting disorders. The protocol of this study was approved by the Research Ethics Committee Faculty of Medicine Universitas Indonesia (No. KET-420/UN2.F1/ETIK/PPM.00.02/2022). Prior to taking part in this study, all subjects provided their written informed consent.

After an overnight fast, venous blood was withdrawn from each subject and collected in 9 mL lithium heparin vacutainer tube and 3 mL vacutainer serum tube. FBG, HbA1c, LDL-cholesterol, HDL-cholesterol, total cholesterol, triglyceride, and high sensitivity C-reactive protein (hs-CRP) were measure using standard routine laboratory techniques at Prodia Clinical Laboratory, Jakarta. Lithium heparin blood was used for peripheral blood mononuclear cells (PBMC) isolation.

PBMC Isolation and PBMC-derived Macrophages Differentiation

PBMC was isolated from phosphate buffer saline (PBS)-diluted heparinized blood using Ficoll-Paque Plus (GE Healthcare Life Science, Chicago, IL, USA) at a 1:1 v/v ratio. Then, it was centrifuged at 400 g for 20 mins at room temperature without break to generate 3 layers. The middle layer that contained buffy coat was pipetted and transferred

to a new conical tube. The cell suspense was washed twice with PBS. In brief, PBMC were cultured at 2 x 10^6 cells/mL in 24 well-plate containing complete medium Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Oxford, UK), 1% penicillin-streptomycin (Gibco), 1% Amphotericin B (Gibco), and 10% fetal bovine serum (FBS) (Gibco) at 37°C CO_2 5% overnight. Then, 50 ng/mL macrophage colony-stimulating factor (M-CSF) (Gibco) was added, and the cell suspension was cultured at 37°C CO_2 5% for 48 hours. The cell suspension was rinsed with RPMI 1640 medium 3 times. Following rinsing, the complete medium and 50 ng/mL M-CSF was added. The medium was changed every 2 days until 7 days when macrophages differentiation was observed.(19) In the 8th day, the medium was changed to complete medium and added 15 µg/mL ox-LDL (Thermo Fisher Scientific, Waltham, MA, USA), or 10 µL LPS (Sigma, St. Louis, MO, USA), or complete medium as a control. The cell suspension was incubated for 24 hours and followed by cell and supernatant harvesting using TrypLE Select enzyme (Thermo Fisher Scientific).

Flowcytometric Analysis

The macrophages were washed in 300 µL 2.5% bovine serum albumin-PBS (stain buffer) and centrifuged 250 g for 5 mins room temperature (RT). Supernatants were discarded and cells were resuspended in 20 µL cocktail of FITC anti-human CD36 Antibody (Cat. #336204, Biolegend, San Diego, LA, USA) and APC mouse monoclonal anti-human ABC1 (Cat. #sc-53482, Santa Cruz Biotechnology, Dallas, TX, USA) and incubated for 30 mins in dark RT. The cells were washed 2 times in stain buffer then centrifuges at 250 g for 5 mins RT. Cell associated fluorescence was determined by using a flow cytometer FACS Canto II (BD Bioscience, Franklin Lakes, NJ, USA). The mean fluorescence intensity was used to represent the data. Flowcytometry data were analyzed using FlowJo V10 software (BD Company, Ashland, OR, USA).

Multiplex Immunoassay

In vitro IL-1β and IL-10 production was measured using Luminex multiplex assay (R&D Systems, Minneapolis, MN). In brief, 20 µL of sample supernatants or standards followed by premixed beads were added into 96 well plate then plate was incubated for 2 hours RT on the plate shaker. The plate was washed using 200 µL washing buffer 3 times. Approximately 20 µL antibody detection was added into each well then it was incubated for 1 hour RT on the plate shaker. Without washing, 20 µL streptavidin-Phycoerythrin was added the incubated for 30 mins RT on the plate shaker. Lastly, the plate was washed 3 times and 150 µL Sheat Fluid Plus were added. The plate was read using Luminex 200 (Luminex Corporation, Austin, TX, USA).

Assay for CD36 and ABCA1 on Macrophages

First, single cell was filtered using forward scatter (FSC)-A versus FSC-H then the single cells were obtained from diagonal located cell population. Using FSC and side scatter (SSC) display, we established the location of macrophages. Then, we identified CD36 and ABCA-1 macrophages. Positive rates of antigens were measured using fluorescence minus one method (Supplementary 1 & 2).

Results

Subjects Characteristics

The characteristics of 13 T2DM subjects and 11 healthy subjects were presented in Table 1. Metabolic factors such as BMI, FBG, and HbA1c; as well as insulin resistance marker, including triglyceride glucose index and hs-CRP were significantly higher in T2DM group. Even though HDL and LDL concentration were not significantly different compared to healthy subjects, triglyceride and triglyceride/HDL ratio were increased in T2DM subjects with p=0.007 and p=0.004, respectively. Moreover, In T2DM, LDL/HDL ratio and triglyceride/HDL ratio were more than normal reference range, thus it indicated that there was increased risk of coronary atherosclerosis heart disease in this group.

We found a strong negative correlation between HDL and triglycerides level in T2DM (r=-0.72; $p \leq 0.001$). The higher is the HDL level, the lower is the triglyceride level. Healthy donors were mostly distributed in areas of high HDL cholesterol with low triglyceride levels, while the T2DM subjects were mostly distributed in areas of low HDL followed by high triglyceride levels (Supplementary 3A). Albeit insignificant, T2DM subjects had a lower HDL/LDL ratio compared to healthy subjects and the HDL/LDL ratio of T2DM subjects was below the median line (Supplementary 3B).

Microscopic Features of PBMCs and Macrophages

During culture, monocyte cells adhere firmly to the surface of the culture well so they were not discarded when medium replacement. M-CSF stimulated PBMC showed the morphological changes from day 0 to day 7 (Figure 1). Monocytes derived macrophages showed more granules in the cytoplasm, were slightly elongated and adherent to the culture plate. Microscopic observation using an

Table 1. Characteristics of subjects.

Characteristics	Healthy Subjects (n=11)	T2DM Subjects (n=13)	*p* -value
Gender, n			
Male	2	9	0.019***
Female	9	4	
Age (years), mean±SD	48.73±6.34	46.69±4.50	0.369
BMI (kg/m^2), mean±SD	24.09±3.02	29.15±4.18	0.003**
FBG (mg/dL), median (min-max)	91 (72-97)	145 (87-338)	0.002**
HbA1c (%), mean±SD	5.41±0.20	8.00±2.02	0.001**
LDL (mg/dL), mean±SD	149.82±41.30	162.23±33.47	0.424
HDL (mg/dL), median (min-max)	56 (35-82)	46 (37-67)	0.082
Triglyceride (mg/dL), median (min-max)	82 (57-195)	145 (60-513)	0.007**
Triglyceride Glucose Index, mean±SD	4.47±0.19	5.07±0.44	0.000***
Triglyceride/HDL ratio, median (min-max)	1.52 (0.82-3.52)	3.35 (0.9-12.62)	0.004**
AIP, mean±SD	0.20±0.25	0.60±0.33	0.004**
LDL/HDL ratio, mean±SD	2.84±1.13	3.61±0.97	0.085
hs-CRP (mg/L), median (min-max)	1.00(0.2-8.4)	3.90 (0.1-17.7)	0.035*

AIP: atherogenic index of plasma, calculated using log (triglyceride/HDL). *Significant if $p<0.05$, **Significant if $p<0.01$, ***Significant if $p<0.001$. Percentage of subjects of each gender was calculated using Chi square test. Age, BMI, HbA1c, LDL, Triglyceride Glucose Index, AIP, LDL/HDL were presented as mean±standard deviation, and the significances were calculated using unpaired Student's t-tests. FBG, HDL, Triglyceride, Triglyceride/HDL, and hs-CRP were presented as median (minimum-maximum), and the significances were assessed using Mann Whitney test.

inverted microscope is difficult to distinguish macrophage development and morphology between healthy donor and T2DM subjects.

Reduced CD36-ABCA1+ Macrophages Population in T2DM

Regardless the stimuli, macrophages population was dominated with CD36-ABCA-, whereas CD36+ABCA+ macrophages population was the least (Table 2). However, there was no difference of CD36 and ABCA1 proportion between healthy donor and T2DM group and among stimuli, that were ox-LDL, LPS, and control. To determine the ABCA expression between healthy donor and T2DM after ox-LDL stimulation, we divided CD36+ABCA1+, CD36+ABCA1-, CD36-ABCA1+, and CD36-ABCA1- populations into 2 categories (lower and higher proportion) using median or mean as cut-offs. For T2DM, we used 0.69, 9.87, 4.77, and 84.27 as cut-off of CD36+ABCA1+, CD36+ABCA1-, CD36-ABCA1+, and CD36-ABCA1- populations, respectively. Then, for healthy donors, we used 0.61, 11.6, 6.22, and 79.96 as cut offs of CD36+ABCA1+, CD36+ABCA1-, CD36-ABCA1+, and CD36-ABCA1-, respectively. We found that T2DM subjects were more likely to have low proportion of CD36-ABCA+ macrophages compared to healthy donors (p=0.041, Figure 2C). Meanwhile, the proportion of CD36+ABCA- was not different between healthy donors and T2DM subjects (Figure 2B).

In the analysis of macrophage response to ox-LDL stimulation, macrophages expressing low or high CD36+ ABCA1+ had the same proportion in non-T2DM and T2DM subjects. The proportion of low CD36+ABCA1- and low expressions of CD36-ABCA1+ macrophage in T2DM subjects was shown to have a high proportion (76.9% and 69.2%). High expression of CD36-ABCA1+ macrophages in non-T2DM subjects had the highest proportion (72.7%) while in T2DM subjects it was only 30.8%. In T2DM subjects, macrophages stimulated with ox-LDL predominantly showed low expression of CD36+ABCA1- while in non-T2DM subjects they showed low and high expression in almost the same proportions. Furthermore, macrophages expressed predominantly high CD36-ABCA1+ in non-T2DM subjects while CD36-ABCA1+ tended to be low in T2DM subjects (Figure 2A).

CD36-ABCA1+ Macrophages After Ox-LDL Stimulation is Correlated with CAD Risk Markers

To analyze whether CD36-ABCA1+ macrophages had an atheroprotective function, we correlate CD36-ABCA1+

macrophages population after ox-LDL stimulation with metabolic parameters, which also marker for glucose homeostasis and atherosclerosis. Regardless the T2DM status, the proportion of $CD36^{-}ABCA1^{+}$ macrophages had a significant negative correlation with glucose homeostasis and insulin resistance markers, including

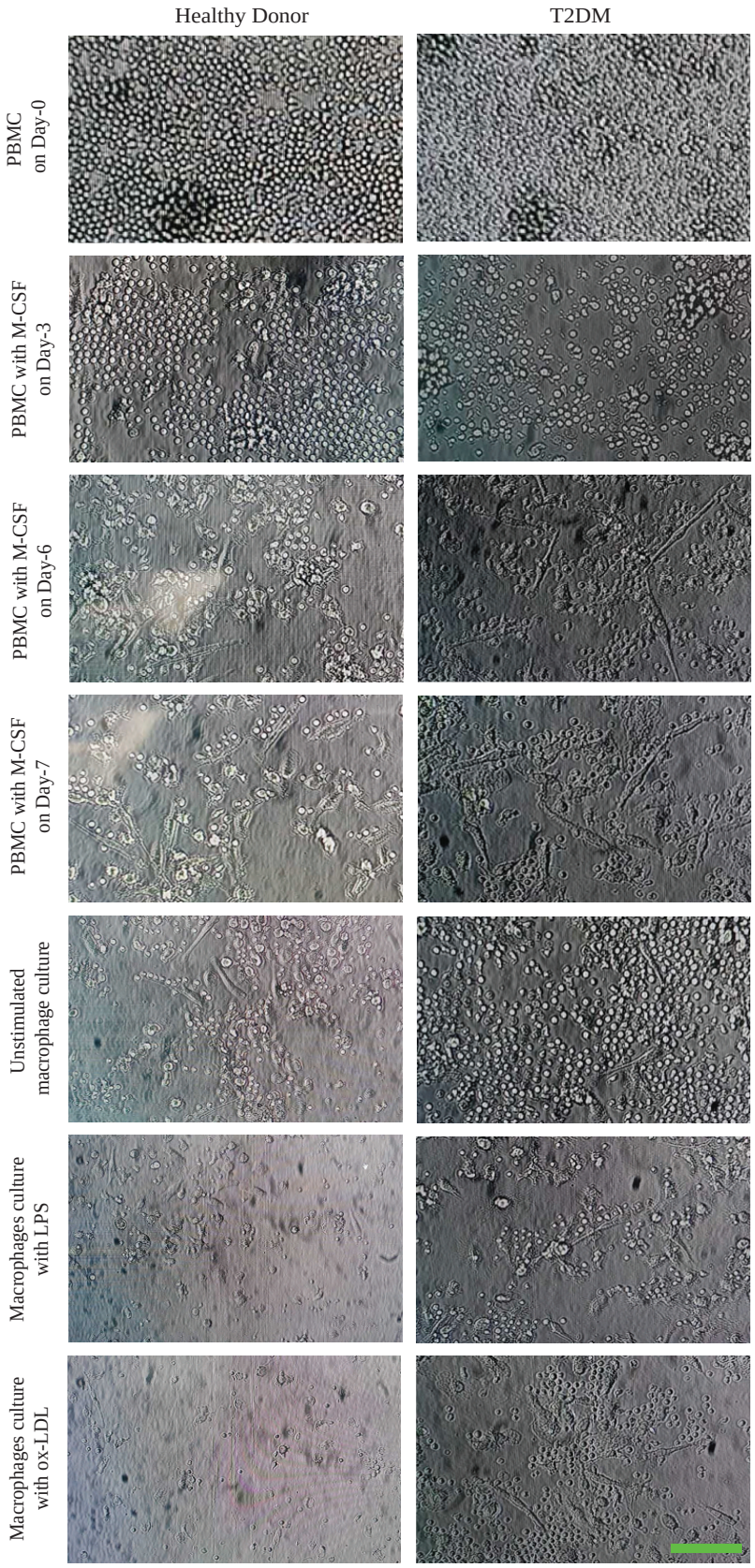

Figure 1. Characteristics of macrophages in healthy donor dan T2DM subjects. The stimulation by LPS or ox-LDL was conducted after the macrophages culture differentiation by 24 h incubation with LPS or ox-LDL. Green bar: 10 μm.

Table 2. The proportion of CD36 and ABCA1 expression on macrophages of healthy donors and T2DM subjects.

Marker	Stimuli	Healthy Subjects (n=11)	T2DM Subjects (n=13)	*p* -value
CD36$^+$	Ox-LDL, median (min-max)	13.01 (2.09-26.95)	10.96 (2.96-29.39)	0.691
	LPS, mean±SD	12.56±9.57	9.75±6.40	0.401
	Unstimulated, median (min-max)	17.27 (1.52-32.00)	9.99 (2.64-34.19)	0.521
ABCA1$^+$	Ox-LDL, mean±SD	5.30±2.92	5.70±2.16	0.146
	LPS, mean±SD	6.47±2.58	5.78±2.63	0.528
	Unstimulated, mean±SD	7.66±2.14	6.50±1.97	0.185
CD36$^+$ABCA1$^+$	Ox-LDL, median (min-max)	0.61 (0.36-2.66)	0.69 (0.31-2.34)	0.776
	LPS, median (min-max)	0.49 (0.14-2.11)	0.75 (0.15-2.05)	0.82
	Unstimulated, mean±SD	1.16±0.67	1.05±0.58	0.691
CD36$^+$ABCA1$^-$	Ox-LDL, median (min-max)	11.6 (1.67-26.50)	9.87 (2.65-28.90)	0.649
	LPS, mean±SD	11.69±9.07	8.94±6.44	0.395
	Unstimulated, median (min-max)	15.6 (1.22-30.9)	9.47 (2.20-32.7)	0.531
CD36$^-$ABCA1$^+$	Ox-LDL, mean±SD	6.22±2.29	4.77±1.90	0.107
	LPS, mean±SD	5.6±2.02	4.97±2.21	0.473
	Unstimulated, mean±SD	6.5±1.9	5.45±1.727	0.171
CD36$^-$ABCA1$^-$	Ox-LDL, mean±SD	79.96±10.17	84.27±6.10	0.213
	LPS, mean±SD	81.80±9.95	85.25±5.81	0.305
	Unstimulated, mean±SD	77.37±11.19	83.31± 8.18	0.148

The proportion of CD36$^+$ with ox-LDL stimulation and without stimulation, the proportion of CD36$^+$ABCA1$^+$ with ox-LDL and LPS stimulation, the proportion of CD36$^+$ABCA1$^-$ with ox-LDL stimulation and without stimulation were presented as median (minimum-maximum), and the significances were assessed using Mann Whitney test. The proportion of CD36$^+$ with LPS stimulation, the proportion of ABCA1$^+$ with ox-LDL, LPS, and unstimulated, CD36$^+$ABCA1$^-$ with LPS stimulation, the proportion of CD36$^-$ABCA1$^+$ with ox-LDL, LPS, control, and CD36$^-$ABCA1$^-$ with ox-LDL, LPS stimulation, and unstimulated were presented as mean±standard deviation, and the significances were measured using independent Student T-test.

FBG (r=-0.408, *p*=0.048), HbA1c (r=-0.380, *p*=0.049), triglyceride glucose index (r=-0,518, *p*=0.009), and hs-CRP (r=-0.556, *p*=0.005). Moreover, it also had a negative correlation with atherogenic markers such as triglyceride (r=-0.417, *p*=0.043), triglyceride/HDL, and LDL/HDL, but had positive correlation with HDL (r=0.540, *p*=0.007). CD36$^-$ABCA1$^+$ macrophages did not show any significant correlation with age, BMI, and LDL (Table 3).

To analyze the relationship between triglyceride/HDL ratio and CD36$^-$ABCA1$^+$ macrophages population in healthy and T2DM subjects, we made scatter plot that x axis represented triglyceride/HDL ratio and y axis represented CD36$^-$ABCA1$^+$ population (Figure 3). We made 4 quadrants with median as a cut-off. Quadrant 1 showed a low proportion of CD36$^-$ABCA1$^+$ macrophage population with a low triglyceride/HDL ratio. Quadrant 2 showed a high proportion of CD36$^-$ABCA1$^+$ macrophages with a low triglyceride/HDL ratio. Quadrant 3 showed a high proportion of CD36$^-$ABCA1$^+$ macrophages population with a high triglyceride/HDL ratio while quadrant 4 shows a low proportion of CD36$^-$ABCA1$^+$ macrophage population with a high triglyceride/HDL ratio. Healthy donors were more widely distributed in quadrant 1 and 2, which are the area of low triglyceride/HDL ratio. Meanwhile, T2DM subjects were abundant in quadrant 3 and 4, which are the area of high triglyceride/HDL ratio. The graph also showed a small proportion of non-patterned proportions in the two ratio models above, that was, non-diabetic subjects have a high triglyceride/HDL ratio, while diabetic subjects have a low one.

High IL-1β/IL-10 Ratio After LPS and Ox-LDL Stimulation in T2DM

The concentration of IL-1β and IL-10 from ox-LDL or LPS stimulated or unstimulated macrophages culture did not show any significant differences between healthy donors and T2DM subjects (Supplementary 4). However, after categorizing the IL-1β/IL-10 ratio based on their median (0.18 for T2DM subjects and 0.12 for healthy donors), it was found that most of the T2DM subjects

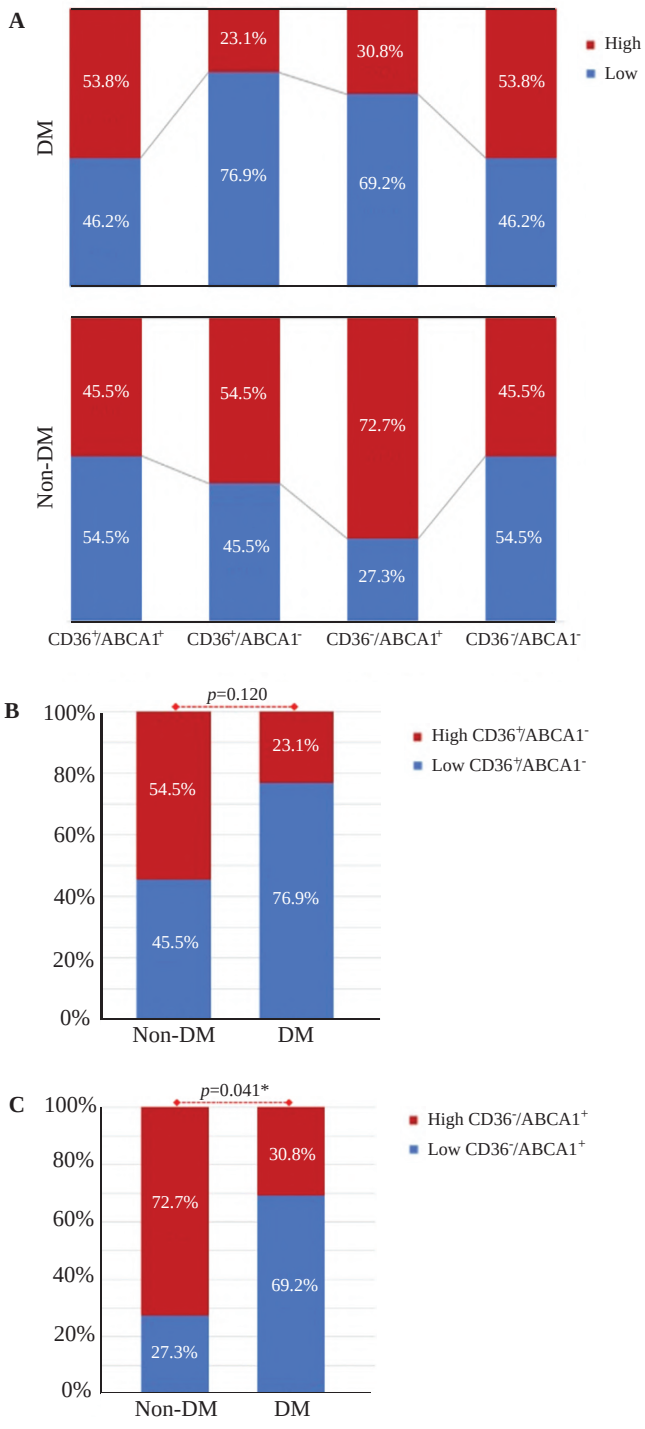

Figure 2. Characteristics of macrophage response to ox-LDL in healthy donors and T2DM subjects. A: The proportion of CD36 and ABCA1 macrophages after ox-LDL stimulation. Low and high proportion of $CD36^{+}ABCA1^{+}$ macrophages are the same in healthy donors and T2DM subjects. B: Comparison of the proportions of $CD36^{+}ABCA1^{-}$ macrophages between healthy donors and T2DM subjects. C: Comparison of the proportion of $CD36^{-}ABCA1^{+}$ macrophages between healthy donors and T2DM subjects. As much as 76.9% of T2DM subjects had low proportion of $CD36^{+}ABCA^{-}$ and 69.2% of T2DM subjects had low proportion of $CD36^{-}ABCA1^{+}$ macrophages. As much as 72.7% healthy donors had high proportion of $CD36^{-}ABCA1^{+}$ macrophage, whereas only 30.8% T2DM subjects had that. All of comparisons were calculated using Chi square test.

Table 3. Correlation between $CD36^{-}ABCA1^{+}$ macrophages after ox-LDL stimulation and metabolic parameters.

Metabolic Parameters	*r*	*p*- value
$CD36^{-}ABCA1^{+}$		
Age	0.141	0.51
BMI	-0.146	0.496
FBG	-0.408	0.048*
HbA1c	-0.38	0.049*
LDL	0.363	0.081
HDL	0.54	0.007**
Triglyceride	-0.417	0.043*
Triglyceride glucose index	-0.518	0.009**
Triglyceride/HDL	-0.495	0.014*
AIP	-0.495	0.014*
LDL/HDL	-0.558	0.005**
Hs-CRP	-0.556	0.005**

*Significant if $p<0.05$, **Significant if $p<0.01$. All data were calculated using Spearman's rank correlation coefficient.

had high IL-1β/IL-10 ratio after the ox-LDL and LPS stimulation ($p=0.02$ and $p=0.05$, respectively). Conversely, the healthy donor group was dominated by low IL-1β/IL-10 ratio (Figure 4).

The T2DM subjects were mostly distributed in quadrant 2 with low proportion of $CD36^{-}ABCA1^{+}$ macrophages followed by a high IL-1β/IL-10 ratio and conversely the healthy donors were widely distributed in quadrant 4 with high expression of $CD36^{-}ABCA1^{+}$ macrophages followed by low IL-1β/IL10 ratio (Figure 5).

Discussion

This current study shows some significant findings. First, atherosclerosis risk markers such as atherogenic index of plasma (AIP), triglyceride/HDL, LDL/HDL, hs-CRP were higher in T2DM. Second, the majority of T2DM subjects had low proportion of $CD36^{-}ABCA1^{+}$ macrophage population which also this population was correlated with glucose homeostasis, insulin resistance, and atherosclerosis markers. Third, low proportion of $CD36^{-}ABCA1^{+}$ macrophages, accompanied with higher proinflammatory response (IL-1β/IL-10 ratio) after ox-LDL and LPS stimulation were also observed in T2DM subjects.

Insulin resistance, which characterized by hyperinsulinemia and defective insulin signaling is a main feature of T2DM. Insulin resistance could induce the alteration of carbohydrate, lipid, and protein metabolism, that eventually lead to atherosclerosis via chronic inflammation,

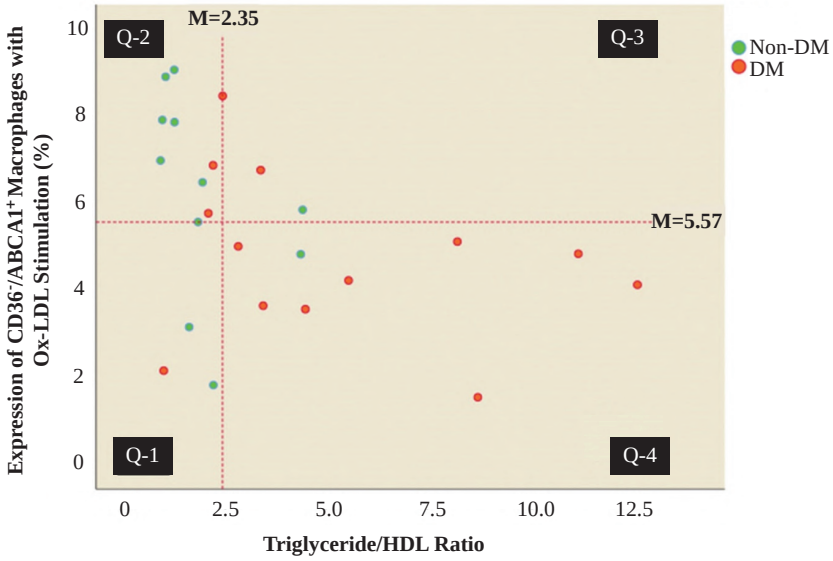

Figure 3. Correlation of triglyceride/HDL ratio with expression of CD36-ABCA1+ macrophages with ox-LDL stimulation. M: median, Q: quadrant.

dyslipidemia, advance glycation end product generation, and oxidative stress.(20) Insulin resistance contributes to dyslipidemia by increased influx of fatty acids to the livers and increased catabolism of HDL-C.(21) Therefore, it is common that T2DM patients have 2-4 folds risk of CAD.

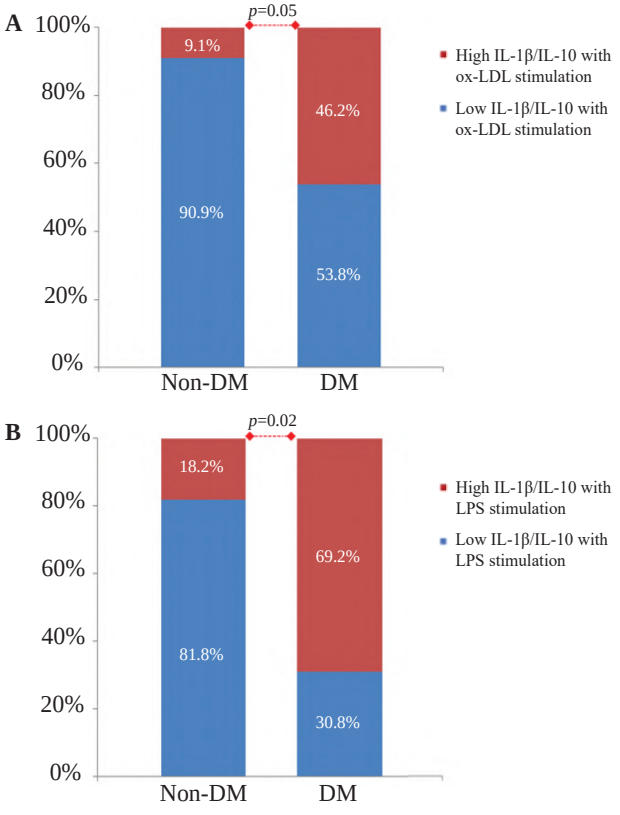

Figure 4. Comparison of IL-1β/IL-10 ratio from ox-LDL stimulated and LPS stimulated macrophages culture of healthy donors and T2DM subjects. A: ox-LDL-stimulated macrophages culture; B: LPS-stimulated macrophages culture.

(22) In accordance with the previous studies, we found T2DM subjects are in high risk of CAD due to increase the atherosclerosis risk markers such as AIP, triglyceride/HDL ratio, LDL/HDL ratio, and hs-CRP.(23)

In addition to LDL-C, parameters for assessing the risk of CAD can also use Apo B and non HDL-C. Apo-B and non HDL-C have been considered better predictors of CAD risk assessment than LDL-C. Non-HDL-C has been shown to be correlated better with Apo-B and has a diagnostic value as a risk factor that is comparable to or higher than Apo-B. Based on ROC analysis, there was no discernible difference between Apo-B and non-HDL-C in predicting the risk of CAD. Furthermore, non-HDL-C and Apo-B were equivalent in predicting future coronary heart disease risk. Another investigation employing validated discriminant ratios demonstrated that non-HDL-C and Apo-B performed similarly in diabetes patients. According to the Emerging Risk Factors Collaboration study, non-HDL-C and Apo-B were the most accurate indicators of CAD risk. As a result, both Apo-B and non-HDL-C have become reliable markers in CAD risk prediction beyond LDL-C. Non-HDL-C can be easily identified from a typical lipid profile panel, in contrast to Apo-B and does not require the additional cost of making it available for clinical decision making. In addition, the average time to report Apo-B was about four times longer than non-HDL-C.(24)

Insulin resistance also affects lipid metabolism in macrophages.(25) High dose of insulin can induce macrophage foam cell formation by upregulating CD36 and downregulating ABCA1, which then promote lipid accumulation in ox-LDL stimulated macrophages. Other study also reported that *in vitro* hyperglycemia could induce

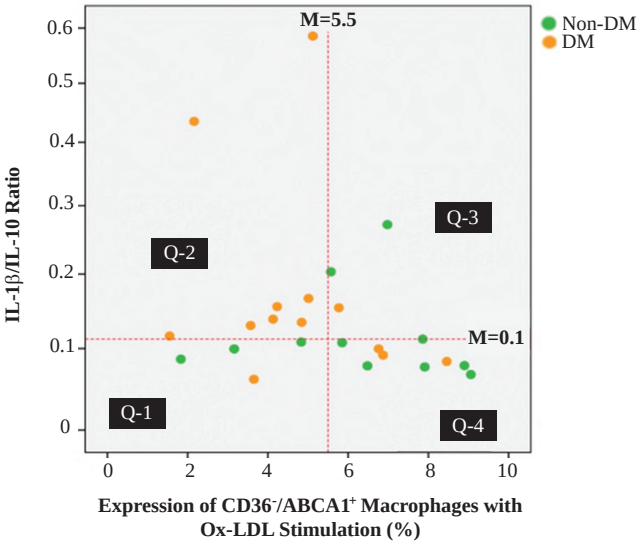

Figure 5. Correlation between CD36$^-$ABCA1$^+$ expression and IL-β/IL-10 ratio. M: median, Q: quadrant.

CD36 messenger ribonucleic acid (mRNA) expression and upregulation of CD36 could increase FFA uptake.(26,27) Increased CD36 expression in pancreatic β cell also reflected β cell disfunction.(27) Opposite to those studies, tumor necrosis factor-α (TNF-α), an inflammatory cytokine that increase in T2DM could suppress the membrane and mRNA expression of CD36. Interestingly, in this study, neither CD36$^+$ABCA1$^+$ or CD36$^+$ABCA1$^-$ expression was altered after ox-LDL stimulation in T2DM. This discrepancy happened may due to antidiabetic that all our subjects used, such as Metformin. Metformin reduces lipid uptake by inhibiting scavenger receptors expression, including SRA, CD36, And LOX1.(28) Therefore, the proportion of CD36$^+$, CD36$^+$ABCA$^+$, and CD36$^+$ABCA1$^-$ did not differ between T2DM subjects and healthy donors.

We found that CD36$^-$ABCA1$^+$ macrophages were markedly decreased in T2DM subjects. ABCA1 expression is regulated by peroxisome proliferation-activated receptor-γ.(29) Due to its contribution to form nascent HDL, defects in ABCA1 cause impairment of apo-A1 mediated lipid efflux and HDL deficiency. ABCA1 expression and function were reduced in T2DM and this expression was negatively correlated with glucose homeostasis. In line with previous study, not only glucose homeostasis (FBG and HbA1c), the result of study showed that proportion CD36$^-$ABCA1$^+$ macrophages were also negatively correlated with insulin triglyceride glucose index and CAD risk markers such as AIP, triglyceride/HDL ratio, LDL/HDL ratio, and hs-CRP and positively correlated with HDL level.(30)

IL-1β is a proinflammatory cytokine that related to metabolic syndrome and diabetes. The *in vitro* stimulation with high concentration of glucose and ox-LDL activated NF-kB through tool like receptor 4 and which then trigger IL-1β expression in macrophages.(31) Conversely, IL-10 is a potent anti-inflammatory cytokine, that can strongly inhibit IL-1β activation. IL-1β/IL10 ratio reflects pro- and anti-inflammatory response. Our Macrophages of T2DM subjects had a high inflammatory capacity, reflected by high IL-1β/IL10 ratio after LPS and ox-LDL stimulation. Whereas, healthy donors were dominated by low IL-1β/IL-10 ratio either in LPS or Ox-LDL stimulation. Obesity and T2DM were known to have macrophage imbalance, which characterized by more dominant of M1 "pro-inflammatory" macrophages than M2 "anti-inflammatory" macrophages.(32)

Instead as cholesterol exporter, ABCA1 could suppress IL-1β, IL-6, and TNF-α in LPS-stimulated macrophages culture via STAT3 pathway.(8) Notably, in our study, reduced proportion of CD36$^-$ABCA1$^+$ macrophage was correlated with high IL-1β/IL-10 ratio in T2DM. However, the mechanism of CD36$^-$ABCA$^+$ reduces systemic inflammatory marker still needs to be elaborated, hence further study is necessary..

Conclusion

The results of this study showed that T2DM subjects had a low proportion of CD36$^-$ABCA1$^+$ macrophage population which also correlated with glucose homeostasis, insulin resistance, and atherosclerosis markers. In addition, low proportion of CD36$^-$ABCA1$^+$ macrophages were also accompanied with higher proinflammatory response (IL-1β/IL-10 ratio) after ox-LDL and LPS stimulation. Therefore, reduced proportion of CD36$^-$ABCA1$^+$ macrophages followed with high IL-1β/IL-10 is associated with a higher risk factor of the development of CAD in T2DM subjects.

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Authors Contribution

NI and HW contributed to conception, design, and acquisition; critically drafted the manuscript; revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. ARP and DLT contributed to acquisition, critically drafted the manuscript, revised the manuscript, gave final approval, and agreed to be accountable for all aspects of work ensuring integrity and accuracy. RK contributed to interpretation, critically revised the manuscript, gave final approval, and agreed to be accountable for all aspects of work ensuring integrity and accuracy. All authors contributed to the article and approved the submitted version.

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