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Dendritic cell activation is blunted in patients with coronary artery disease and diabetes mellitus

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ABSTRACT

Background: It has been shown that functional status of dendritic cells (DCs) in diabetic patients with unstable angina pectoris (UAP) are more mature and activated than diabetic patients without coronary artery disease (CAD) and none diabetic patients with UAP. Accordingly we aimed to assess the activation of DCs in patients with CAD with/and without Diabetes Mellitus (DM) and compare to those in subjects with normal coronary arteries (NCA).
Materials and methods: Twenty three patients with severe CAD who were scheduled to coronary artery by-pass grafting surgery and 6 patients with angiographycally NCAs were included in the study. Activation of peripheral blood DCs have been analyzed by flow cytometric measures of CD86 activation.
Results: In patients with CAD and without DM, DC activation significantly increased after stimulation of oxidesized LDL (135 ± 121 vs 248 ± 197 $p = 0.024$). However this activation didn't significantly increased in patients with CAD and DM (100 ± 20 vs 120 ± 97, p = 0,54). Patients with NCAs and without DM showed marked activation of CD86 after stimulation with ox-LDL.
Conclusion: We have documented that DC activation, upon stimulation of ox-LDL has blunted in patients with CAD compared to patients with NCAs. Moreover this defective activation is more pronounced in those with diabetic patients with CAD.

1. Introduction

Tremendous achievement in understanding the molecular biology of atherosclerosis and related signaling pathways has been gained in last decades. Over all, Diabetes Mellitus constitutes a major contributing factor for every aspects of atherosclerosis. Dendritic cells (DCs) have been initially identified as potent antigen-presenting cells (APCs) that play a key role in induction of the innate immune response.[1] They are not only essential in launching immune reactions against harmful antigens, but also in maintaining immune tolerance and adaptive immune systems.[2–4] Dendritic cells have been shown to be involved in the pathogenesis of atherosclerosis. Decreased circulating DC precursors in patients with coronary artery disease (CAD) have been reported in literature almost a decade ago.[5,6] However, the DC activation status needs to be verified, since the numerical changes may result from the altered expression of the subset markers during activation.[7] Upon stimulation by circulating oxLDL or other atherosclerosis-related modified proteins, blood DCs may become activated, upregulate chemokine receptors such as CCR-7, and then travel towards lymphoid organs or inflamed tissues, such as atherosclerotic plaques. Brussel et al[8] has demonstrated that decreased numbers of peripheral blood dendritic cells in patients with CAD are associated with diminished plasma Flt3 ligand levels and impaired plasmacytoid dendritic cell function. Recently it has been shown that functional status of DCs in patients with Diabetes Mellitus (DM) and unstable angina pectoris is more mature and activated than patients with DM but without CAD.[9] Functional status of DCs in patients with unstable angina pectoris and without DM is less mature and less activated compared to those patients with DM and unstable angina pectoris.[9]

Accordingly, we aimed to assess the activation of monocyte and plasmacytoid -derived DCs in patients with CAD with/and without Diabetes Mellitus who were scheduled to coronary artery by-pass grafting

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surgery and compare to those in subjects with normal coronary arteries (NCAs).

2. Materials and methods

2.1. Study population

The study population consisted of 29 patients who had undergone coronary angiography. Of whom 23 patients with severe and diffuse coronary artery disease were scheduled to coronary artery by-pass grafting surgery and the remaining 6 patients had angiographycally normal coronary arteries. Patients with CAD were divided into two groups as having DM (10 patients) or not having DM (13 patients). All patients had type II diabetes. The diagnosis of type 2 Diabetes Mellitus was made according to the criteria of the American Diabetes Association.[10] Patients who had acute coronary syndrome or recent myocardial infarction with preceding one month, autoimmune diseases, malignancies, chronic or acute infections, asthma, severe heart failure (NYHA class 3 and 4) and advanced liver or renal diseases were excluded. Blood samples were obtained before coronary artery by-pass grafting operation in CAD group and at least 48 h after coronary angiography in those with NCAs. Blood samples were obtained into EDTA containing tubes from all subjects via antecubital vein puncture soon after admission. White blood cell analysis and biochemical analysis were performed by using routine protocols.

The protocol was approved by the hospital Ethics Committee and written informed consent was obtained from all patients. The study was performed according to the principles of the Declaration of Helsinki.

2.2. In-vitro generation of human immature monocyte derived dendritic cells using CD14 magnetic bead

We used monocyte and plasmacytoid-derived DCs to access their activation status. Enumeration of peripheral blood mononuclear cells and generations of DC were performed as described.[11] 15 mL of blood was collected from all subjects. Briefly, Assay –buffer was prepared (pH: 7.2) with RPMI 1640 (Omega) medium added with 10% fetal calf serum (BSA) and 2 mM EDTA. Mononuclear cell pellet was incubated in 300 μL assay buffer. 100 μL FcR blocking reagent and 100 μL non DC depletion cocktail was added and centrifuged 10 min. 1100 rpm. Cell pellets than incubated in 6 mL assay buffer. Pellet than was transferred to MAC S magnetic separator (MACs human CD 14 microbeads (MiltenyiBiotec)) and washed with 3 mL buffer solution. Cell suspension was centrifuged 10 min. in 1800 rpm. Than 100 μL DC Enrichment Cocktail was added. CD14 marked cell was separated and the rest was collected. Pellet was separated in two parts. One was control and the other was for incubation and stimulation with ox-LDL.

2.3. Monoclonal antibodies

Phycoerythrin (PE)-conjugated CD11c, PE conjugated CD141, peridininchlorophyll protein (PerCP)-conjugated anti- CD86, fluorescent isothiocyanate (FITC)-conjugated anti-CD-80, anti-CD-123, anti-CD14 and (FITC) conjugated lineage cocktail (lin), Allophycocyanin (APC)-conjugated HLA-DR were purchased from Becton Dickinson (USA). The Lin contains monoclonal antibodies (mAbs): CD3 (T cells), CD14 (monocytes/macrophages), CD19 (B lymphocyte), CD56 (NK Natural killer cells).

2.4. Characterization of the surface membrane phenotype of human DC

Monoclonal antibodies to the following surface proteins of DC were used: CD86, CD45, CD11c, CD141, CD14, CD80, CD 123, HLA-DR, LIN (BD). We used 4 tubes for flow cytometer: 1- CD123-FITC, CD11c-PE, CD45-APC, CD86-PerCP Cy 5.5. 2- CD11c-PE, HLA-DR-APC, CD80 FITC.

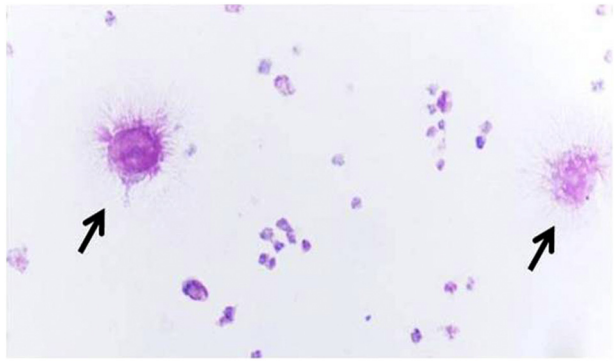

Fig. 1. Hematoxylen&Eosin staining ox-LDL stimulated DCs: Black arrows showing activated DCs.

3- CD141-PE, CD14-FITC, CD45-APC, CD86-PerCP Cy 5.5. 4- Lin-FITC, HLA-DR-APC 300 μL pellet were added into each tube (FACs tubes 5 mL (BD Falcon)) and 5 μL monoclonal antibody were used. 2 mL PBS was added into each tubes and vortexed 5 min. 1500 rpm. HLADR+ Lin- cells were accepted as Dendritic cell (Fig. 3).

2.5. Ox-LDL preparation

2 mg/mL LDL (β-lipoprotein, Sigma) was incubated with CuSO4 18 h with 37 °C. Oxidation was terminated by adding with 0.5 mM EDTA.

2.6. Cell culture

Cells that incubated with GM-CSF and TNF-α were harvested and counted. Non adherent cells were gently washed out with MACs Buffer saline (DPBS) (BD). Cell pellets were cultured in RPMI medium (RPMI1640) with PBS (Bovine serum albumin10%). Dendritic cells (5 × 105) were seeded in each well of 6-well plates, cultured in carbon dioxide oven. On day 2cells were collected for ox-LDL treatment (20 μL for each well).

2.7. Methods for assessing human DC activation

Cell pellets were transferred to RPMI 1640(%5) medium added with 10 IU/mL heparin. Than cells were cultured in medium 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/mL IL-4. Adherent cells were cultured for two days. Hematoxylen-Eosin staining of activated DCs are presented in Fig. 1.

We examined the expression level of CD86 to access the maturation state of DCs (Fig. 4). DCs (1 × 106 cells) were harvested and blocked with 10% normal goat serum for 15 min at 4 °C, washed and then stained

Table 1
Clinical characteristics and CD86 activity of patients with NCAs and CAD.

	NCAs (n = 6)	CAD (n = 23)	CAD (n = 23)	
			DM(+) (n = 10)	DM(−) (n = 13)
Age	60 ± 8	65 ± 8	65 ± 9	66 ± 8
Gender (female)	2/6	7/23	3/10 (30%)	4/13 (30%)
Smoking	2/6 (33%)	6/23 (26%)	3/10 (30%)	3/13 (23%)
Hyperlipidemia	2/6 (33%)	9/23 (43%)	5/10 (50%)	5/11 (38%)
Hypertension	2/6 (33)	9/23 (43%)	5/10 (50%)	5/11 (38%)
CD86 activity				
Before ox-LDL	142 ± 69	120 ± 92	100 ± 20	135 ± 21
After ox-LDL	547 ± 560	192 ± 68*	120 ± 98	247 ± 193¶

CAD; coronary artery disease, NCA; normal coronary artery, DM; Diabetes Mellitus.
* p = 0.01 vs NCAs.
¶ p = 0.05 vs DM (+).

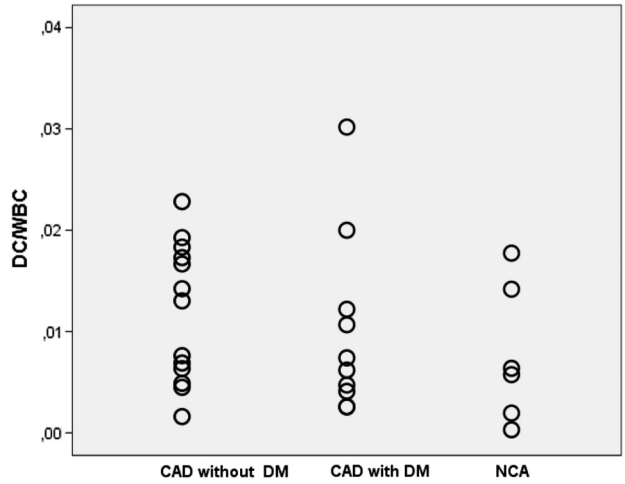

Fig. 2. Scatter diagram showing DC/WBC ratio as percentages in CAD patients with and without DM and control subjects ($p > 0.05$): DC; dendritic cell, CAD; coronary artery disease, DM; diabetes mellitus, NCA; normal coronary arteries.

with FITC-conjugated antibodies against CD86 for 30 min at 4 °C. Cells stained with the appropriate isotype-matched IgG were used as negative controls. After immunofluorescence staining, cells were analyzed by FACS Calibur using CellQuest software (BD Bioscience San Jose, CA 95131-1807, 2007).

2.8. Statistical analysis

Continuous variables are expressed as mean ± sd, and categorical variables are expressed as counts or percentages. For categorical variables Chi-square or Fisher's exact *t*-test were used where appropriate. Comparison of continuous variables were compared by using nonparametric Mann-Whitney *U* test and Mann Whitney U test. Kruskal-Wallis one-way analysis of variance used compare nonparametric variables between groups. A p value < 0,05 was considered to be statistically significant.

3. Results

Study population and clinical characteristics are presented in Table 1. There were not significant differences between the patients with CAD and subjects with NCAs regarding the age, gender, smoking status and hyperlipidemia ($p > 0.05$). All the patients with NCAs were nondiabetics. Additionally clinical parameters were also comparable between patients with and without DM. The absolute number and percent of DCs within the total leukocyte population was similar in patients with CAD ($8.6 \pm 5.6 \times 10^6$/L and 1.18%) and normal controls ($7.4 \pm 6.5 \times 10^6$/L and 1.35%, $p = 0.65$). Percents of DC among three groups namely, CAD with DM, CAD without DM, and normal subjects were also found to be comparable by using Kruskal-Wallis one-way analysis of variance (Fig. 2). Baseline DC activation of patients with CAD (23 patients) did not differ from those of normal subjects (142 ± 69 vs 120 ± 92 respectively $p = 0,58$, Table 1), However, fluorescence activated cell sorter (FACS) analysis showed stimulation of DCs with ox-LDL resulted in almost four fold increase in the activity of CD86 (Fig. 3). Although it was statistically insignificant it was likely to be significant regarding the low number of control subjects. While the baseline activities were comparable; the mean activation of DC after ox-LDL stimulation in patients with NCAs was significantly higher than in those with CAD (Table 1). In patients with CAD and without DM, DC activation significantly increased after stimulation of ox-LDL (135 ± 121 vs 248 ± 197 $p = 0.024$). However this activation did not significantly increased in patients with CAD and DM (100 ± 20 vs 120 ± 97, $p = 0, 54$, Fig. 4). Detection of fractions of peripheral blood DCs and expression of CD 86 on dendritic cells after stimulation of Ox-LD ox-LDL by standardized 3-color flow cytometry were shown in Figs. 5 and 6.

4. Discussion

The main finding of our study is that while DCs of patients with CAD and without DM have shown significant CD86 activation, diabetic patients with CAD and without DM have not shown such activation upon stimulation with oxidized LDL. Interestingly, patients with NCA have demonstrated marked elevation of CD86 activation, although the difference is tended to be significant. Additionally, the number and percentage of DCs are comparable between patients with CAD and subjects with NCAs.

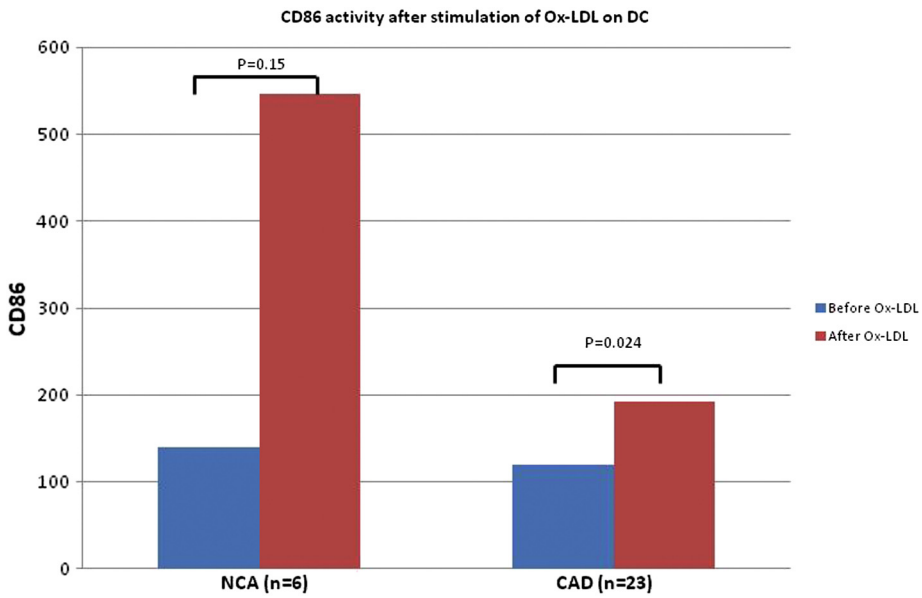

Fig. 3. Bar diagram of CD86 expression activity patients with and without CAD before and after Ox-LDL stimulation.

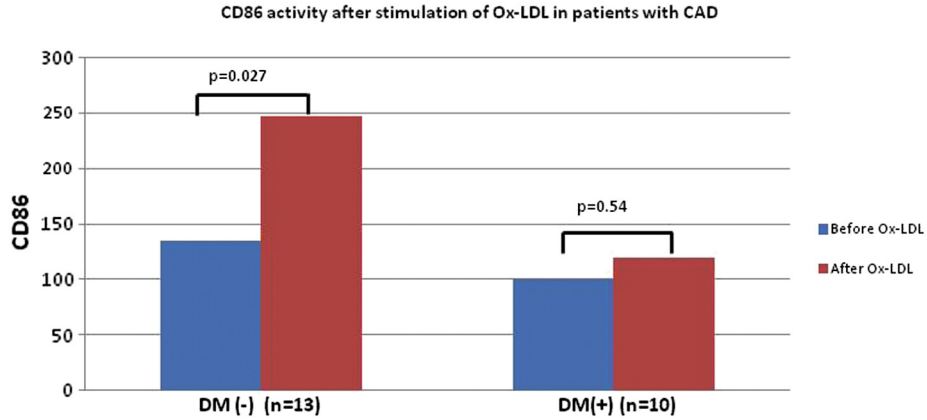

Fig. 4. Bar diagram of CD86 expression activity in CAD patients with and without Diabetes Mellitus before and after Ox-LDL stimulation.

Atherosclerosis is a complex disease mainly accompanied by chronic inflammatory process. As the most potent APCs, dendritic cells are important players responsible for the induction of immunity and atherosclerosis.[12–18] Human monocyte– derived dendritic cell-mediated lipid antigens delivery, and inflammatory cytokines are essential for the initiation and progression of atherosclerosis.[15–18] Innate and adaptive immunities have been linked to vascular inflammation in the pathogenesis of atherosclerosis in which macrophages, monocytes and DCs play major and crucial role.[19] Although the involvement of APCs in atherosclerotic process has been well demonstrated in literature, the main mechanism of DCs in atherosclerosis and DM is largely unknown and literature reveals controversial results. This controversy mainly comes from methodology of the studies evaluating the role of DCs in normal and pathologic conditions. Type of DCs, resident or circulatory cells, clinical status of patients such as diabetic vs non-diabetic, unstable angina or stable angina, activation or maturation markers of DCs such as CD4, Cd11b, CD86, etc. used in the studies might have led to prevent comprehensive understanding of DC in pathologic or normal conditions.

Atherogenic factors in the vascular wall such as oxidized LDL cholesterol,[20] advanced glycation end products,[11] nicotine,[21] and insulin[22] have been shown to induce the maturation of DCs. Direct evidence of relationship between DCs and atherosclerosis has been reported by Bobryshev et al[23] by showing accumulation of DCs in atherosclerotic lesions. However, combined deficiency of CD80 and CD86 in *Ldlr*−/− mice has resulted in reduced early and to some extent advanced atherosclerotic lesion formation, implicating protective role of CD80 and CD86 against atherosclerosis.[24] Moreover, impaired plasmacytoid dendritic cell function have been documented in patients

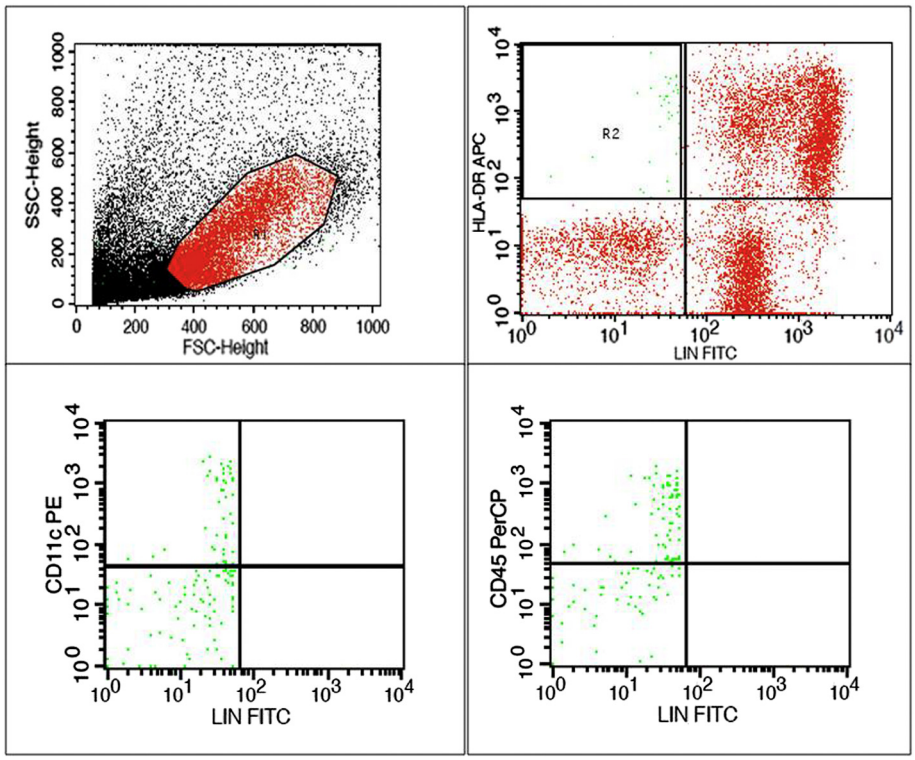

Fig. 5. Detection of fractions of peripheral blood dendritic cells (DCs) by standardized 3-color flow cytometry. Peripheral blood samples were collected and the cells were stained with APC-conjugated anti-HLA-DR, PE-conjugated anti-CD11c, PerCP-conjugated CD45, and FITC-conjugated lineage markers (lin1) containing anti-CD3, CD15, CD16, CD19, CD20, and CD56. After lysis of erythrocytes, the cells were analyzed by three-color flow cytometry: All mononuclear cells gated(R1); cells were gated on region R1, a dot blot of lineage marker (x axis) versus HLA-DR (y axis) was used to define the region of Lin-cells; DC was defined as Lin1−, HLA-DR+, FITC: fluorescein isothiocyanate; FSC-H: forward scatter; PE: phycoerythrin; In region R2, circulating DCs were detected according to their specific staining.

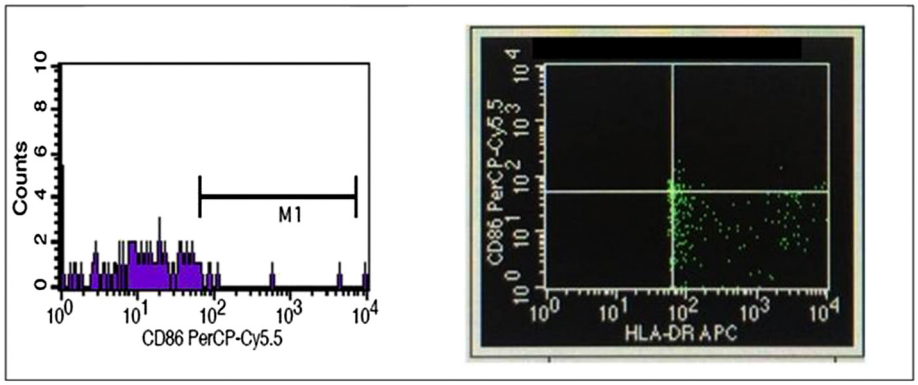

Fig. 6. Expression of CD 86 on dendritic cells after stimulation of Ox-LD ox-LDL-induced morphological and phenotypic change in monocyte-derived DCs. Immature DCs were treated with different concentrations of ox-LDL in DC medium supplemented with 50 ng/mL GM-CSF and 50 ng/mL IL-4 for 48 h. Cell surface molecules (CD86, and HLA-DR) were examined by FACS analysis.

with coronary artery disease.[8] In terms of DM it is more complicated to understand the effects of DCs on the process of atherosclerosis. Lu et al[22] has suggested that hyperinsulinaemia can promote DC activation and up-regulate the expression of the scavenger receptors, which can increase the oxLDL-uptake capacity of DCs. The results of the present study indicate that one of the mechanisms by which insulin promotes atherogenesis is mediated by its effects on DCs. On the other hand, less production of CCL2 and reduced expression of CD86 by peritoneal phagocytes and splenic DCs have been documented by Venturini et al in murine models of DM.[25]

Corrales et al[26] has reported that type 2 diabetic men with atherosclerotic complications display both quantitatively and functionally impaired immunological responses by circulating APCs. They have also commented that decreased patterns of inflammatory cytokine production by these cells may subsequently influence the downstream of inflammatory response mediated by APC and by other cells such as T cells. On the contrary, functional status of DCs in diabetic patients with unstable angina pectoris have been found to be more mature and activated than diabetic patients without CAD and none diabetic patients with UAP evidenced by the upregulation of costimulatory molecule CD86 and the enhanced capability of DCs to stimulate T-cell proliferation and cytokine production.[9] Moreover, they have reported decreased number of circulatory DCs in diabetic patients with unstable angina compared to nondiabetics with unstable angina. On the contrary we have found that DCs are comparable between patients with and without CAD which underlights that functional status rather than the qualitative status of DCs may play a pivotal role in the pathogenesis of atherosclerotic process. However study population in which patients with acute coronary syndrome included is not comparable to our study population.

Because of the limitation in small population of our study, further detailed studies in patients with CAD and/or DM in different clinical subpopulation including both stable and unstable CAD will no doubt lead to improve our knowledge of DCs in different spectrum of atherosclerotic process and its complications.

5. Conclusion

We have documented for the first time that DC activation, upon stimulation of ox-LDL has blunted in patients with CAD compared to patients with NCAs. Moreover this defective activation is more pronounced in those patients with CAD and DM. On the contrary, patients with NCAs and without DM have shown the marked activation of DCs after stimulation of ox-LDL. These results underline the possible deleterious effect of diminished activation of monocyte and plasmacytoid derived DCs in the pathogenesis of atherosclerosis especially in DM. However this issue needs to be clarified by studies including large number of patients with different clinical stages and presentations of the atherosclerosis.

List of abbreviation

DC	Dendritic cells
CAD	coronary artery disease
DM	Diabetes Mellitus
NCA	normal coronary arteries
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage- colony stimulating factor
WBC	White blood cell

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Authors' contributions

Study conception and design: Gulay Yetkin, Aysegul Atak Yucel,
Acquisition of data: Gulay Yetkin, Ishak Ozel Tekin, Hakan Atalay,
Analysis and interpretation of data: Gulay Yetkin, Ishak Ozel Tekin, Mustafa Yılmaz, Ertan Yetkin Drafting of manuscript: Gulay Yetkin, Ertan Yetkin, Aysegul Atak Yucel.
Critical revision: Aysegul Atak Yucel, Ishak Ozel Tekin, Mustafa Yılmaz.

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