

## The effect of ox-LDL and platelets on macrophages, M2 macrophage polarization, and foam cell formation

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### Original Article

#### Abstract

**BACKGROUND:** The accumulation of oxidized LDL (ox-LDL) in macrophages in association with platelet activity leads to the formation of foam cells, which play a key role in the pathophysiology of atherosclerosis and coronary artery diseases (CAD). Here, in this study, we aimed to investigate the simultaneous effect of ox-LDL and platelets on foam cell formation, as well as modification in cell markers.

**METHOD:** First, the U937, a human monocytic cell line, was cultured in RPMI-1640. Then, isolated platelets were co-cultured with the U937 and exposed to ox-LDL (80 µg/ml) to evaluate the impact of ox-LDL on foam cell formation using Oil red O (ORO) staining. Also, the expression of foam cells' surface markers and *CD36*, *ABCA1*, *SR-B1*, *ACAT1*, and *LXRα* genes, which are involved in macrophage metabolism and ox-LDL uptake, was measured by flow cytometry and real-time PCR, respectively.

**RESULTS:** Our findings suggest that platelets promoted foam cell formation (ORO-positive cells), accompanied by a higher level of CD163+ M2 macrophages. Furthermore, the expression of *CD36*, *ABCA1*, *SR-B1*, *ACAT1*, and *LXRα* genes, which are implicated in cholesterol accumulation in macrophages, was significantly upregulated in the ox-LDL+ platelets group compared to the control ( $P < 0.05$ ). Moreover, the up-regulation of *CD36*, *ABCA1*, and *SR-B1* genes in the ox-LDL+ platelets group was more accentuated compared to the ox-LDL group ( $P < 0.05$ ).

**CONCLUSIONS:** Owing to the positive effector role of platelets in the formation of foam cells and CD163+ cells, it could be assumed that platelets play a dual role in the development of these cells.

**Keywords:** ox-LDL, Platelets, Macrophages, Foam cells, Atherosclerosis

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#### Introduction

Atherosclerosis refers to the pathological build-up of fats in arteries, which is the first-line risk factor of CAD, taking a great death toll globally<sup>1,2</sup>. Upon atherosclerosis, the arterial lumen is occluded, thereby, contributing to the development of myocardial infarction (MI) and stroke<sup>3</sup>. Multiple cellular and molecular factors are involved in the pathogenesis of atherosclerosis and plaque formation including

foam cells, lipid-laden macrophages, and the derived cells<sup>3-5</sup>.

According to the current literature, multiple factors are implicated in the formation of foam cells including endothelial barrier activation under inflammatory conditions and elevated levels of modified LDL (e.g., ox-LDL) in the sub-endothelial layer. Subsequently, immune cells, in particular, monocytes, are translocated to the area<sup>6</sup>. Monocytes differentiate into macrophages, which then, release pro-

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inflammatory or anti-inflammatory cytokines or promote the uptake of ox-LDL through scavenger receptors such as CD36 and SR-A. The failure to metabolize the large quantity of ox-LDL leads to lipid accumulation in macrophages and their conversion into foam cells<sup>6,7</sup>. Various macrophage properties, particularly, surface markers such as CD68+, CD163+, and CD146+ are involved in this process<sup>8-10</sup>. Platelets play a role in atherosclerotic inflammation and plaque formation through different mechanisms such as promoting ox-LDL uptake by monocytes/macrophages<sup>11-13,14-16</sup>. The current study was carried out to explore the simultaneous effect of ox-LDL and platelets on foam cell formation. Furthermore, we aimed for determining how surface markers such as CD86 and CD163 and the expression of *CD36*, *ABCA1*, *SR-B1*, *LXR $\alpha$* , and *ACAT1* genes can be modulated upon the conversion of macrophages into foam cells.

## Materials and Methods

### *Oxidation of LDL*

According to the protocol described by Khiabani et al, ox-LDL was prepared<sup>17</sup>. In brief, 10 units of LDL (1mg/mL) (Pishtaz teb, Iran) were mixed with 1 unit of CuSO<sub>4</sub> solution (5  $\mu$ M), then, incubated at 37° for 24 hours. Subsequently, the mixture was put into a dialysis bag and dialyzed against EDTA (5 mM) solution at room temperature for 2 hours and PBS (PH=7.4, 10 mM) at 4 °C for 24 hours, respectively. All steps were conducted on the rotator. Ultimately, the absorbance and concentration of prepared ox-LDL were detected using a spectrophotometer (HALO VIS- 20) at 360 nm wavelength and standard ox-LDL (Thermo Fisher Scientific, USA) with 2.5 mg/ml concentration.

### *Platelet preparation*

Platelets were prepared based on the protocol described by Hamdipour et al.<sup>17</sup>. In brief, healthy volunteers' blood samples were taken into Acid Citrate Dextrose solution-A (ACD-A) (1:4), then, centrifuged at 200  $\times$

g for 15 min to obtain platelet-rich plasma (PRP). Subsequently, the PRP was diluted with Tyrodes-HEPES- buffer (HEPES-2.5 mM; NaCl-150 mM; KCl-1 mM; NaHCO<sub>3</sub>-2.5 mM; NaH<sub>2</sub>PO<sub>4</sub>-0.36 mM; glucose-5.5 mM; BSA-1 mg/ml; PH 6.5), then, centrifuged at 800  $\times$  g for 10 minutes. After removing the supernatant, the platelet pellet was resuspended in Tyrodes-HEPES buffer (PH7.4, enriched with 0.9 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>).

### *U937 Cell culture*

The U937 cell line was obtained from the cell bank of the Iranian Institute of Pasteur, then, cultured in RPMI-1640 solution containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere and passaged every two days.

### *U937 cell differentiation*

U937 cells were cultured in 6-well plates for flow cytometry and real-time PCR and 48-well plates for ORO staining (1 $\times$ 10<sup>6</sup> cells/ml). These cells were then incubated with phorbol 12-myristate 13-acetate (PMA) (80 ng/mL) (Santa Cruz Biotechnology, USA) for 24 hours to yield macrophages. Subsequently, medium of the PMA-treated cell was refreshed, then, incubated for 24 hours. Afterward, ox-LDL (80  $\mu$ g/ml) and isolated platelets (monocyte to platelet 1:100) were added to the medium, then, incubated for 4 days. to evaluate the effect of ox-LDL on foam cell formation and explore platelets' effects on the formation of lipid-droplet cells. Finally, three modified cell lines including PMA-treated cells (control group), PMA+ ox-LDL (ox-LDL group), and PMA+ ox-LDL+ platelets (ox-LDL +platelets group) were determined. In two days intervals, half of the medium was refreshed.

### *Oil Red O staining*

At the end of incubation time, the cells were washed with PBS 3 times, then, fixed with 10% formalin for 30 minutes. Subsequently, these cells were treated with 60% isopropanol for 1 min and stained with ORO (Sigma-Aldrich,

USA) for 30 minutes. Thereafter, ORO solutions were removed by 60% isopropanol within 15 seconds. Finally, the cells were rinsed with PBS 3 times and observed by 10× and 40× magnification light microscope (Olympus) with the Optika camera (Italy). The image analysis was performed using Image J software.

#### *Evaluation of cell surface markers*

Flow cytometry was employed to determine the expression of surface markers in cells. First, the cells were washed with PBS 3 times and harvested, then, fluorescent-conjugated antibodies against CD4-FITC (Exbio, Czech Republic), CD33-FITC (Dako, Denmark), CD14-PE (Beckman Coulter, USA), CD11c-FITC (Dako, Denmark), CD86-PerCP (Abcam, USA), and CD163-FITC (R&D Systems, UK), as well as their isotype controls were added to each sample of suspended cells and incubated for 30 minutes at 4 °C in dark. Finally, cells were fixed with 0.5% paraformaldehyde and re-suspended in PBS and each marker's percentage was evaluated compared to its isotype control.

#### *cDNA synthesis and real-time PCR*

To remove platelets, cells were washed with PBS 3 times, then, harvested and the total RNA was extracted using the Trizol RNA extraction protocol (Sigma-Aldrich, USA). The isolated RNA was reverse transcribed into cDNA using the cDNA synthesis kit (Thermo Fisher Scientific, USA) and the primer sequences (Table 1). Subsequently, using real-time PCR cyclor instrument (Rotor-Gene Q, QIAGEN, Germany), the expression of *CD36*, ATP-binding cassette A1 (*ABCA1*), scavenger receptor class B type 1 (*SR-B1*), acetyl-CoA acetyltransferase 1 (*ACAT1*), and *LXRα* (*NR1H3*) genes was evaluated. To determine the relative expression of genes of interest, a  $2^{-\Delta\Delta Ct}$  formula with the housekeeping ABL gene as internal control was utilized.

#### *Statistical analysis*

All experiments were repeated in triplicate and the mean  $\pm$  standard deviation (S.D.) of three independent tests was calculated. For

data analysis, the unpaired two-tailed Student's t-test method was applied using SPSS (version 16.0) and GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The p-value of  $<0.05$  indicated minimal statistical significance in all experiments.

## Results

LDL was oxidized to produce ox-LDL in a valuable concentration (1.60 mg/ml). Table 2 represents ox-LDL and LDL concentrations.

#### *PMA induced the differentiation of U937 cells to macrophages*

PMA was shown to induce monocytic cell conversion into macrophages with a spindle-shaped appearance, thus, validating U937 cell differentiation (Figure 1A). Further, we revealed that the expression of CD4 was significantly downregulated ( $P \leq 0.05$ ) in macrophages (4.21%) compared to U937 cells (98.6%). Also, the expression of CD14 and CD11c was 12.8% and 87.4% in macrophages and 3.59% and 31.8% in U937 cells, respectively ( $P \leq 0.05$ ). In addition, the expression of CD86 and CD163 was 23.2% and 43.1% in macrophages and 7.27% and 57.3 % in U937 cells, respectively, ( $P \leq 0.05$ ) (Figure 1B).

#### *Increased formation of foam cells induced by ox-LDL and platelets addition to the medium*

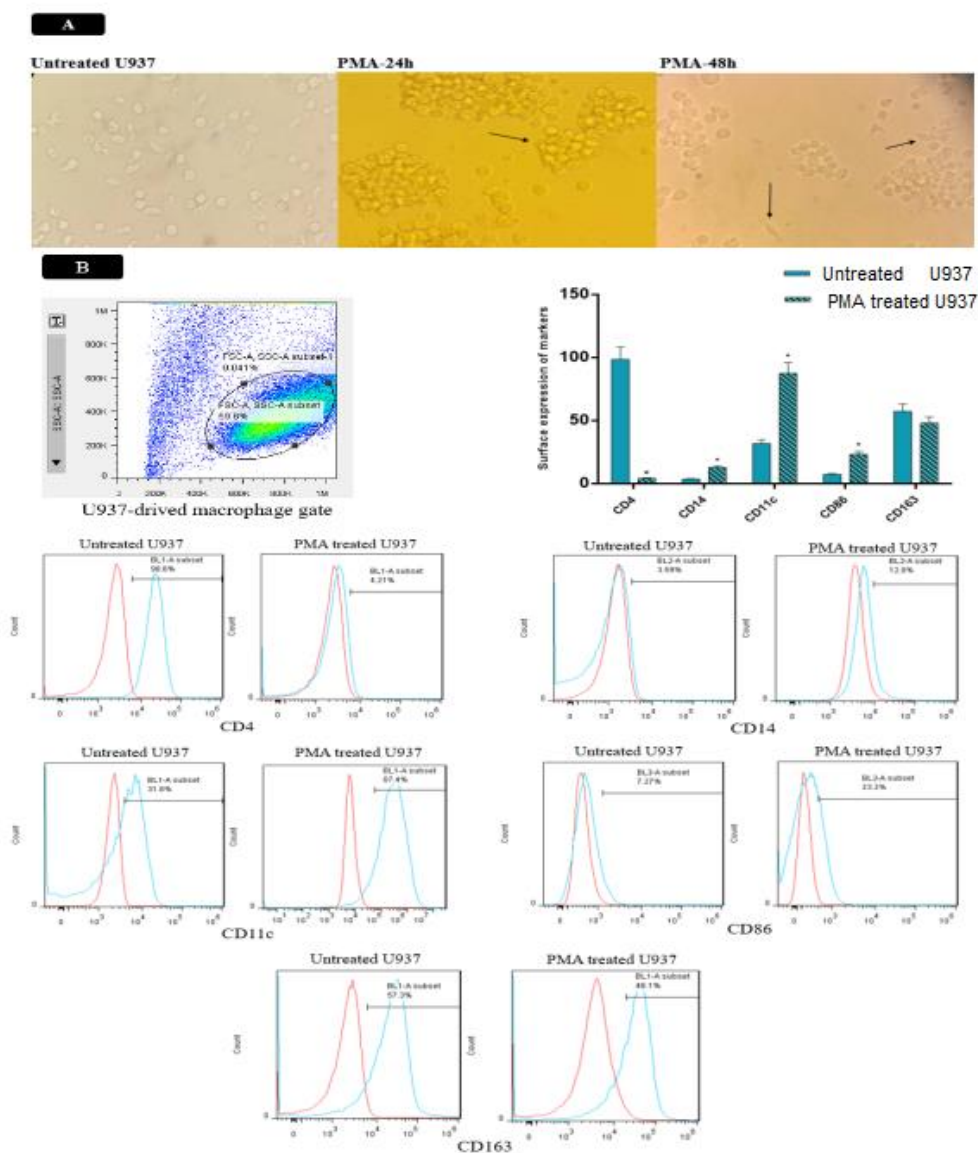
ORO staining indicated that foam cells were more detectable in the ox-LDL group compared to the control. Also, cells co-treated with ox-LDL and platelets were more susceptible to foam cell formation compared to ox-LDL and control groups (Figure 2).

#### *Alteration of surface markers induced by ox-LDL and platelet treatment*

Our findings revealed that the expression of CD86 was downregulated in cells treated with ox-LDL (19.3%) and co-treated with ox-LDL and platelets (13.8%) compared to the control (23.2%), which was significant regarding the presence of platelets compared to other groups ( $P \leq 0.05$ ). Also, the expression of CD163 was

**Table 1.** Primer sequences used for RT-PCR

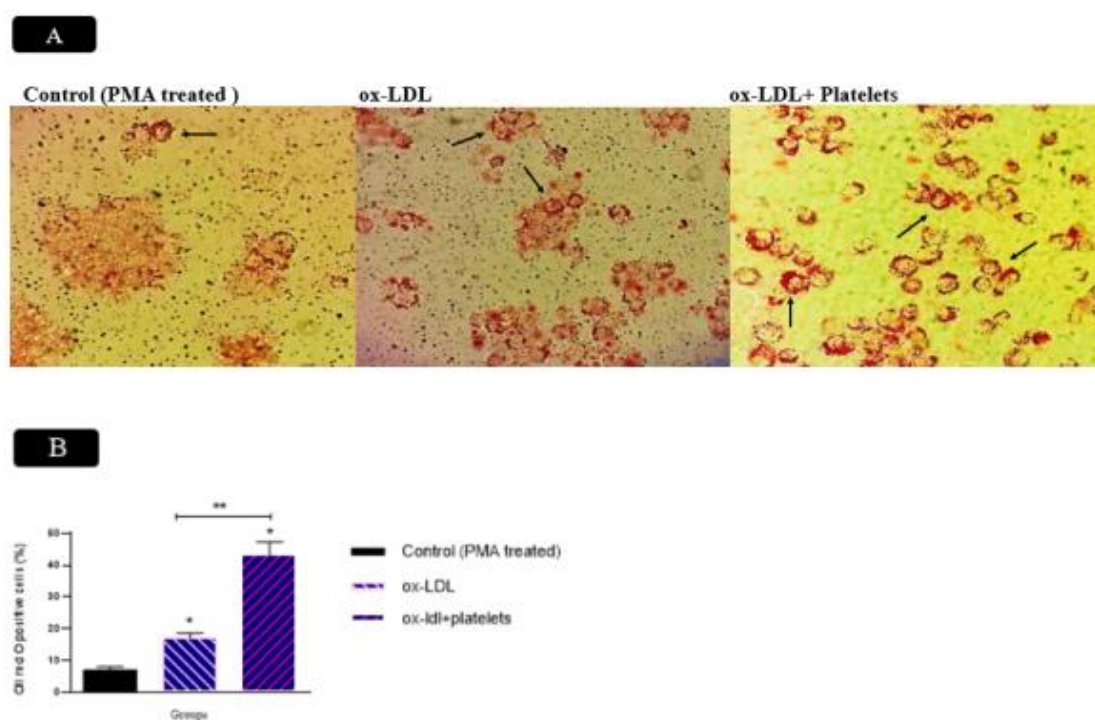
| GENE         | Forward Primer (5'-3')   | Reverse Primer (5'-3')  | Size (bp) |
|--------------|--------------------------|-------------------------|-----------|
| ABCA1        | GGCAATCATCAGGGTGCTGACG   | CCGCAGAAAGATGTCATCAACG  | 97        |
| CD36         | GCAGCAACATTCAAGTTAAGC    | AGCCTCTGTTCCAAGTATAG    | 158       |
| SR-B1        | TTGCCAACGGTCCATCTAC      | CAGTTTGTCCAATGCCTGCG    | 288       |
| ACAT1        | TCTACTCCATGTACCACCATAAAC | CATAAGCGTCCTGTTCAATTCG  | 298       |
| LXR $\alpha$ | ACAACCTGGGAGTGAGAGT      | AACATCAGTCGGTCATGGGG    | 295       |
| ABL          | CTCTTGGTGCCTGAGAGTGAG    | GACGTAGAGCTTGCCATCAGAAG | 115       |



**Figure 1.** Morphological and surface marker assessment after PMA-treatment: Induction of adhesive feature and spindled form of macrophage (1-A). Reduction expression level of CD4 and increased level of CD14, CD11c and CD86 in U937 cells through 48 hrs after PMA treatment ( $P \leq 0.05$ ) and the descriptive augmentation of CD163 expression was observed. \*, represent significant changes from the control group (1-B). (Red histogram represent isotype control; Blue histogram represent Target CD markers)

**Table 2.** Absorbance and concentration of ox-LDL and LDL

| Name of material   | Absorbance (OD) | Concentration (mg/mL) |
|--------------------|-----------------|-----------------------|
| Preparation Ox-LDL | 0.53            | 1.49                  |
| Native LDL         | 0.32            | 1.00                  |
| Standard ox-LDL    | 0.14            | 2.50                  |



**Figure 2.** Evaluation of ox-LDL and platelet effect on foam cell formation. (A) Oil red O staining was performed 4 days after incubation of cells with desired agents. Ox-LDL and platelets had the inducer effect in the increased number of lipid laden cells (cells with red particles inside) and this was more seen in ox-LDL+ platelets group Vs. ox-LDL and control groups ( $P \leq 0.05$ ). Magnification: 400 $\times$ . (B) Bar diagram displaying a comparison of ORO positive cells between different groups, images were analyzed by Image J software. Each experiment was done in triplicate. \*, \*\* represent significant changes from the control and ox-LDL group respectively

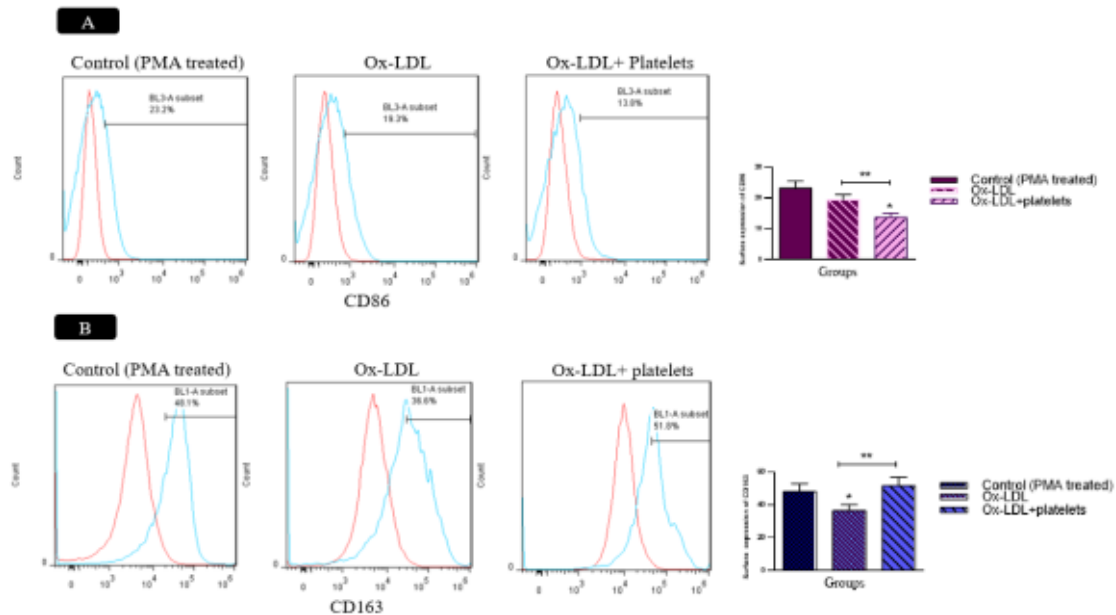
downregulated in the ox-LDL group (36.6%) compared to the control (48.1%) ( $P < 0.05$ ), while its expression was upregulated (51.8%) in the co-treated group compared to the ox-LDL group ( $P \leq 0.05$ ) (Figure 3).

#### *The expression of genes involved in foam cell formation*

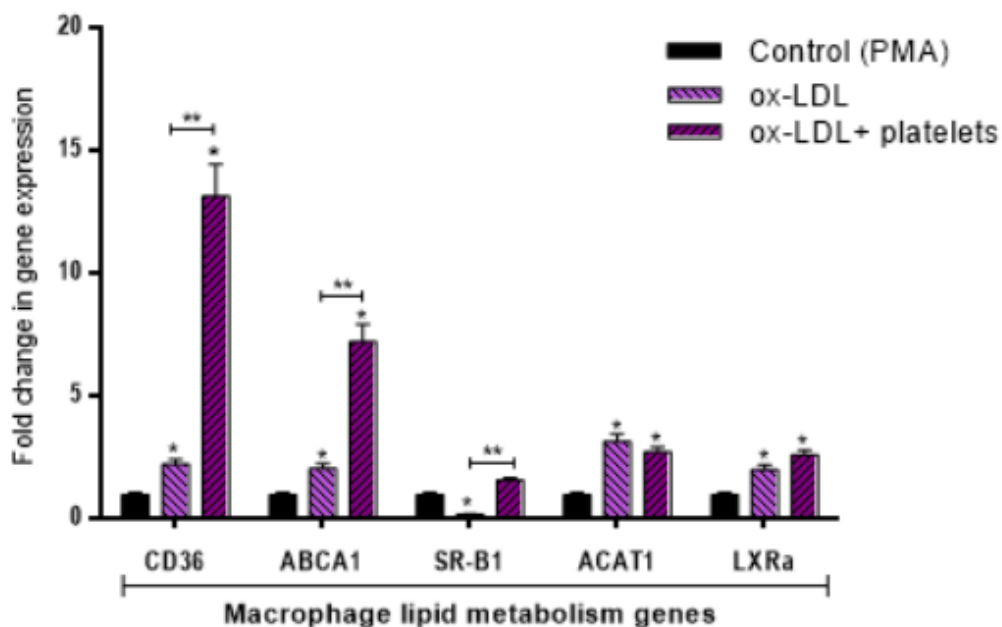
Our data revealed that the expression of *CD36*, *ABCA1*, *ACAT1*, and *LXR $\alpha$*  genes was upregulated in ox-LDL-treated and co-treated groups compared to the control ( $P \leq$

0.05). Besides, the expression of *CD36* and *ABCA1* genes was significantly upregulated in the presence of platelets compared to the ox-LDL group ( $P \leq 0.05$ ).

Furthermore, the expression of *SR-B1* genes was significantly downregulated in the ox-LDL group compared to the control, while its expression was significantly upregulated in the co-treated group compared to the ox-LDL group ( $P \leq 0.05$ ) (Figure 4).



**Figure 3.** The fluctuation of CD86 and CD163 expression during foam cell formation. (A) The reduction of CD86 in ox-LDL and ox-LDL+ platelets ( $P \leq 0.05$ ) samples in comparison with the control group. (B) CD163 revealed the decrease in the ox-LDL group ( $P \leq 0.05$ ) and the significant increase in the presence of platelets compared with the ox-LDL group ( $P \leq 0.05$ ). The results are given from three independent experiments. \*, \*\* represent significant changes from the control and ox-LDL group respectively. (Red histogram represent isotype control; Blue histogram represent Target CD markers)



**Figure 4.** mRNA expression level of genes related to lipid loading in macrophages. Relative expression of desired genes was analyzed Vs. their housekeeping ABL by measuring the cycle thresholds (Ct). Each test was done in triplicate and values are given as mean  $\pm$  SD of three independent experiments. \*, \*\* represent significant changes from the control and ox-LDL group respectively

## Discussion

Multiple factors are implicated in atherosclerosis development, thus, making it a complex pathological process and a multifactorial disorder<sup>3, 18</sup>. The ox-LDL constitutes a major risk factor in the development of atherosclerosis by mediating foam cell formation from macrophages<sup>19</sup>. Platelets are yet another potential risk factor for atherosclerosis, which promote the recruitment of immune cells to atherosclerotic lesions<sup>20</sup>. Given the inflammatory state of atherosclerosis, platelet-monocyte interaction is a key event in the progression of atherosclerosis<sup>11</sup>. Nonetheless, altered gene expression and modified surface markers of macrophages during the development of atherosclerosis remain largely elusive. Therefore, the current study aimed to decipher the role of ox-LDL and platelets in the conversion of U937-derived macrophages into foam cells, as well as explore the expression of macrophage's surface markers. Our findings suggest that ox-LDL and platelets promoted the formation of foam cells in cultured U937-derived macrophages by modulating cell-surface phenotype and gene expression of macrophages. However, the effect of platelets on the formation of foam cells was more accentuated than ox-LDL. In this regard, Badrnya and coworkers revealed that platelets promote ox-LDL uptake by macrophages, leading to increased foam cell formation<sup>15</sup>.

In the current study, CD163 overexpression, which is a dominant marker of M2 (anti-inflammatory) macrophages, and the formation of foam cells in the presence of platelets were shown (Figure 3). Yet, the key question is whether platelets could lead to atherosclerosis progression by enhancing foam cell formation or prevent atherosclerosis by polarizing M2 macrophages.

Thus far, it was believed that M2 macrophages appear in atherosclerotic plaques, thereby, contributing to foam cell formation<sup>21, 22</sup>. Also, Van Tits and associates reported that CD163+ cells were more susceptible to the build-up of ox-LDL and conversion into foam cells<sup>23</sup>. Also, Chatterjee et al.

showed that co-culturing platelets with blood monocytes led to the polarization of CD163+ macrophages through the CXCL12-CXCR4/CXCR7 interaction, ultimately, inducing their differentiation into foam cells<sup>10</sup>. Hence, we hypothesize that the co-existence of CD163+ macrophages and foam cells could be stemmed from the sustained co-culture of platelets with M2 macrophages, thereby, acting as a positive effector in the conversion of M2 macrophages into foam cells.

To explain these findings, we highlighted the expression of genes involved in foam cell formation including lipid influx (*CD36*), efflux (*LXRα*, *ABCA1*, *SR-B1*), and cholesterol esterification (*ACAT1*)<sup>24-26</sup>. In this sense, the expression of *CD36* and *ACAT1*, which participates in de novo synthesis of cholesterol ester as the intracellular form of lipid droplets<sup>27</sup>, was significantly upregulated after treatment with ox-LDL and co-treatment with ox-LDL and platelets. Furthermore, Rana et al. showed that the expression of *CD36* was upregulated in macrophages treated with ox-LDL, mainly through interleukin-1 receptor-associated kinase-1 (IRAK1) and STAT1 signaling<sup>28</sup>. Also, in another independent study, Li and colleagues reported that *ACAT1* was significantly upregulated in THP-1-derived foam cells, while downregulated after treatment with ACAT 1 inhibitors<sup>29</sup>. Hence, increased upregulation of *CD36* in the presence of platelets might be linked to platelets' role in enhancing ox-LDL uptake and foam cell formation.

In terms of lipid extraction genes, significant upregulation of *ABCA1* and *LXRα* genes and downregulation of *SR-B1* gene were demonstrated after ox-LDL treatment. In line with this, previous reports revealed that *LXRα* and *ABCA1* are components of the same pathway<sup>25</sup>. Su et al. also revealed that the expression of *ABCA1* mRNA in foam cells shifted proportionately to different ox-LDL doses so that the highest and lowest expression was achieved at 50 mg/L and 125 mg/L concentrations, respectively<sup>30</sup>. Therefore, 80 µg/ml of ox-LDL, used in our study, likely indicated the average expression of the *ABCA1* gene. In contrast, He and

colleagues revealed that the expression of *ABCA1* and *LXRα* genes was downregulated in THP-1-derived foam cells through receptor-interacting protein 140 (RIP140) signaling<sup>31</sup>. Also, in concomitant with our result, Qin Ma et al. found that the expression of *SR-B1* mRNA was downregulated after ox-LDL treatment, while upregulated upon apigenin-7-O-β-D-glucuronide (AG) treatment<sup>32</sup>.

However, upregulation of these genes ensuing ox-LDL treatment was also associated with the exposure to platelets likely through platelets-induced polarization of M2 macrophage, as well as platelets' effects on cholesterol efflux.

### Conclusion

Overall, the findings of this study suggested the dual activity of platelets, which could depend on various factors, resulting in either the progression or retardation of atherosclerotic plaques. Therefore, further studies are urgent to cast more light on the role of platelets in atherosclerosis and plaque formation.

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### Authorship and Conflict-of-Interest Statement

The authors declare that they have no potential conflict of interest; XX performed the experiment, analyzed the data, and wrote the paper.

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