



Electronegative LDL Induces M1 Polarization of Human Macrophages Through a LOX-1-Dependent Pathway

Shwu-Fen Chang,¹ Po-Yuan Chang,² Yuan-Chun Chou,³ and Shao-Chun Lu^{3,4}

Abstract— In response to environmental stimuli, monocytes undergo polarization into classically activated (M1) or alternatively activated (M2) states. M1 and M2 macrophages exert opposing pro- and anti-inflammatory properties, respectively. Electronegative low-density lipoprotein (LDL) (LDL(-)) is a naturally occurring mildly oxidized LDL found in the plasma of patients with hypercholesterolemia, diabetes, and acute myocardial infarction, and has been shown to involve in the pathogenesis of atherosclerosis. In this study, we examined the effects of LDL(-) on macrophage polarization and the involvement of lectin-like oxidized LDL receptor-1 (LOX-1) in this process. THP-1 macrophages were treated with native LDL (nLDL) or LDL(-), and then the expression of M1/M2-related surface markers and cytokines were evaluated. The results show that treatment with LDL(-) resulted in profound increase in proinflammatory cytokines, IL-1 β , IL-6, and TNF- α , and M1-surface marker CD86; however, M2-related cytokines, IL-10 and TGF- β , and M2-surface marker CD206 were not changed by LDL(-). Untreated or nLDL-treated cells were used as control. LDL(-)-induced M1 polarization and secretion of proinflammatory cytokines were diminished in LOX-1 knockdown cells. Taken together, the results show that LDL(-) promotes differentiation of human monocytes to M1 macrophages through a LOX-1-dependent pathway, and explore the contribution of LDL(-) and LOX-1 to the development of chronic inflammation in atherosclerosis.

KEY WORDS: Electronegative LDL; LOX-1; Macrophage polarization.

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Abbreviations *Arg-1*, Arginase-1; *IL*, Interleukin; *KD*, Knockdown; *LDL*, Low-density lipoprotein; *LDL(-)*, Electronegative LDL; *LOX-1*, Lectin-like oxidized LDL receptor-1; *nLDL*, Native LDL; *NLRP3*, NOD-, LRR-, and pyrin domain-containing protein 3; *NF*, Nuclear factor; *oxLDL*, Oxidized low-density lipoprotein; *SR*, Scavenger receptors; *STEMI*, ST-segment elevation myocardial infarction; *TGF*, Transforming growth factor; *TNF*, tumor necrosis factor

INTRODUCTION

Macrophages play a central role in the pathogenesis of atherosclerosis. During the early stage of atherosclerosis, circulating monocytes undergo differentiation into macrophages in the subendothelial space, where macrophages take up oxidized low-density lipoprotein (oxLDL) *via* scavenger receptors, accumulating cholesterol, finally convert into foam cells, which is a hallmark of atherosclerosis [1]. In response to environmental stimuli, monocytes differentiate into classically activated (M1) or alternatively activated (M2) states [2]. M1 macrophages are oriented to produce proinflammatory cytokines such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α and function as immune surveillance, whereas M2 macrophages mostly serve to resolve inflammation and promote tissue remodeling through expression of factors like arginase-1 (Arg-1), transforming growth factor (TGF) β , and IL-10 [2, 3]. Considering the phenotypic plasticity and pleiotropic roles of macrophages, the transmigration and transformation of monocytes to form cells, the polarization status of macrophage and the signals involved to trigger this change, appear to be important to explore the pathogenesis of atherosclerosis.

The oxidation of LDL is thought to occur primarily in the intimal space, and the oxLDL displays increased electrophoretic mobility and higher atherogenic properties. However, mildly oxidized electronegative LDL (LDL(-)) can be isolated from the plasma of patients with hypercholesterolemia, type II diabetes, and ST-segment elevation myocardial infarction (STEMI) [4–7], suggesting that oxLDL may already form in plasma *in vivo*. Several studies investigated the effect of oxLDL on macrophage differentiation using the *in vitro* generated oxLDL, by exposure of LDL to Cu₂SO₄; however, reports focused on macrophage differentiation affected by the *in vivo* oxidized LDL have not been addressed so far.

Oxidative modification of LDL often associates with an increase in net negative charge. Differences in the electrical charge of LDL can be differentiated by separating the LDL(-) from native LDL (nLDL) with an anion-exchange chromatography [8, 9]. We have recently reported that LDL(-), isolated from atherogenic diet-fed hamsters, induced the production of TNF- α in Kupffer cells [10], and LDL(-), isolated from atherogenic diet-fed rabbits, activated nuclear factor (NF)- κ B and MAPKs and induced the productions of IL-1 β , IL-6, and TNF- α in macrophages [11]. In addition, LDL(-), isolated from plasma of patients with STEMI, activated NF- κ B and NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3)

inflammasome, subsequently increased IL-1 β production in human macrophages [9]. Altogether, these results strongly suggest that naturally occurring LDL(-) can be one of the determinants mediating the inflammatory processes, which then exaggerate the pathogenesis of atherosclerosis.

Lectin-like oxidized LDL receptor-1 (LOX-1) has been reported to be a major oxLDL receptor in endothelial cells. Later studies have shown that LOX-1 is also expressed in macrophages and smooth muscle cells [12]. Moreover, LDL(-)-induced proinflammatory effects were diminished upon blocking LOX-1 with a neutralizing antibody or shRNA knockdown [13]. In addition, a high cholesterol diet-induced plaque formation, atherosclerosis, proinflammatory, and pro-oxidant signals were reduced in LOX-1 knockout mice [14]. These results suggest that LDL(-) exerts its biological effects through a LOX-1-dependent pathway. Upregulation of LOX-1 in endothelial cells has been reported in many pathophysiological events, such as inflammation and atherogenesis [15]. In addition, we have demonstrated that LOX-1 is associated with inflammation in high fat-diet induced non-alcoholic steatohepatitis [10]. These results indicate that LOX-1 may be associated with different aspects of macrophage biology. Binding of LDL(-) to LOX-1 leads to intracellular signaling and activates multiple downstream events, including the feedforward elevation of LOX-1 expression [15], which would further promote the subsequent responses. Our previous report demonstrated that LDL(-) induced expressions of IL-1 β , IL-6 and TNF- α in macrophages is LOX-1-dependent [11]. In this study, we further investigated whether LDL(-) contributed to macrophage polarization, and examined the involvement of LOX-1 in this process.

MATERIALS AND METHODS

Materials

RPMI medium, FBS, and penicillin/streptomycin were obtained from Gibco BRL/Life-Technologies (Rockville, MD, USA). Dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cholesterol, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). Human IL-1 β , IL-6, and TNF- α ELISA kits were obtained from R&D systems (Minneapolis, MN, USA). Rabbit anti-human CD86 and anti-human CD206 antibodies were obtained from Abcam

(Cambridge, UK). M-MLV reverse transcriptase was obtained from Promega (Madison, WI, USA).

Cell Culture and Treatment

THP-1, a human monocytic leukemia cell line, was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and was maintained as previously described [9, 13]. Briefly, cells were maintained in the RPMI 1640 medium containing 2 mM glutamine, 100 U/ml penicillin-streptomycin, and 10% FBS. For experiments, cells were differentiated into macrophages by addition 160 nM PMA for 2 days, and then refreshed in the culture medium without PMA for another 24 h. Then, the medium was replaced with serum-free RPMI 1640, and the cells were treated with nLDL or LDL(-) for 24 h or as indicated. Control cells were treated with phosphate-buffered saline (PBS) in all experiments or as indicated. After the indicated culture period, medium was collected, and levels of cytokines in the medium were determined.

Animals and Diets

Sixteen-week-old male New Zealand white rabbits (~2 kg) were kept for an acclimation period of 2 weeks. After adaptation, rabbits were fed with an atherogenic diet (chow diet supplemented with 5% lard and 0.25% cholesterol) for 2 months as described previously [11], after that blood was collected from the marginal ear veins with tubes containing EDTA, and centrifuged at 1400×g at 4 °C for 10 min. Plasma was transferred to new tubes and subjected to LDL isolation.

LDL Preparation and LDL(-) Purification

LDL was isolated by sequential NaBr density gradient ultracentrifugation as described previously [11, 13]. Isolated LDL was then resolved into nLDL and LDL(-) by anion-exchange chromatography on a fast protein liquid chromatographic system (AKTA explorer; GE, Uppsala, Sweden) using UnoQ12 column as described previously [11, 13]. All lipoprotein isolations were carried out immediately after blood was obtained. Precautions were taken to prevent all LDL preparations from endotoxin contamination and further oxidation. Protein concentrations were estimated by the Bradford method (DC Protein Assay Reagent, Bio-Rad, Hercules, CA, USA).

LOX-1 Knockdown in THP-1 Cells

The pLKO.1-shRNA plasmids encoding short hairpin RNA (shRNA) targeting LacZ (5'-CGCGATCGTAATCA

CCCGAGT-3') and human LOX-1 (5'-GCTCGGAAGCT-GAATGAGAAA-3') were obtained from the National RNAi Core Facility at Academia Sinica, Taipei, Taiwan. The knockdown experiments were performed as described in previous studies [9, 13].

RNA Isolation and Quantification

Total RNA was isolated by TRIzol reagent according to the manufacturers' instructions. First-strand cDNA was reverse-transcribed and the levels of Arg-1, CD36, CD86, CD163, CD206, IL-1 β , IL-6, LOX-1, scavenger receptors (SR)-A, SR-B1, TGF- β , TNF- α , and GAPDH were determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Sequence of primers is listed in Table 1.

Immunofluorescence Staining

Cells were first fixed with 4% paraformaldehyde followed by blocking in citric acid buffer containing 10% FBS at room temperature for 1 h. CD86 and CD206 expressions were determined using rabbit anti-human CD86 and CD206 antibodies. Cell nucleus were revealed using DAPI. Detailed methods were described in previous study [10].

Flow Cytometry Analysis

For flow cytometry analysis, the cells were scratched with PBS containing 0.02% EDTA, and centrifuged to obtain the cell pellet. After incubation at 4 °C for 1 h with 1:20 dilution of Alexa Fluor® 488 anti-human CD86 antibody (IT2.2 #305413) or PE anti-human CD206 antibody (15-2 #321105), as well as the relative isotype controls (BioLegend Inst. San Diego, CA, USA), cells were washed three times with cold PBS and fixed with 4% paraformaldehyde. Afterwards, unstained samples were prepared for cell size (FSC) and granularity (SSC) assessments, and data of total 10⁴ cells were collected and analyzed with FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA).

Quantification of Cytokines in the Medium

Culture medium was collected and analyzed for specific cytokines using Quantikine human IL-1 β , IL-6, TNF- α , IL-10, and TGF- β ELISA kits (R&D Systems) according to the manufacturers' protocol.

Table 1. Sequences of the Primers Used in This Study

Arg-1	Forward	5'-TCC AAG GTC TGT GGG AAA AG-3'
	Reverse	5'-ATT GCC AAA CTG TGG TCT CC-3'
CD36	Forward	5'-GAG AAC TGT TAT GGG GCT AT-3'
	Reverse	5'-TTC AAC TGG AGA GGC AAA GG-3'
CD86	Forward	5'-GAA CCT AAG AAG ATG AGT-3'
	Reverse	5'-TCC AGA ATA CAG AAG ATG-3'
CD163	Forward	5'-AAG CAA CTG GGA TGT CCA AC-3'
	Reverse	5'-ATG TCA CGC CAG CAT CTT C-3'
CD206	Forward	5'-GGC GGT GAC CTC ACA AGT AT-3'
	Reverse	5'-ACG AAG CCA TTT GGT AAA CG-3'
GAPDH	Forward	5'-CAT GAG AGT ATG ACA ACA GCC T-3'
	Reverse	5'-AGT CCT TCC ACG ATA CCA AAG T-3'
IL-1 β	Forward	5'-GCT GAG GAA GAT GCT GGT TC-3'
	Reverse	5'-CGT GCA CAT AAG CCT CGT TA-3'
IL-6	Forward	5'-TGT GAA AGC AGC AAA GAG GCA CTG-3'
	Reverse	5'-ACA GCT CTG GCT TGT TCC TCA CTA-3'
LOX-1	Forward	5'-GTG CTT GTC TTT GGA TGC CA-3'
	Reverse	5'-AGT GGG GCA TCA AAG GAG AA-3'
SR-A	Forward	5'-CCA GGG ACA TGG AAT GCA A-3'
	Reverse	5'-CCA GTG GGA CCT CGA TCT CC-3'
SR-BI	Forward	5'-ACT TCT GGC ATT CCG ATC AGT-3'
	Reverse	5'-ACG AAG CGA TAG GTG GGG AT-3'
TGF- β	Forward	5'-CAA GGA TCT GGG CTG GAA GTG GA-3
	Reverse	5'-CCA GGA CCT TGC TGT ACT GCG TGT-3'

Statistical Analyses

The data was shown as mean \pm standard deviation. Differences between groups were evaluated by ANOVA and were considered significant at $p < 0.05$.

RESULTS

LDL(-) Induces the Expression of M1 Marker CD86 But Not M2 Marker CD206 in THP-1 Cells

Surface and mRNA expressions of the prototypical M1 marker, CD86, and the M2 marker, CD206, were analyzed using immunofluorescence staining and real-time RT-PCR to reveal the LDL(-) effect on THP-1 polarization. The results showed that the control cells expressed low levels of both CD86 and CD206 at protein and mRNA levels (Fig. 1). Treatment with 20 $\mu\text{g/ml}$ of LDL(-) for 24 h significantly increased the CD86 immunoreactive signal (Fig. 1a) but not the CD206 signal (Fig. 1b) on cell surface, while nLDL had no discernible effect on both CD86 and CD206 surface markers. Flow cytometry analysis also showed that LDL(-) increased CD86 surface

marker (Fig. 1c) but not CD206 surface marker (Fig. 1d), and nLDL had no effect on both CD86 and CD206 surface markers (Fig. 1c, d). Moreover, LDL(-) significantly increased the mRNA level of CD86 but not the CD206 mRNA, while nLDL significantly increased the level of CD206 mRNA, but not the CD86 mRNA (Fig. 1e, f). These results show that LDL(-) but not nLDL can increase CD86 expression at both mRNA and protein levels in THP-1. To exclude that the effects of LDL(-) were due to endotoxin contamination, LDL(-) (10 $\mu\text{g/ml}$) and LPS (100 ng/ml) were incubated with polymyxin B (PMB; 10 $\mu\text{g/ml}$) for 1 h before treated to THP-1 cells. LPS-induced IL-1 β secretion was abolished by PMB, confirming the blockade of endotoxin. However, LDL(-)-induced IL-1 β production was not reduced by PMB (Supplementary Fig. 1); the results indicate that the effects of LDL(-) were not due to contamination with endotoxin.

LDL(-) Induces the Expressions of M1-Related Proinflammatory Cytokines

The effects of nLDL and LDL(-) on the expression of M1- and M2-type cytokines, mRNA levels of IL-1 β , IL-6,

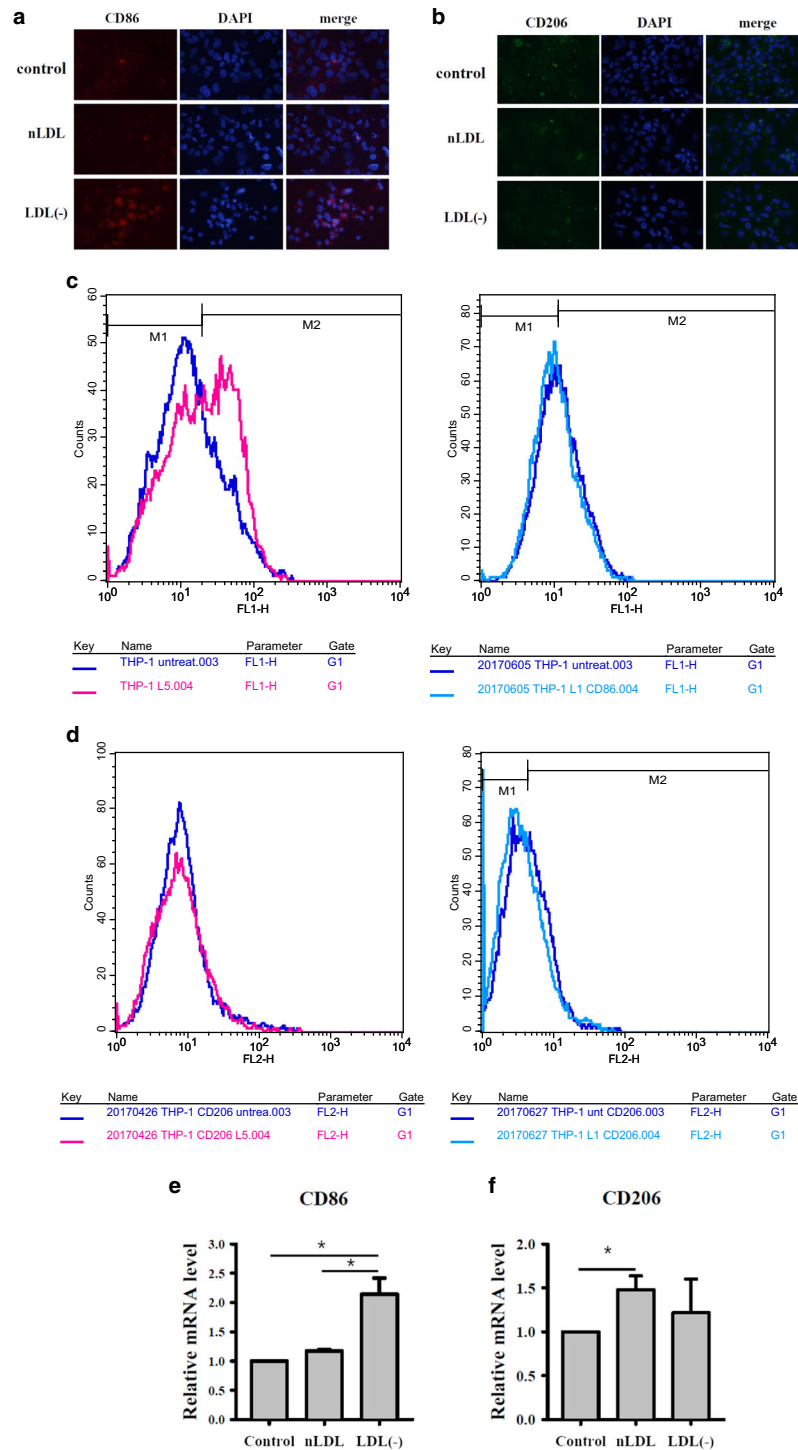


Fig. 1. Effects of nLDL and LDL(-) on the expression of CD86 and CD206 on the THP-1. THP-1 cells were incubated with PBS (control), 20 μ g/ml of nLDL or LDL(-) for 24 h. Cells were then stained with (a) CD86 (red) or (b) CD206 (green) antibodies. DAPI was counterstained to reveal cell nuclei. Photos are at original magnification $\times 200$. Flow cytometry analysis was performed to determine the effects of LDL(-) (left panel, red) and nLDL (right panel, light blue) on surface expression of CD86 (c) and CD206 (d). The mRNA levels of (e) CD86 and (f) CD206 were determined by RT-qPCR, normalized to the levels of GAPDH mRNA, and expressed relative to levels in the control cells (relative value = 1). Values are the means \pm SD for at least 3 independent experiments. $*p < 0.05$.

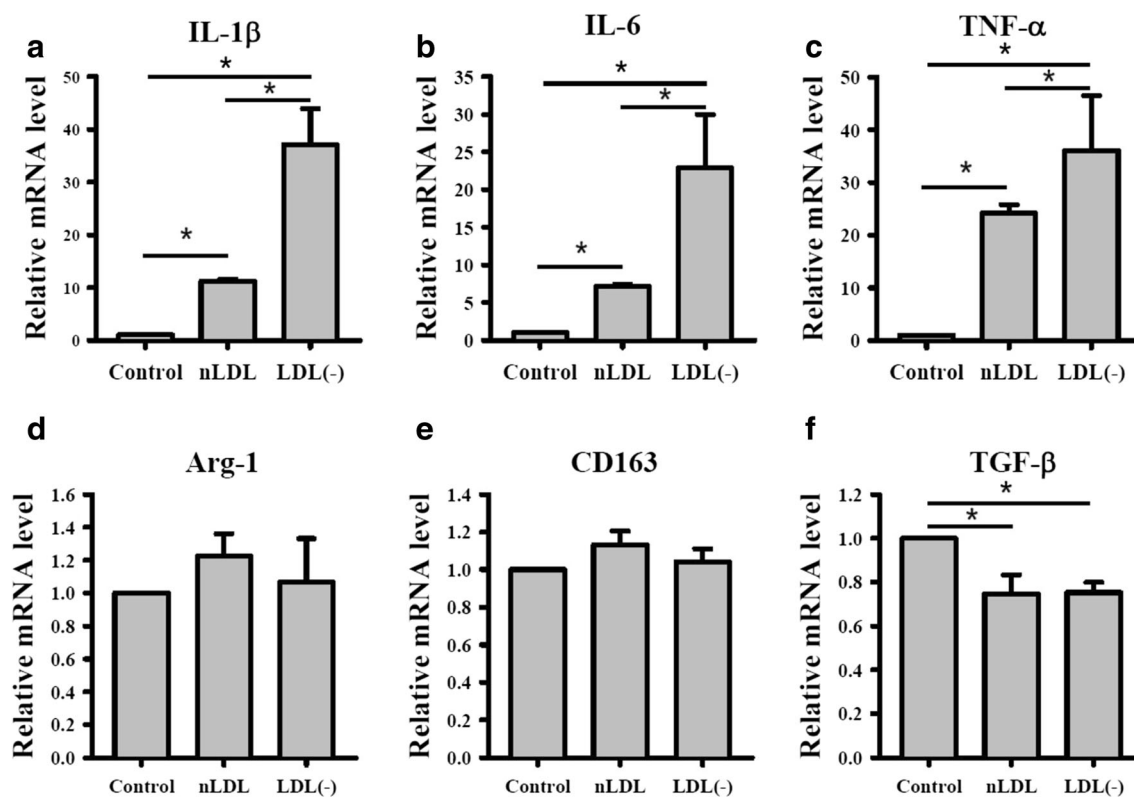


Fig. 2. Effects of nLDL and LDL(-) on mRNA expressions of M1 and M2 cytokines in THP-1 macrophages. THP-1 cells were treated with PBS (control), 20 μ g/ml of nLDL or LDL(-) for 6 h. The level of M1 related cytokines IL-1 β , IL-6, and TNF- α (a-c), and M2-related cytokines Arg-1, CD163, and TGF- β (d-f) mRNA expressions were determined by RT-qPCR, normalized to the levels of GAPDH mRNA, and expressed relative to the levels in the control cells (relative value = 1). Values are the means \pm SD for at least 3 independent experiments. * p < 0.05.

TNF- α , Arg-1, CD163, TGF- β , and GAPDH were determined by RT-qPCR. The results show that both nLDL and LDL(-) upregulated mRNAs of the three M1-type cytokines, IL-1 β , IL-6, and TNF- α , and LDL(-) exhibited further profound effect on these three cytokine expressions compared to nLDL (Fig. 2a-c). Moreover, there were only basal levels of Arg-1 and CD163 mRNAs detected in nLDL- or LDL(-)-treated cells (Fig. 2d, e), and TGF- β mRNA levels were reduced in either nLDL or LDL(-)-treated cells. The productions of cytokines in LDL(-)-treated THP-1 cells were then measured by ELISA using untreated cells as a control. Results showed that LDL(-) significantly increased the secreted protein levels of the M1-type cytokines, including IL-1 β , IL-6, and TNF- α (Fig. 3a-c). Although both nLDL and LDL(-) significantly enhanced IL-10 secretion, there was no obvious alteration of the secreted TGF- β found in either nLDL or LDL(-)-treated cells (Fig. 3d, e). These results indicated that LDL(-) preferentially induced classical activation of macrophages, the

M1 type, and upregulated pro-resolving cytokine IL-10, but not the M2 markers including Arg-1, CD163, and TGF- β .

The Effects of nLDL and LDL(-) on the Expression of Scavenger Receptors

SRs such as CD36, LOX-1, SR-A, and SR-B1 have been reported to mediate the uptake of oxLDL in macrophages [16]. We then examined the effects of nLDL and LDL(-) on the expression of these receptors in THP-1 macrophages. Figure 4 shows that neither nLDL nor LDL(-) affected the expressions of SR-A, SR-B1, and CD36 at the mRNA level (Fig. 4a, c, d); however, LDL(-), but not nLDL, significantly upregulated the LOX-1 mRNA (Fig. 4b). Moreover, immunofluorescence staining revealed LDL(-) treatment resulted in a marked increase of the LOX-1 immunogenic signal, while nLDL treatment had no effect on the signal of LOX-1 (Fig. 4e).

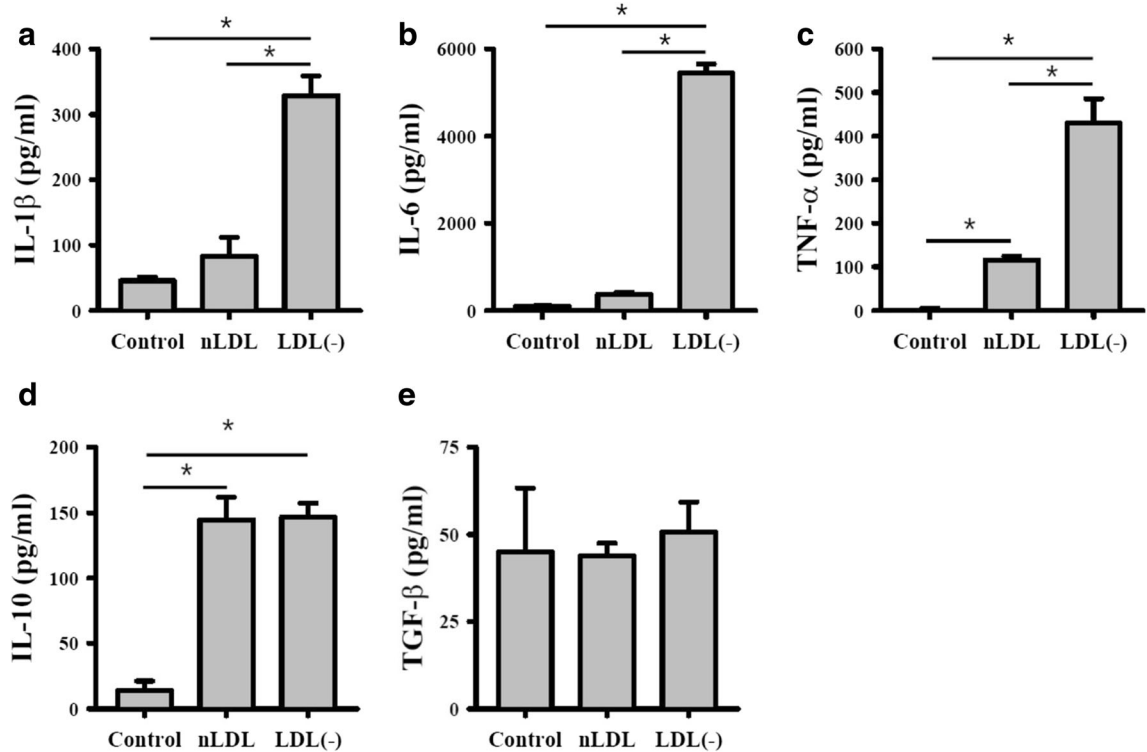


Fig. 3. Effects of nLDL and LDL(-) on the production of M1 and M2 cytokines in THP-1. THP-1 cells were treated with PBS (control), 20 μ g/ml of nLDL or LDL(-) for 24 h. Culture medium was collected and M1 cytokines (TNF- α , IL-1 β , and IL-6) (a-c) and M2 cytokines (IL-10 and TGF- β) (d-e) were determined by ELISA. Values are the means \pm SD for at least 3 independent experiments. * p < 0.05.

LOX-1 Involved in LDL(-)-Triggered M1 Polarization of THP-1 Macrophages

We and others have shown that LDL(-) induced production of proinflammatory cytokines through a LOX-1-dependent pathway in macrophages [9, 15]. We then asked whether the LOX-1 also involved in LDL(-)-triggered macrophage polarization toward the M1 type. The LOX-1 knockdown (LOX-1 KD) THP-1 was generated by RNA interference as described previously [9]. The LOX-1 mRNA was significantly reduced in the LOX-1 KD cells compared to that of the control knockdown (lacZ KD) cells (Fig. 5a). Consistent with previous findings, LDL(-) treatment increased CD86 on cell surface but did not affect CD206 compared to that in the PBS treated control of the lacZ KD cells (Fig. 5b, c). However, LOX-1 KD reduced LDL(-)-increased CD86 expression and did not affect the CD206 level (Fig. 5b, c). Flow cytometry analysis also showed that LDL(-) increased CD86 surface marker in LacZ (control) knockdown cells, but not in LOX-1 knockdown cells (Fig. 5d). Moreover, the LDL(-)-induced IL-1 β , IL-6, and TNF- α productions were significantly

reduced in the LOX-1 KD cells compared to those in the lacZ KD cells, although the reduction did not reach the basal level as detected in the control cells (Fig. 5e-g). These results suggest that LDL(-)-triggered M1 activation, switching the expressions of subtype-specific surface markers and the productions of proinflammatory cytokines, should be mostly through a LOX-1-dependent pathway.

DISCUSSION

In this study, we showed that a naturally occurring LDL(-) promoted macrophage polarization toward an M1 phenotype. LDL(-) significantly increased the generation of proinflammatory cytokines including IL-1 β , IL-6, and TNF- α and the expression of M1-specific surface marker CD86; however, it had no effect on the expression of Arg-1 and TGF- β and M2-surface markers such as CD206 and CD163. Moreover, LDL(-) elevated the expression of LOX-1 but did not alter the expression of other scavenger

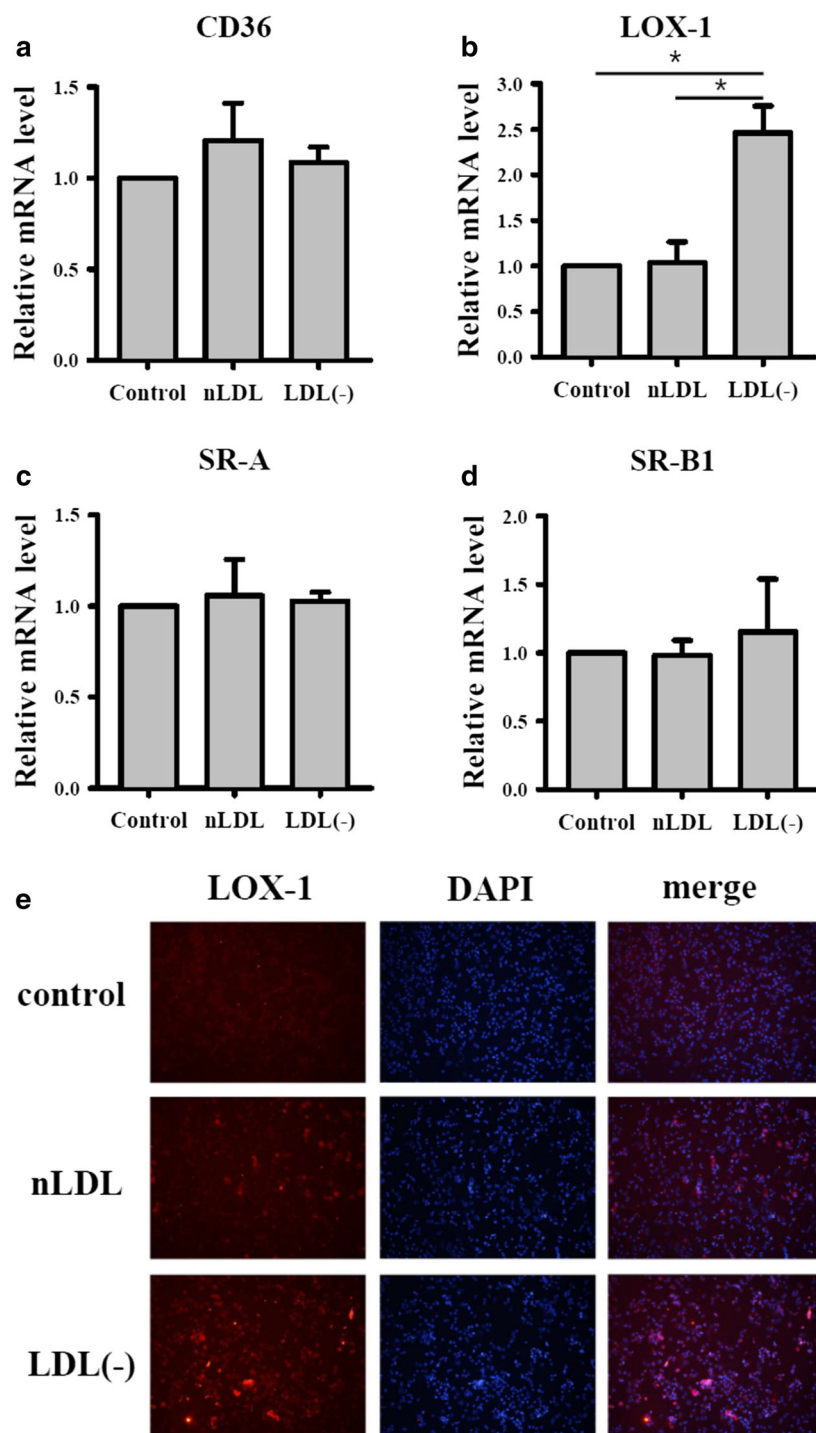


Fig. 4. Effects of LDL(-) on the expressions of various scavenger receptors. THP-1 cells were treated with PBS (control), 20 μ g/ml of nLDL or LDL(-) for 6 h. The SR-A (a), SR-B1 (b), CD36 (c), and LOX-1 (d) mRNA expressions were determined by RT-qPCR, normalized to the levels of GAPDH mRNA, and expressed relative to levels in the control cells (relative value = 1). (e) Immunofluorescence staining of LOX-1 in THP-1 macrophages treated with PBS (control), nLDL or LDL(-) for 24 h. DAPI was counterstained to reveal cell nuclei. Photos are at original magnification \times 200. Values are the means \pm SD for at least 3 independent experiments. * p < 0.05.

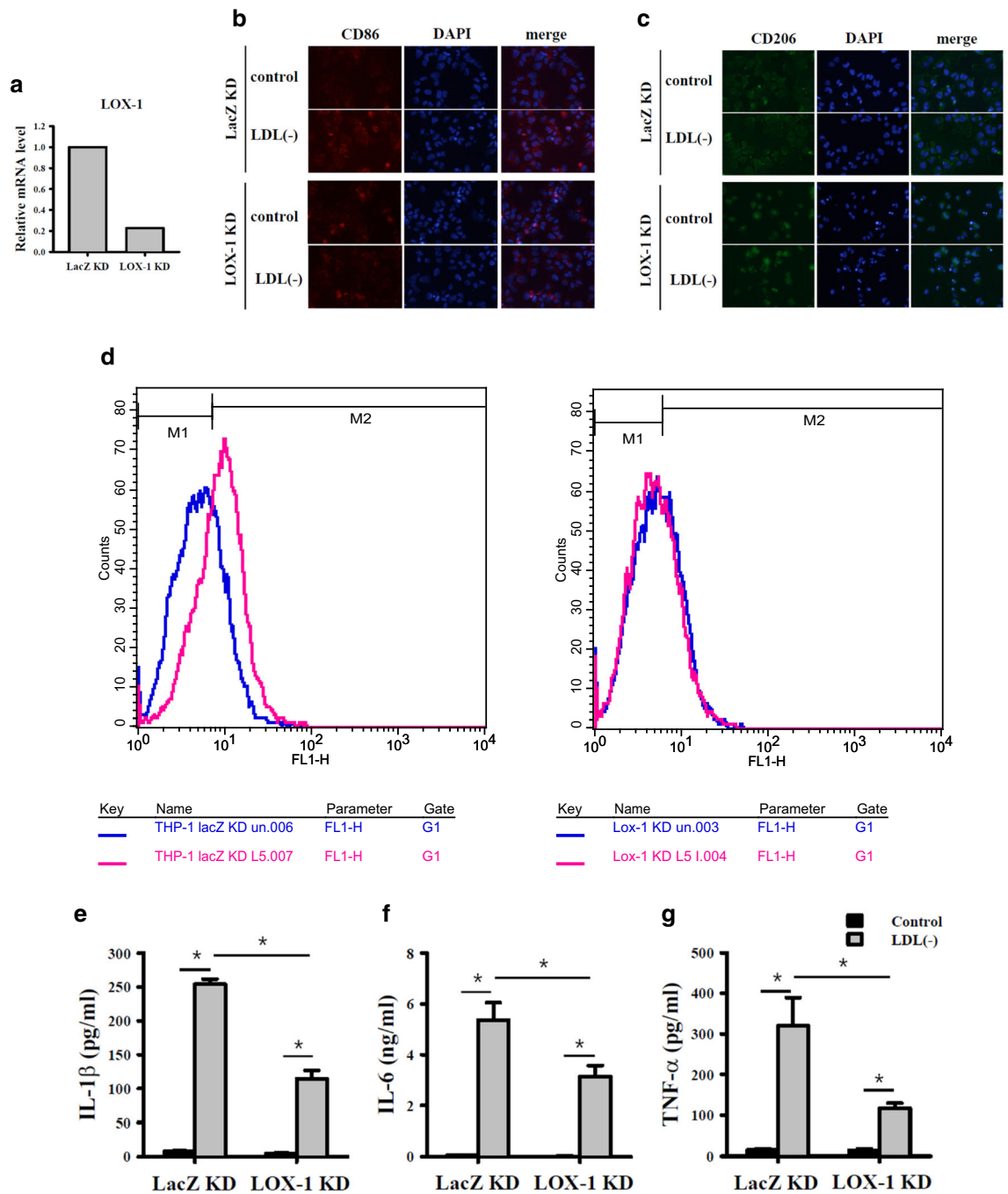


Fig. 5. Knocking down of LOX-1 diminished M1 cytokine expression and polarization in the THP-1. THP-1 cells were infected with Lentivirus carrying LacZ shRNA (shLacZ) or LOX-1 shRNA (shLOX-1) and selected with 10 μ g/ml of puromycin for 10 days. The knockdown efficiency was determined by measuring the LOX-1 mRNA level by RT-qPCR (a). Macrophages were treated with PBS (control), 20 μ g/ml of nLDL or LDL(-) for 24 h and then immunofluorescence stained with CD86 and CD206 antibodies (b, c). Photos are at original magnification \times 200. Flow cytometry analysis was performed to determine the effects of LDL(-) on surface expression of CD86 (d) in LacZ- (right panel) and LOX-1- (left panel) knockdown cells, control (blue) and LDL(-) (red). Levels of IL-1 β , IL-6, and TNF- α in the medium were determined by ELISA (e-g). Values are the means \pm SD for at least 3 independent experiments. * p < 0.05.

receptors, such as SR-A, SR-B1, and CD36. Knockdown of LOX-1 significantly decreased the LDL(-)-induced expressions of the M1-related surface marker and cytokines. Taken together, the results suggest that LOX-1 should play a critical role in the LDL(-) triggered macrophage M1 polarization. Higher plasma levels of LDL(-) were found in patients with hypercholesterolemia, type II diabetes, metabolic syndrome, and STEMI [4–7]. These patients are all in high risk of atherosclerosis and pathological conditions linked to CVDs. During the early stage of atherosclerosis, monocytes are recruited into the sub-endothelial space and differentiate into macrophages. In this study, we provide evidence showing that naturally occurring LDL(-) was able to trigger PMA-differentiated THP-1 polarization toward M1 phenotype. In addition, LDL(-) has been shown to induce the productions of G-CSF and GM-CSF [13], which are critical in mobilization of bone marrow-derived progenitor cells into the peripheral blood and increase circulating leukocytes. Therefore, plasma LDL(-) may increase the number of monocytes and further induce their differentiation toward M1 macrophages. These results strongly suggest that LDL(-) may play a pivotal role in the development of atherosclerosis.

Few studies have investigated the effects of oxLDL on macrophage polarization; however, the results are not consistent. Rios et al. showed that oxLDL, which was generated by exposure of LDL to 20 μ M CuSO₄ for 18 h at 37 °C, induced macrophage differentiation and activation toward the M2 phenotype through CD36 and platelet-activating factor receptor (PAFR)-dependent pathways [17]. Seo et al. investigated the effects of oxLDL with different degree of oxidation on macrophage differentiation [18] and found that low-oxidized LDL (low-oxLDL) promoted THP-1 differentiation to M1 type, but high-oxidized LDL (hi-oxLDL) induced M2 macrophage polarization. The low-oxLDL and hi-oxLDL were defined by TBAR value (TBARs of 8.7–11.8 and 55.7–75.6 nmol/mg protein for low oxLDL and high oxLDL, respectively). The results suggest that the oxidation degree of LDL may be critical for its effect on macrophage polarization. The TBARS of the LDL(-) used in our study was < 1 nmol/mg protein [11]. Compared to the results obtained by Seo et al. using low oxLDL, we found that naturally occurring LDL(-)-induced much higher expressions of IL-6, TNF- α , and CD86 in THP-1. Moreover, LDL(-) significantly induced the expression of LOX-1 but the low-oxLDL did not influence the expression of LOX-1, which is known to be expressed at higher levels in M1 [19]. Oh et al. reported that M2 macrophages expressed high levels of CD36 and SR-A1 [20], which were not affected by LDL(-). LDL(-)

has been shown to be unique and crucial for inflammation responses and the pathogenic development of atherogenesis [21, 22]. Our previous study demonstrated that hamster LDL(-) induced high level TNF- α production in primary Kupffer cells through LOX-1 and NF- κ B dependent pathways [10]. Furthermore, STEMI LDL(-) also enhanced IL-1 β , IL-6, and TNF- α expressions in THP-1 macrophages through a LOX-1 dependent pathway [13]. In this study, we show that rabbit LDL(-) promoted THP-1 macrophages toward the M1 phenotype by increasing the expressions of M1-surface marker CD86 and proinflammatory cytokines IL-1 β , IL-6, and TNF- α through a LOX-1 dependent pathway. LDL(-) clearly triggered activation of NF- κ B and multiple downstream events and production of proinflammatory cytokines [11, 13]. All together, these results suggest that LDL(-) isolated from STEMI patients and experimental animals may all be capable of modulating macrophages toward M1 lineage and inducing inflammatory response. In addition to the upregulation of those proinflammatory cytokines, we found that IL-10 was upregulated by both nLDL and LDL(-), and the result was consistent with other reports [16]. Though IL-10, representing an anti-inflammatory cytokine, was able to offset the LDL(-)-induced inflammatory response [23], we did not detect LDL(-) increased the expressions of the M2 markers CD206 and TGF- β (Figs. 2 and 3), implying that M1 polarization may dominantly contribute to LDL(-)-activated macrophage lineages. M2b, a subset of M2 macrophages, produces both inflammatory and anti-inflammatory cytokines including IL-10, IL-1 β , TNF- α , and CD86 [24]. In our study, we observed the elevation of those M1 and M2b shared features in LDL(-) treated cells; therefore, LDL(-) may play multiple immunoregulatory roles in macrophage polarization.

Scavenger receptors belong to a large family of membrane-bound receptors that are initially thought to bind and internalize oxLDL and other modified LDL [25]. However, current studies show that scavenger receptors also bind to a variety of ligands including endogenous proteins and pathogens [26]. Different scavenger receptors bind differently to LDL with various degree of oxidation, and among these receptors, LOX-1 and CD36 are considered as the major receptors for moderately oxidized LDL [16]. In this study, we demonstrated that LDL(-) induced the expression of LOX-1, but not other major scavenger receptors, such as CD36, SR-A, and SR-B1. All the results support that LOX-1 is a major receptor that mediate LDL(-)-induced M1 polarization. Our previous studies have shown that LOX-1 but not CD36 was responsible for the LDL(-)-induced expression of IL-1 β , G-CSF, and

GM-CSF [9, 13]. However, involvement of other scavenger receptors cannot be excluded. The C-terminal domain of LOX-1 is highly conserved in many species. Structural studies revealed that the cysteine in the C-terminal domain might provide positive charge to capture the negative charge on oxidized lipids and modified apoB100 on LDL [27, 28]. Thus, similar modification on rabbit LDL(−) is likely to be recognized by human LOX-1, subsequently triggering downstream inflammatory responses. The idea was supported by Kakutani et al. who demonstrated that mildly copper-oxidized rabbit LDL had higher interaction with LOX-1 than the higher oxidized rabbit LDL [29]. These results emphasize the importance of LOX-1 in the LDL(−)-induced responses.

CONCLUSIONS

In summary, LDL(−) is induced in hypercholesterolemic rabbits that were fed with an atherogenic diet [11]; compared to nLDL, LDL(−) is potent in triggering macrophage polarization toward M1 phenotype through a LOX-1-dependent pathway. Therefore, the proinflammatory roles of LDL(−) may further link hypercholesterolemia with atherosclerosis.

FUNDING INFORMATION

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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