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*Original Article*

# Inflammatory cytokine secretion and toll-like receptor 4 activation in retinal pigment epithelium induced by oxidized low-density lipoprotein

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**Abstract:** Native and oxidized (OX) low-density lipoprotein (LDL) may contribute to the pathogenesis of age-related macular degeneration (AMD). In this study, we investigated the effects of lipoproteins, including n-LDL and OX-LDL, on the expression of inflammation factors, interleukin-6 (IL-6) and interleukin-1β (IL-1β), in vivo and in cultured retinal pigment epithelial (RPE) cells. The potential role of Toll-like receptor 4 (TLR4) was preliminarily explored. Fifteen male Sprague-Dawley rats were randomized into three groups and injected intravenously with PBS, low n-LDL (1 mg/kg), and high n-LDL (4 mg/kg) for 14 days. Immunohistochemistry analysis of retina sections was conducted to detect IL-6 and IL-1β. ARPE-19 cells were incubated with 10-100 mg/mL n-LDL or OX-LDL for 24 h. Reverse transcription polymerase chain reaction was used to detect IL-6, IL-1β and TLR4 mRNA levels in ARPE-19. IL-6 and IL-1β protein expression was measured by enzyme-linked immune sorbent assay. Activation of TLR4 and extracellular signal-regulated kinase (ERK) protein were evaluated by western blot analysis. One-way analysis of variance was used to compare differences. As a result, circulating LDL increased both IL-6 and IL-1β expression in rat retina tissue. OX-LDL treatment increased IL-6 and IL-1β expression in ARPE-19 cells, and activated TLR4-ERK signaling pathway. In conclusion, LDL activates inflammation cytokines in rat retina. OX-LDL induces inflammation promotion and TLR4-ERK signaling pathway upregulation in cultured RPE cells.

**Keywords:** Age-related macular degeneration, oxidized low-density lipoprotein, retinal pigment epithelium, inflammation, toll-like receptor 4

## Introduction

Age-related macular degeneration is the most popular cause of irreversible blindness in people over the age of 55 in developed countries [1]. The pathological features of AMD include a relationship with age, and the presence of pigmentary disturbances, drusen, thickening of Bruch's membrane (BrM), and basal laminar deposits, followed by a secondary deterioration of the photoreceptors [2, 3]. However, the underlying etiology of AMD seems multifactorial and needs to be further elucidated [4, 5].

Oxidative stress has been associated with many aging disorders, including atherosclerosis (AS), Alzheimer's disease (AD) and AMD [6, 7]. The retina suffers from a low-grade chronic oxidative insult, which increases in level with advancing age. In the aging macular, a large amount of oxidized phospholipid (oxPL) is generated resulting from sunlight exposure and high oxygen content [8]. OxPL induces lipid accumulation in aged retinal pigment epithelial (RPE) cells and leads to membranous basal deposits onto the BrM [9]. The apolipoprotein B-containing particles pool in the BrM, forming esterified and unesterified cholesterol-enriched lipid deposits named drusen [10]. A variety of oxidation-modified proteins and lipids have been detected in drusen and BrM [11, 12]. By binding to RPE and macrophages, oxPL initiates inflammation and activates downstream inflammatory cascades [13, 14]. Local inflammation could promote drusenformation, RPE degeneration and BrM destruction, boosting multiple pathophysiological events of AMD [15].

In our previous study, continuous intravenous injection of low-density lipoprotein (LDL) induced AMD-like alternations in rat retina, such as RPE changes, thickening of the BrM, retinal

TUNEL-positive cells, inflammatory cell infiltration and decreased retinal sensitivity [16]. In vitro studies showed that oxidized-LDL (OX-LDL) treatment induced ARPE-19 cell apoptosis, vascular endothelial growth factor (VEGF)/pigment epithelium derived factor (PEDF) ratio and matrix metalloproteinase-2 (MMP-2)/tissue inhibitors of metalloproteinase-3 (TIMP-3) ratio dysregulation [17, 18]. LDL-induced inflammatory response in the retina is not analyzed yet. A pathogen-associated molecular pattern receptor, TLR4, has been proved to initiate chronic OX-LDL induced inflammatory response in AS and AD [19-21]. TLR4 and its coreceptor CD14 have been found to be expressed by a variety of ocular tissues and cells including RPE cells [22]. In this study, we tested the hypothesis that in ARPE-19, TLR4 was activated by OX-LDL and mediated inflammation cytokines. In order to further evaluate the inflammatory impact of OX-LDL in the retina and RPE cells, we investigated important inflammation cytokine expression as well as TLR4 activation in the RPE and the underlying pathways.

## Materials and methods

This study was approved by the Ethical Committee of the School of Medicine, Shanghai Jiaotong University, shanghai, China. It was conducted according to the Declaration of Helsinki principles, with written informed consent obtained from all study participants.

### *Animals*

All experimental and raising procedures were conducted as previously described [16]. Fifteen healthy male Sprague-Dawley rats weighing 350 to 380 g were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. Human LDLs were purchased from AppliChem (Darmstadt, Germany). Rats were randomized into PBS, low native LDL (n-LDL; 1 mg/kg body weight), and high n-LDL (4 mg/kg body weight) groups containing 5 rats each. The rats were injected intravenously with PBS, low n-LDL, and high n-LDL in the tail vein for 14 days under aseptic conditions.

### *Immunohistochemistry*

To prepare tissue samples, rats were sacrificed by an overdose of anesthesia, and their eyeballs were enucleated and fixed in 4% paraformaldehyde for 24 hours at 4°C. Fixed retinal tissues were embedded in paraffin, and 5-μm sections were cut through the optic disk. Sections were used for immunohistochemistry (IHC) analysis with the following antibodies: rabbit anti-IL-6 (1:400, Novus Biologicals, Littleton, Colorado, USA) and anti-IL-1β (1:100, Novus Biologicals). After incubation with goat anti-rabbit IgG secondary antibodies (1:500, Novus Biologicals), sections were incubated with 3,39-Diaminobenzidine (DAB).

### *ARPE-19 cell culture*

ARPE-19 cells were seeded at $1\times10^4$ cells/100 μL/well and cultured in Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were grown to 70-80% confluence and placed in serum-free medium (SFM) for 24 h. The cells were then treated with SFM, native LDL or OX-LDL (0-100 mg/L) for 24 h.

### *Lipoprotein preparation and oxidation*

Lipoproteins were isolated from healthy donors by gradient ultracentrifugation (1.019<d< 1.063) [23]. Briefly, blood for lipoprotein isolation was collected in EDTA (1 mg/mL) after 12 h of fasting. After density adjustment with KBr, LDL was isolated from plasma via preparative ultracentrifugation (50,000 rpm, 22 hr, Beckman ultracentrifuge, type 50 rotor). LDL preparations were washed by ultracentrifugation, dialyzed against phosphate-buffered saline (PBS) solution containing 1 mmol/L EDTA, passed through an Acrodisc filter (0.22-μm pore size, PALL, New York, USA) to remove aggregates, and then stored under nitrogen in the dark. LDL oxidation was achieved by incubating freshly isolated LDL (diluted in PBS, pH 7.4, to a final concentration of 300 μg/mL in the presence of 10 μmol/L $Cu^{2+}$) at 37°C for 18 hr. The reaction was quenched by the addition of 200 μmol/L EDTA and 40 μmol/L butylhydroxytoluene (BHT). OX-LDL was dialyzed against PBS containing 200 μmol/L EDTA and 40 μmol/L BHT.

### *Quantitative polymerase chain reaction (qPCR)*

Total RNA was extracted from tissue in 3.5-cm Petri dishes using RNeasy Mini Kit (Qiagen).

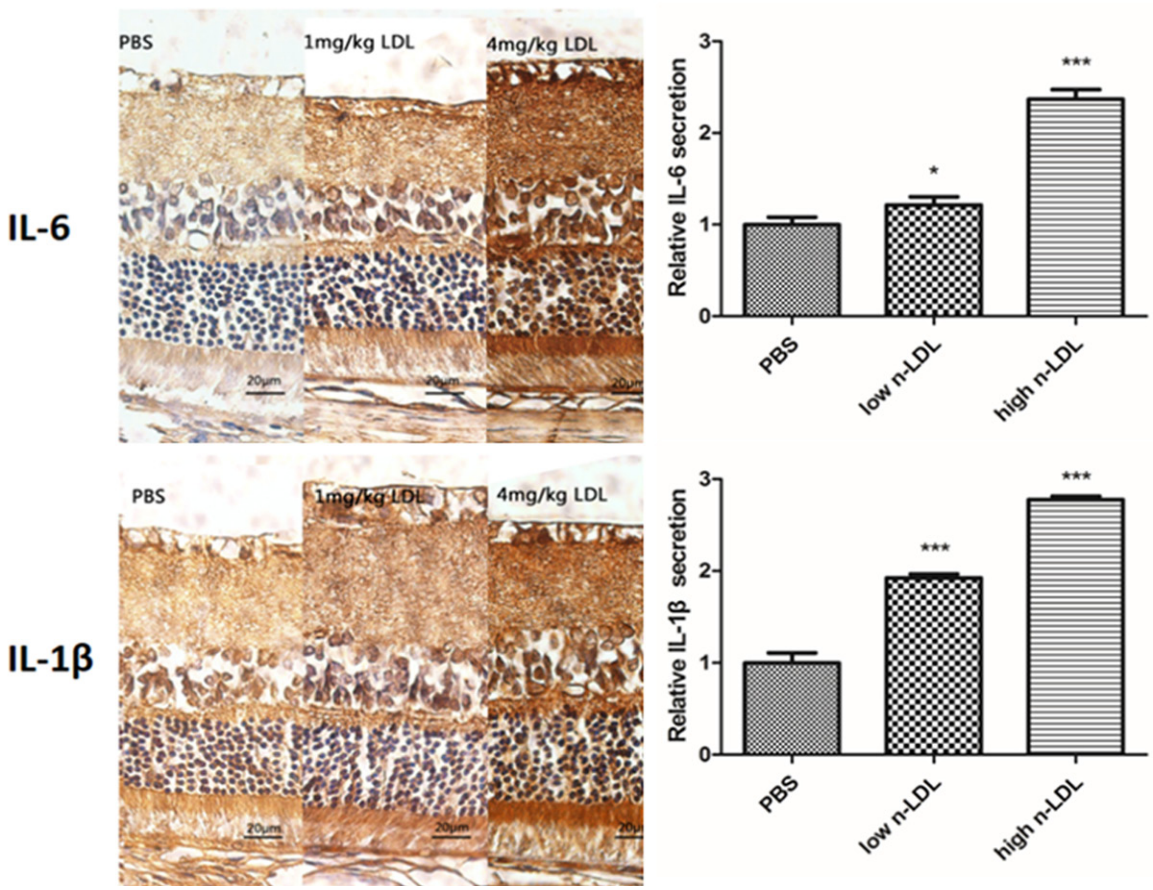


**Figure 1.** IL-6 and IL-1β IHC analysis of rat retina sections after the 14-day intravenous LDL treatment. The expression levels of IL-6 and IL-1β detected by IHC were determined by assessing its staining using software image pro-plus 6.0. The results were showed as integrated optical density (IOD)/area. Three different sections and five different fields in each section have been detected. (n = 3, Mean ± SD, asterisk* indicates P<0.05 versus PBS, ***P<0.01).

Structural integrity of the RNA samples was confirmed by electrophoresis on 1% Tris-acetate-EDTA agarose gels. Yield and purity were determined photometrically. After RNA isolation, mRNA was transcribed into cDNA using the PrimeScript RT Master Mix (Takara-Bio, Kusatsu, Shiga, Japan). This cDNA was subjected to qPCR. Quantification of human TLR4, IL-6 and IL-1β mRNA was performed using specific sense/antisense primers as follows: TLR4, 5'-CCT GTG CAA TTT GAC CAT TG-3'/5'-AAG CAT TCC CAC CTT TGT TG-3'; IL-6, 5'-CAA TGA GGA GAC TTG CCT GG-3'/5'-GGC ATT TGT GGT TGG GTC AG-3'; IL-1β, 5'-TGG CAA TGA GGA TGA CTT GT-3'/5'-GTG GTG GTC GGA GAT TCG TA-3'; and β-actin, 5'-CAC GAA ACT ACC TTC AAC TCC-3'/5'-CAT ACT CCT GCT TGC TGA TC-3'.

The QuantiNova SYBR Green PCR kit (Qiagen, Hilden, Germany) and a real-time PCR cycler (ABI ViiA7 Real-Time PCR Systems, Applied Biosystems Life Technologies, Foster City, CA, USA) were used according to the manufacturer's instructions. The mean fold-change in TLR4, IL-6 or IL-1β mRNA expression relative to that of β-actin expression was determined using the $2^{-\Delta\Delta CT}$ method.

*Elisa*

The IL-6 concentrations in cell media were measured with a Valukine IL-6 enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, Minneapolis, MN, USA). A Valukine IL-1β ELISA Kit (R&D Systems) was purchased to quantify the levels of IL-1β in cell media. Serial dilutions of recombinant human IL-6 and IL-1β were included in all assays to serve as standards.

*Western blotting*

ARPE-19 cells grown in 10-cm dishes were homogenized in lysis buffer [50 mmol/L Tris-

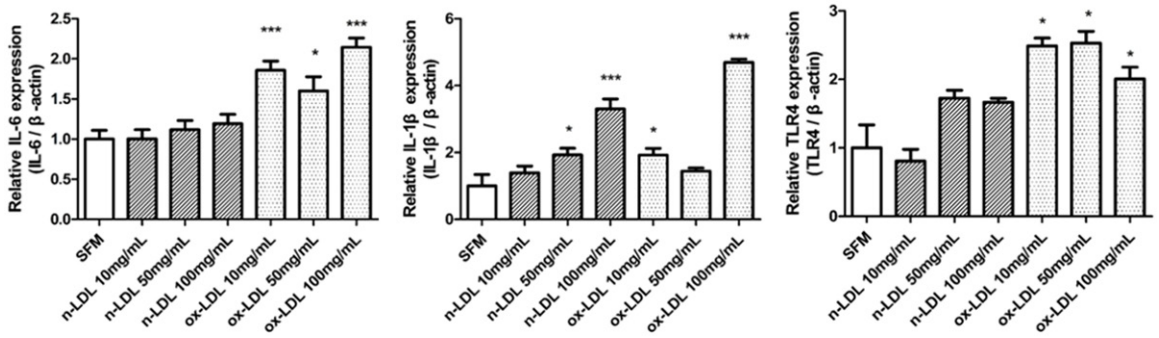


**Figure 2.** Analysis of effects of lipoprotein on IL-6, IL-1β and TLR4 mRNA expression in cultured human ARPE-19 cells. Cells were treated with variant concentrations of LDL or OX-LDL for 24 h under serum-free conditions. The mean fold change in IL-6, IL-1β and TLR4 mRNA expression relative to that of β-actin expression was determined using the method. Data represent the mean ± SD of three independent experiments (Asterisk* indicates P<0.05 versus SFM, ***P<0.01).

HCl, pH7.4, 10% glycerol, 2 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L $MgCL_2$, 50 mmol/L glycerophosphate, 2 mmol/L $Na_3VO_4$ 20 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 1% nonidet P-40] and centrifuged (12,000 rpm, 15 min, 4°C). The protein concentrations in the supernatants were measured using the Bio-Rad DC protein assay. Protein from each sample (50 μg) was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a polyvinylidine difluoride filter membranes (EMD Millipore, Bedford, MA, USA).The sheet was blocked in 5% nonfat dried milk solution and incubated overnight with a partially purified rabbit anti-TLR4 monoclonal antibody (R&D Systems). Polyclonal antibodies directed against phospho-ERK1/2, phospho-JNK1/3, phospho-p38 MAPKs, phospho-NF-κB (1:1000; Cell Signaling Technology, Danvers, MA, USA) were used to analyze the activation of intracellular signaling during OX-LDL-induced RPE cell death. β-actin expression was examined using a monoclonal anti-β-actin antibody (Cell Signaling Technology) as an internal loading control.

*Statistical analysis*

All experiments were performed at least three times. For each experiment, the mean and standard deviation were determined from three independent experiments. The SPSS software (ver. 13.0; Chicago, Illinois, USA) was used to analyze differences among group means via one-way analysis of variance (ANOVA). *P*-value less than 0.05 were deemed to indicate statistical significance.

## Results

*LDL-treated rat retina present evident inflammatory cytokine secretion*

The main inflammatory cytokines participating in AMD, IL-6 and IL-1β, were generally detected in the LDL-treated rats' retina, including photoreceptor layer, and the choriocapillaris-BrM-RPE complex. Immunohistochemistry analysis of serial sections showed expressed high levels of IL-6 and IL-1β in the two LDL groups (P<0.01). High n-LDL group (4 mg/kg body weight) retina presented higher IL-6 and IL-1β than low n-LDL group (1 mg/kg body weight) (P<0.05) (**Figure 1**).

*OX-LDL promotes IL-6, IL-1β and TLR4 mRNA expression in ARPE-19*

Real-time PCR was used to quantify IL-6, IL-1β and TLR4 mRNA expression after treatment with LDL or OX-LDL at concentrations of 10, 50 or 100 mg/mL. mRNA expression in cells treated with SFM was used as the baseline (i.e., set at 1.0) (**Figure 2**). IL-6, IL-1β and TLR4 mRNA expression increased after OX-LDL treatment significantly, while not in a dose-dependent way. Whereas IL-6 and TLR4 expression increase in LDL treatment is insignificant.

OX-LDL increases IL-6, IL-1β secretion, promotes TLR4 protein expression and activates ERK phosphorylation.

To determine total IL-6, IL-1β protein levels released into the culture medium after treatment with lipoproteins, an ELISA was used. The basal level of IL-6 and IL-1β proteins in untreat-

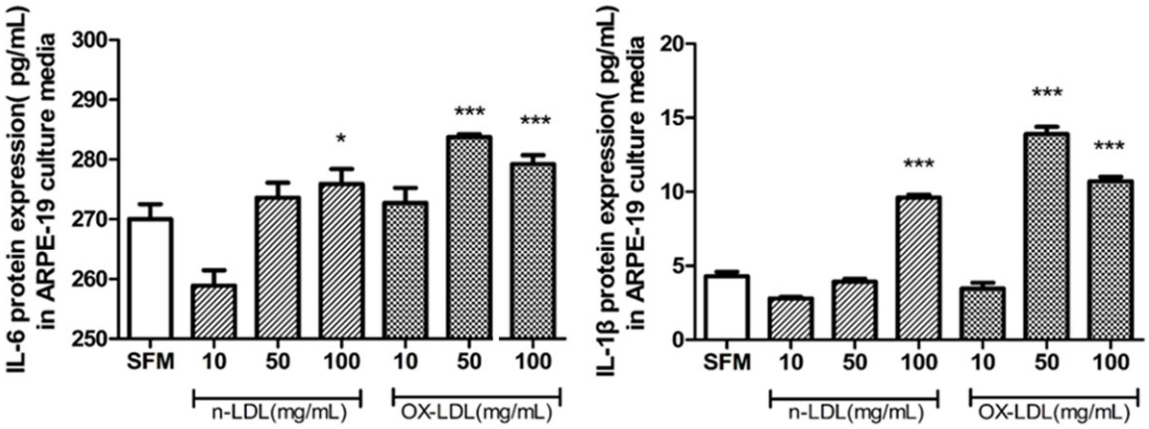


**Figure 3.** IL-6, IL-1β protein secretion by RPE cells were treated with 10, 50, and 100 mg/mL n-LDL or ox-LDL for 24 hours and analyzed by ELISA; 100 mg/mL n-LDL and 50, 100 mg/mL OX-LDL increased both IL-6 and IL-1β protein secretion. Data represent the mean ± SD of three independent experiments with similar results (Asterisk* indicates P<0.05 versus SFM, ***P<0.01).

ed control cells was 270.0 ± 2.50 pg/mL and 4.286 ± 0.301 pg/mL, respectively. Treatment of RPE cells with 100 mg/mL n-LDL and 50, 100 mg/mL OX-LDL increased both IL-6 and IL-1β protein secretion (**Figure 3**). Western blot analysis shows that TLR4 protein expression in ARPE-19 cells increased after treatment with 50 or 100 mg/mL LDL and OX-LDL (**Figure 4**). To examine the role of MAPK signal transduction in LDL- and OX-LDL-induced TLR4 expression, the effects of LDL and OX-LDL on MAPKs were analyzed in ARPE-19 cells. ERK 1/2 was phosphorylated by OX-LDL with the significant increase stimulated by 50 and 100 mg/mL OX-LDL (**Figure 4**), whereas the levels of phosphorylated p38 and JNK didn't change (not shown in this article).

## Discussion

In this study, the inflammation response after LDL treatment of rat retina is observed, and the potential relation between LDL-induced inflammation upregulation of RPE cells and TLR4 signaling pathways is preliminarily investigated.

The exact etiology of AMD is still unclear. It has been suggested that the hallmark lesions of AMD, drusen and basal linear deposits, evolve from the lipid wall [24]. The lipid wall results from a continuous accumulation of neutral lipids in BrM through adulthood [25]. Circulating human plasma LDL could go through fenestrated junctions in the choriocapillaris endothelium, either staying in BrM, or crossing BrM to reach RPE [26, 27]. In our previous study, many morphologic features similar to human AMD were observed in LDL-induced rat retina degeneration [16]. These features include incrassation of BrM, inflammatory infiltration, and photoreceptor apoptosis. RPE cells have been reported to express both LDL receptors and its coreceptor, CD36, and plasma LDL could be internalized by the RPE in vivo and in vitro [26, 28]. As formerly demonstrated, LDL and OX-LDL make various biological effects on RPE resembling AMD pathogenic changes in vitro, such as accelerated senescence, apoptosis, extracellular matrix accumulation, oxidative stress, VEGF upregulation, and inflammation [17, 29, 30]. OX-LDL was reported to induce multiple transcriptional alterations in genes related to inflammation, mediated by multiple signaling pathways [30, 31].

Inflammation is now thought to be a key event in human AMD. A large range of adverse conditions followed by inflammation instigators, such as infection and tissue injury, trigger the recruitment of leukocytes and plasma proteins and affect the tissue site [32]. Tissue stress or malfunction similarly induces an adaptive response, which is referred to as para-inflammation. This response is intermediate between the basal homeostatic state and an inflammatory response. Para-inflammation is probably responsible for the chronic inflammatory conditions that are associated with modern human diseases, including AMD [33]. Retina cells in

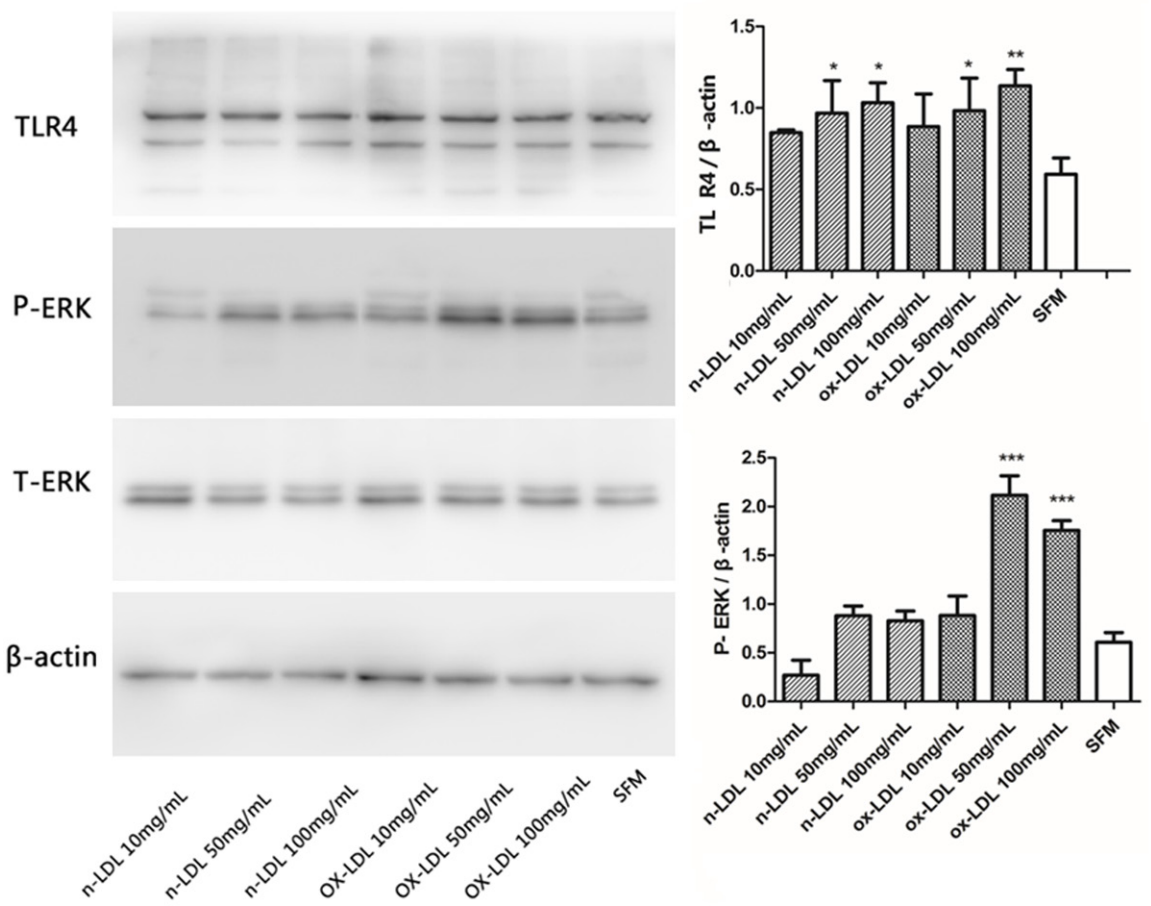


**Figure 4.** Effects of LDL and OX-LDL on protein expression levels of TLR4 and phosphorylated ERK (p-ERK). Total lysates were analyzed by western blot with specific anti-TLR4, antiphospho-ERK and anti-ERK antibodies. Equal protein loading was confirmed through the use of an internal control (β-actin antibody). After treatment with 50 mg/mL, 100 mg/mL OX-LDL, TLR4 protein and p-ERK increased. Data represent the mean ± SD of three independent experiments with similar results (Asterisk* indicates $P<0.05$ versus SFM, ***$P<0.01$).

the macular area are under strong oxidative stress with high metabolisms in the long term. Chronic inflammation infiltration result in increased complement activation in RPE and BrM as well as subretinal microglial accumulation [34].

AS and AMD share similar etiology and pathophysiologic features [35, 36]. Toll-like receptors (TLRs) are a family of surface molecules, involving innate immune responses and inflammatory responses, including AS [37]. In all members of TLRs, TLR4 is the most relevant one with AS, playing a substantial role in lipid-mediated para-inflammatory signaling in AS [38]. TLR4 is also expressed by a variety of ocular tissues and cells, including RPE cells [22].

We observed increased activation of inflammation cytokines after LDL treatment both in vivo and in vitro. Furthermore, the OX-LDL-induced upregulation of TLR4-ERK signaling pathway in ARPE-19 is proved.

The mitogen-activated protein kinases (MAPKs: p38 and c-Jun N-terminal kinase, JNK, not extracellular signal-regulated kinase, ERK) have been proved to be involved in OX-LDL upregulated VEGF expression, senescence and apoptosis in RPE [17, 39]. Inhibitors of JNK have explicitly been shown to be protective against retinal neuronal cell death [40]. If a general involvement of JNK in RPE cell death and ERK in RPE cell inflammation in AMD pathology can be verified, the inhibition of them may be an interesting new approach to prevent progression of dry AMD, as it may protect the overlying neuroretina as well. Further studies should be aimed at deciphering these particular pathways

in order to elucidate possible options of intervention.

In summary, we demonstrate in this work the inflammatory responses in circulating LDL-induced AMD-like retina in vivo. The upregulation of inflammatory cytokines in correlation with activated TLR4as well as its downstream signaling pathways in ARPE-19 cells is preliminarily confirmed. Accordingly, this study provides a stronger basis for the role of lipoproteins in the pathogenesis of AMD and implies that deactivation of TLR4 by humanized antibody may effectively attenuate the pathogenesis of AMD.

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## Disclosure of conflict of interest

None

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