Toll-like receptor 2 downregulates the cholesterol efflux by activating the nuclear factor-κB pathway in macrophages and may be a potential therapeutic target for the prevention of atherosclerosis

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Abstract. Atherosclerosis is a chronic inflammatory disease, which is triggered by lipid retention. Toll-like receptor 2 (TLR2) is a novel target for therapeutic intervention in atherosclerosis. In addition, nuclear factor-κB (NF-κB) serves important roles in stress response and inflammation. The present study investigated whether TLR2 is involved in the activation of cholesterol efflux in macrophages by regulating the NF-κB pathway. The human monocyte THP-1 cell line and murine macrophage RAW264.7 cell line were treated with 50 μg/ml oxidized low-density lipoprotein (ox-LDL) for 48 h in order to obtain macrophage foam cells. The cholesterol efflux of the cell lines under exogenous TLR2 treatment was assessed by liquid scintillation counting. Furthermore, the protein and mRNA expression levels of ATP binding cassette transporter A1 (ABCA1), ABCG1 and scavenger receptor B1 (SR-B1) were examined by western blot and quantitative polymerase chain reaction assays, respectively. To detect the effect of NF-κB on cholesterol efflux, the cells were divided into three groups, including the control, 10 ng/ml lipopolysaccharides (LPS; 24 h) and 10 ng/ml LPS + 50 μM pyrrolidinedithiocarbamate (PDTC; 24 h) groups. The results indicated that ox-LDL induced foam cell formation in the THP-1 and RAW264.7 cells, while TLR2 significantly decreased the cholesterol efflux in dose- and time-dependent manners. Accordingly, TLR2 reduced ABCA1, ABCG1 and SR-B1 expression at the transcriptional and translational levels in a dose-dependent manner. In addition, application of PDTC (an NF-κB specific inhibitor) markedly suppressed the LPS-induced downregulation of cholesterol efflux. These data revealed that TLR2 may be involved in the activation of cholesterol efflux in macrophages by regulating the NF-κB signaling pathway.

Introduction

Atherosclerosis is a chronic inflammatory disease, which is triggered by lipid retention in the arterial wall (1). It is considered a benign disease until plaque rupture occurs, leading to severe thrombus formation (2). The characteristic component of the atherosclerotic plaque is the differentiation of monocytes to macrophages that accumulate lipoprotein-derived cholesterol to form foam cells (3). The accumulation of foam cells results in atherosclerotic plaque growth and lipid storage (4). In addition, the accumulation of excess low-density lipoprotein (LDL) cholesterol, which is modified in the oxidant-rich environment, triggers atherosclerosis (5). Oxidized LDL (ox-LDL) induces the apoptosis of smooth muscle cells and macrophages, which can be effectively cleared by macrophages via efferocytosis in early plaques (6). Accompanied by the accumulation of lipids in macrophages, efferocytosis becomes defective and plaque vulnerability is promoted (7).

In order to prevent cholesterol accumulation, an efficient cholesterol efflux mechanism exists in macrophages. High-density lipoprotein (HDL) and its apolipoproteins participate in the transfer of cholesterol from the peripheral tissues and cells to the liver, through a process known as reverse cholesterol transport (RCT) (8). According to previous findings on RCT, the proteins ATP binding cassette transporter G1 (ABCG1), ATP binding cassette transporter A1 (ABCA1) and scavenger receptor B1 (SR-B1) serve key roles in suppressing cholesterol accumulation in macrophages (9).

Toll-like receptors (TLRs) are a family of type I transmembrane glycoproteins that contain extracellular domain with leucine-rich repeat motifs and a Toll/interleukin-1 receptor signaling domain (10). To date, 12 and 10 TLRs have been identified in mice and humans, respectively (11). A previous study demonstrated increased expression levels of TLR1, TLR2 and TLR4 in human atherosclerosis and inflammation with downstream signaling of inflammatory genes (12). TLR2, one of 10 human TLRs, is able to recognize the lipoproteins that are anchored to the bacterial membrane by covalent lipid chains, which are attached to conserved N-terminal...
cysteines (13). Furthermore, TLR2 is considered as a novel target for therapeutic intervention in atherosclerosis, since it mediates responses to lipoproteins derived from multiple pathogens (14).

Nuclear factor-kB (NF-κB) serves important roles in stress response and inflammation (15,16). It contains five family members in mammals, including c-Rel, RelA/p65, RelB, p52 (NF-κB2) and p50 (NF-κB1) (17). Under non-stimulated conditions, p50/p65 NF-κB is sequestered in the cytoplasm (18). However, when the cells are stimulated, it undergoes phosphorylation in the proteasome, leading to gene transcription (19).

In present study, it was hypothesized that TLR2 may be involved in the cholesterol efflux in macrophages. Therefore, the study initially examined the dose-dependent and time-dependent effect of TLR2 on cholesterol efflux in THP-1 and RAW264.7 macrophage-derived foam cells. Subsequently, the dose-dependent effect of TLR2 on the expression levels of genes linked to cholesterol efflux, including ABCA1, ABCG1 and SR-B1, was explored. Finally, the regulatory mechanisms of TLR2 on NF-κB in cholesterol efflux were investigated. The present study provided novel insights for evaluating the potential roles and mechanisms of the TLR2/NF-κB pathway in the development of atherosclerosis.

Materials and methods

Cell culture. The human monocytic THP-1 (cat no. TIB-202) and murine macrophage RAW264.7 cell lines (cat no. SC-6003) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), 0.1% bovine serum albumin (fraction V, fatty acid free; EMD Millipore, Billerica, MA, USA) and incubated in 2 ml serum-free RPMI-1640 containing 0.1% nonessential amino acids in a 5% CO2 chamber at 37°C. Next, the cells were treated with 160 nmol/l oxLDL for 48 h. Briefly, cells were seeded in 96-well plates. For knockdown of TLR2, the cells were transfected with siRNA-TRL2 (GenePharma Co., Ltd., Shanghai, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), and siRNA-scramble (GenePharma Co., Ltd.) was used as the control. After transfection for 48 h, the cells were harvested and used in further experiments.

Cellular cholesterol efflux experiments. For analysis of the cholesterol efflux, cells were initially labeled with 0.2 µCi/ml [3H] cholesterol (PerkinElmer, Inc., Waltham, MA, USA). Following cultivation for 72 h, cells were washed with PBS and incubated in RPMI-1640 medium supplemented with 0.1% (w/v) bovine serum albumin in order to allow for equilibration of the [3H] cholesterol in all the cellular pools. Equilibrated [3H] cholesterol-labeled cells were then washed with PBS and incubated in 2 ml serum-free RPMI-1640 containing 0.1% bovine serum albumin (fraction V, fatty acid free; EMD Millipore, Billerica, MA, USA). Next, 150 µl efflux medium was obtained after a 6 h incubation and passed through a 0.45-µm filter to remove any floating cells. The monolayers were subsequently washed twice in PBS, and cellular lipids were extracted with isopropanol. A liquid scintillation counting method was performed to measure the medium and cell-associated [3H] cholesterol (21). The percentage of efflux was calculated as follows: Cellular cholesterol efflux=[total media counts/(total cellular counts + total media counts)] x100% (22).

High-performance liquid chromatography (HPLC) assay. The lipid analysis was conducted by HPLC as described previously (23). Briefly, the protein concentrations in the cell solution were measured using a BCA kit (Pierce; Thermo Fisher Scientific, Inc.), and 0.1 ml cell lysate was used to measure the free cholesterol (FC) and total cholesterol (TC) levels. Next, the samples were dissolved in 100 µl isopropanol-acetonitrile (v/v, 20:80; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), followed by an ultrasound water bath for 5 min. Subsequently, the samples were used for HPLC analysis (Agilent 1100; Agilent Technologies, Inc., Santa Clara, CA, USA). The cholesterol was eluted with isopropanol-acetonitrile solution (v/v, 20:80) at a speed of 1 ml/min and then detected in terms of the absorbance at 210 nm. The levels of cholesterol ester (CE) were calculated according to the following formula: CE = TC - FC.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The quality of total RNA was assessed by spectrophotometry (A260/280 ratio: 1.8-2.0). cDNA was reverse transcribed from
100 ng RNA using a First-Strand RT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was then performed using a SYBR Green qRT-PCR kit (Thermo Fisher Scientific, Inc.) on an Applied Biosystems 7900HT Real-Time PCR system (Thermo Fisher Scientific, Inc.). The primers used for qPCR were as follows: ABCA1 forward, 5'-GATTGGCTTCCAGATGTTGAGA-3' and reverse, 5'-GTATTTTGCAAGGCTACAGTTTACGTTGACAA-3'; ABCG1 forward, 5'-CAGTGACAGCCATCCGGTCT-3' and reverse, 5'-CGATGAGTCCAGGTACGCTTGCG-3'; SR-BI forward, 5'-GCTGTCTGCTGGAGAGTGC-3' and reverse, 5'-TTCGCGGCTGTGAGTGGTCT-3'; GAPDH forward, 5'-GACACCCTAAGGGCTAGAAC-3' and reverse, 5'-TGATGACGACCAGTGTGA-3'. The PCR conditions for quantification were as follows: 10 min at 95°C, 40 cycles of 10 sec at 95°C, 20 sec at 58°C, and 30 sec at 72°C. qPCR was performed using 2 µl diluted cDNA products, 12.5 µl SYBR Green (Thermo Fisher Scientific, Inc.), 0.5 µl forward and reverse primers (10 µM) and 9.5 µl nuclease-free water in a final volume of 25 µl. GAPDH was used as an internal control and the relative expression of mRNA was calculated using the 2^{-ΔΔCq} method (24).

Western blot analysis. The proteins were isolated from the cells using radioimmunoprecipitation assay lysis and extraction buffer (containing 150 mM NaCl, 25 mM Tris-HCl, pH 7.6, 1% sodium deoxycholate, 1% NP-40, protease inhibitor and 0.1% SDS). The BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) was used to calculate the total protein concentration. The total proteins (50 µg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (EMD Millipore). The membrane was blocked with 5% non-fat dry milk in PBS with 5% Tween-20. Following 3 washes in PBS with 5% Tween-20, the membranes were incubated with primary antibodies against TLR2 (1:1,000; ab108998; Abcam, Cambridge, UK), SR-BI (1:1,000; MAB8114; R&D Systems, Inc., Minneapolis, MN, USA), ABCG1 (1:500; NB400-132), ABCA1 (1:1,000; NB400-105) (both from Novus Biologicals, LLC, Littleton, CO, USA), p-NF-κB (1:500; 8214; Cell Signaling Technology, Inc., Danvers, MA, USA), NF-κB (1:500; MAB72261; R&D Systems, Inc.) and GAPDH (1:200; 4670; Cell Signaling Technology, Inc.) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated IgG secondary antibodies for 1 h at room temperature. The bands were subsequently visualized by enhanced chemiluminescence detection reagents (GE Healthcare Life Sciences, Little Chalfont, UK), and the images were analyzed by the NIH ImageJ software (version 1.47t; National Institutes of Health, Bethesda, MD, USA).

**Results**

**Effect of ox-LDL on foam cell formation in THP-1 and RAW264.7 cells.** Macrophages are known to transform into foam cells when incubated with ox-LDL (25). To evaluate the formation of foam cells, ORO staining and the intracellular cholesterol contents were measured. THP-1 and RAW264.7 cells were incubated with 50 µg/ml ox-LDL for 48 h prior to staining with ORO. As shown in Fig. 1A, THP-1 and RAW264.7 cells treated with ox-LDL exhibited significant accumulation of lipid droplets.

The contents of TC, FC and CE in normal cells and foam cells were also detected following incubation with ox-LDL for 48 h. As shown in Fig. 1B, the results revealed that the TC, FC and CE contents were significantly increased in THP-1 and RAW264.7 macrophage-derived foam cells treated with ox-LDL for 48 h when compared with those in untreated cells. In addition, the contents of TC and CE were markedly upregulated in RAW264.7 macrophage-derived foam cells treated with ox-LDL compared with those in untreated cells (Fig. 1C). These results demonstrated that ox-LDL induced foam cell formation in the THP-1 and RAW264.7 cells.

TLR2 blocks the efflux of macrophage cholesterol in THP-1 and RAW264.7 macrophage-derived foam cells. In order to investigate the role of TLR2 in mediating the efflux of cholesterol, the cellular cholesterol efflux was measured by liquid scintillation counting. As shown in Fig. 2, addition of TLR2 significantly decreased the cholesterol efflux of THP-1 and
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RAW264.7 cells in dose- and time-dependent manners. These data suggested that TLR2 was a negative regulator of cholesterol efflux in THP-1 and RAW264.7 macrophage-derived foam cells.

Knockdown of TLR2 promotes cholesterol efflux in THP-1 and RAW264.7 macrophage-derived foam cells. To further confirm whether TLR2 is a negative regulator of cholesterol efflux, THP-1 and RAW264.7 macrophage-derived foam cells were transfected with TLR2 siRNA. As demonstrated in Fig. 3A and B (left panels), the cells transfected with TLR2 siRNA presented inhibited TLR2 protein expression in comparison with those transfected with scramble siRNA, which confirmed the knockdown of TLR2. Furthermore, knockdown of TLR2 by siRNA significantly increased the cholesterol efflux (Fig. 3; right panels). Thus, these results supported the involvement of TLR2 in the downregulation of cholesterol efflux.

TLR2 inhibits ABCA1, ABCG1 and SR-B1 expression in THP-1 and RAW264.7 macrophage-derived foam cells. ABCA1, ABCG1 and SR-B1 are critical proteins in the regulation of cellular cholesterol homeostasis (9). In the present study, the effect of TLR2 on the mRNA and protein expression levels of ABCA1, ABCG1 and SR-B1 in THP-1 and RAW264.7 macrophage-derived foam cells was examined by RT-qPCR and western blot analysis, respectively. As shown in Fig. 4, TLR2 reduced ABCA1, ABCG1 and SR-B1 expression at the transcriptional and translational levels in a dose-dependent manner.

Effect of TLR2 on NF-κB activation and inhibition of cholesterol efflux by the NF-κB activation in THP-1 and RAW264.7 macrophage-derived foam cells. It has been demonstrated that NF-κB is a downstream molecule of TLR2 (26). Thus, in the present study, it was hypothesized that NF-κB may be involved...
in the role of TLR2 in the downregulation of cholesterol efflux. As shown in Fig. 5A and B, western blot analysis revealed that THP-1 and RAW264.7 macrophage-derived foam cells transfected with TLR2 siRNA exhibited reduced phosphorylation levels of NF-κB (p65). By contrast, overexpression of TLR2 increased the phosphorylation levels of NF-κB (p65). Moreover, upregulation or downregulation of TLR2 had no effect on the protein expression levels of NF-κB.

To detect the effect of NF-κB on cholesterol efflux, liquid scintillation counting was performed. As observed in Fig. 5C and D, the cholesterol efflux was markedly reduced following treatment with lipopolysaccharides (LPS) in THP-1 and RAW264.7 macrophage-derived foam cells. By contrast, the application of PDTC, an NF-κB specific inhibitor, significantly suppressed the LPS-induced downregulation of cholesterol efflux.

**Discussion**

Macrophages take up ox-LDL and other lipids to form foam cells, resulting in early atherosclerosis (27). The aortic atherosclerotic lesion and foam cell formation are accelerated by enhanced cholesterol accumulation (28). In the present study, the effects of TLR2 on macrophage cholesterol efflux and the underlying molecular mechanisms were investigated. In addition, the effect of exogenous TLR2 on cell cholesterol efflux was examined. Intracellular cholesterol efflux was detected in the THP-1 and RAW264.7 macrophage-derived foam cells transfected with TLR2 siRNA. The expression levels of phosphorylated NF-κB (p65) in cells transfected with TLR2 siRNA and TLR2 overexpression vector were also determined. The results of the current study provided convincing evidence for the role of TLR2 in suppressing macrophage cholesterol efflux through targeting NF-κB.

TLR2 represents an attractive therapeutic target in atherosclerosis (29). The proatherogenic effect of TLR2 activation has also been demonstrated to induce intimal hyperplasia and atherosclerotic lesion development (30). Mullick et al (31) revealed that TLR2 participated in the modulation of atherosclerosis in mice, and complete knockdown of TLR2 led to decreased lesion size, while exposure to an exogenous TLR2 significantly exacerbated atherosclerosis. Cao et al (32) identified that *Chlamydophila pneumoniae*-induced macrophage foam cell formation was mediated by TLR2. Similarly, Zhao et al (33) suggested that TLR2 was able to mediate the effect of *C. pneumoniae* on cholesterol homeostasis in human THP-1 macrophages.

Cholesterol efflux transport is mediated by specific proteins, and has been recently demonstrated to be mediated by ABCA1, ABCG1 and SR-B1 (34). ABCA1 mediates the transport of phospholipids, cholesterol and other lipophilic molecules across cellular membranes to lipid-poor HDL apolipoproteins (23). In addition, ABCG1 promotes efflux through redistribution of intracellular cholesterol to the plasma membrane domains accessible for removal by HDL (35). Furthermore, SR-B1 was demonstrated to enhance cell cholesterol influx and cholesterol efflux from HDL, but did not alter cellular cholesterol mass (36). In the current study, the decrease in TLR2-mediated cholesterol efflux in dose-dependent manner was consistent with the downregulated expression of ABCA1, ABCG1 and SR-B1 at the transcriptional and translational levels in THP-1 and RAW264.7 macrophage-derived foam cells.
TLR2 has been demonstrated to use the downstream adaptor MyD88 for signal transmission, and the MyD88-dependent pathway gives rise to activation of the NF-κB transcription factor, which controls proinflammatory gene expression (37). Thus, the molecular mechanisms between TLR2 and NF-κB required to be further investigated. Recent evidence revealed the potential role of NF-κB in atherosclerosis. For instance, activated NF-κB has been identified in macrophages and human atherosclerotic plaques (38), while the genes regulated by NF-κB have been detected to be upregulated in plaques (39). Furthermore, several Toll-like receptors that can signal to NF-κB have also been identified in lesions (40). NF-κB has been demonstrated to mediate the inflammatory role of TLR2 in several diseases, such as dry eye (41,42). However, it remains unclear how the network of TLR2 and NF-κB signaling controls atherogenesis. In the current study, the results demonstrated that the cholesterol efflux was downregulated via the NF-κB activator, LPS. However, cell treatment with PDTC, an inhibitor of NF-κB, reversed the LPS-induced downregulation of cholesterol efflux. Additionally, knockdown of TLR2 attenuated the phosphorylation levels of NF-κB (p65), while overexpression of TLR2 resulted in the opposite tendency. Therefore, the role of TLR2 in reducing cholesterol efflux may partly be through the NF-κB pathway in macrophage-derived foam cells, and it likely contributes to the pathogenesis of atherosclerosis.

In conclusion, the present study provided a novel insight into the role of TLR2 on suppression of cholesterol efflux via downregulation of ABCA1, ABCG1 and SR-B1 expression levels, and indicated that the TLR2 effect is mediated by the NF-κB signaling pathway. Thus, TLR2 may be a potential therapeutic target for the prevention of atherosclerosis.

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