Oxidized Low-Density Lipoprotein Promotes Macrophage Lipid Accumulation via the Toll-Like Receptor 4-Src Pathway

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**Background:** Uptake of oxidized low-density lipoprotein (oxLDL) by macrophages is recognized as a crucial step in the development of atherosclerosis, whereas the precise molecular mechanisms involving it remain to be elucidated.

**Methods and Results:** This study focused on determining the role of toll-like receptor 4 (TLR4) and Src kinase in macrophage lipid accumulation. oxLDL significantly enhanced Src kinase activity and intracellular lipid contents in RAW264.7 macrophages, whereas the small interference RNA-mediated knockdown of TLR4 and Src or chemical inhibition of Src activity blocked oxLDL-induced lipid accumulation. Immunoprecipitation and immunofluorescence studies demonstrated that TLR4 was associated with Src on the plasma membrane upon oxLDL stimulation.

**Conclusions:** The results of the present study suggest an essential role of TLR4-Src signaling in macrophages in the pathogenesis of atherosclerosis. (*Circ J* 2015; 79: 2509–2516)

**Key Words:** Atherosclerosis; Lipid; Macrophage; Src; Toll-like receptor 4 (TLR4)

*Retention of lipoprotein in macrophages constitutes a major cause for coronary artery disease by initiating and enhancing local inflammation.*

Deposition of oxidized low-density lipoprotein (oxLDL) within the subendothelial space of arterial wall recruits circulating monocytes into the intima. Monocyte-derived macrophages absorb excessive lipids and secrete an array of pro-inflammatory cytokines that accelerate the progression of atherosclerosis. A variety of lipoprotein receptors, including CD36 and lectin-like oxidized LDL receptor-1 (LOX-1), have been identified. Previously, we and others have demonstrated that macrophage lipid accumulation is also regulated by toll-like receptor 4 (TLR4).

TLR4 was first identified to play a fundamental role in innate immunity by detecting lipopolysaccharide (LPS) and activating downstream signals, including Src kinase. As a non-receptor tyrosine kinase, Src is the prototype of a family of highly conserved proteins implicated in diverse signaling pathways and cellular processes. Src may function as an ‘integrator’ of multiple endogenous and exogenous stimuli to control cell growth, migration, adhesion and lipid metabolism in macrophages.

In this study, we hypothesized that the TLR4-Src pathway is essential for oxLDL-induced lipid uptake in macrophages. Using immunohistochemistry, we show that phosphorylated Src and TLR4 are increased in macrophages in atherosclerotic plaques. We further demonstrate that Src phosphorylation at tyrosine 418 by TLR4 is involved in macrophage lipid accumulation. These results provide a novel pathway that may advance the understanding of coronary artery disease.

**Methods**

**Reagents and Antibodies**

Oxidized LDL (Serotec, Oxford, UK) was used to stimulate...
Triton X 100, Hematoxylin, AEC Staining Kits, Neutral red, 3, 3′-Diaminobenzidine, and the BCIP®/NBT Liquid Substrate System were purchased from Sigma-Aldrich (MO, USA). The primary antibodies used included: β-actin, tumor necrosis factor α-associated factor (TRAF) 6, Fyn, Lyn, CD36 and LOX-1 (Cell Signaling, MA, USA), p-Src Tyr418, Src (Abcam, MA, USA), and TLR4 (Invitrogen, CA, USA). HRP-, AP-, Alexa 549-, Alexa 647- and Alexa 488-conjugated antibodies (Cell Signaling) were used as secondary antibodies. The immunohistochemical antibodies used included CD68, TLR4 and Src (Abcam).

**Clinical Samples**

Human femoral arteries with atherosclerotic plaques were

![Figure 1. Expression of toll-like receptor 4 (TLR4) and Src in atherosclerotic lesions. (A) Human femoral arteries with atherosclerotic lesions and normal internal thoracic arteries were examined by immunohistochemical analysis. Paraffin-embedded sections were stained with hematoxylin and eosin, anti-CD68, anti-TLR4, anti-p-Src, and control immunoglobulin G (IgG) as negative control. (B) Immunofluorescence staining of the artery with 4,6-diamidino-2-phenylindole (DAPI) (blue), rabbit anti-Src or rabbit IgG (green), mouse anti-TLR4 or mouse IgG (red), goat anti-CD68 or goat IgG (pink), respectively. A gray color in the merge indicates co-localization. Results are representative of 3 independent experiments. AS, atherosclerosis; Con, control.](image)
obtained from 3 patients undergoing leg amputation. The internal thoracic arteries (n=3) were obtained and used as normal arteries. The study protocol was approved by the Ethics Committee of Rui Jin Hospital, Shanghai Jiaotong University School of Medicine, and written informed consent was obtained from all patients and donors.

Cell Culture
Primary human monocytes were isolated from buffy coats derived from healthy donors. Cells were maintained in Roswell Park Memorial Institute medium (RPMI) supplemented with 20% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, and 2% L-glutamine for 5 days. RAW264.7 cells (ATCC, VA, USA) were seeded in 6-well plates at a density of 1.0×10^6 cells per well and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS).

Oligonucleotide and Plasmid Transfection
Transient transfection of RAW264.7 cells with a negative control, TLR4-, Src- or TRAF6-specific siRNA (100 nmol/L per 10^5 cells) was conducted by using Lipofectamine reagent (Invitrogen).

Assessment of Intracellular Lipids and Total Cholesterol
After oxLDL stimulation, cultured macrophages in 6-well plates were washed with PBS (Gibco, NY, USA) and then fixed in 4% paraformaldehyde/PBS for 15 min. After rinsing with ddH2O, macrophages were stained with 0.3% Oil red O (ORO) in 60% isopropanol for 10 min. After rinsing with PBS for another 4 times, cells were photographed under a microscope (Olympus Microsystems) at 40x magnification. Five randomly selected fields of 3 independent experiments were analyzed and showed typical images in each group. Quantification of the ORO staining was then performed by eluting-stained oil droplets in 100% isopropanol for 10 min. Optical density was then measured at 500 nm wavelengths and equalized with the cell numbers analyzed by microscope. Quantifications of total cholesterol were performed following the protocols from the manufacturer (Biovision, CA, USA). Cholesterol ester was hydrolyzed into free cholesterol by using cholesterol esterase. Total cholesterol was then detected by using a colorimetric method and expressed as relative values compared to total protein concentration (n=3).

Immunohistochemistry
Human femoral arteries (n=3) and internal thoracic arteries (n=3) were used for histological and immunohistochemical analysis. Samples were fixed in 4% paraformaldehyde overnight, embedded with an optimal cutting temperature (OCT) compound, and cut into serial cryosections (6 μm thickness). Sections were used for hematoxylin and eosin staining or immunohistochemistry analysis with the following antibodies: anti-Cd68 (1:50), anti-TLR4 (1:50) or anti-Src (1:50). After incubation with horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated secondary antibodies (1:100), membranes were blocked with 5% BSA in TBS-Tween 20 (TBST). Immunoblotting was then performed using antibodies diluted in 1% BSA TBST. Antibodies against TLR4 (1:500 dilution), Src (1:1,000 dilution), Fyn (1:1,000 dilution) and Lyn (1:1,000 dilution) were used. Images were captured and the density of each band was analyzed with GelDoc software (Bio-Rad, Munich, Germany).

Pretreatment With PP2 and PP3
RAW264.7 cells were treated with PP2 (10 μmol/L) or PP3 (10 μmol/L) for 30 min prior to the addition of oxLDL. PP3 served as negative control for PP2.
YANG K et al.

**Results**

**TLR4 and Src Co-Localize in Macrophages in Atherosclerotic Lesions**

To investigate the expression and cellular localization of TLR4 and Src in atherosclerotic lesions, immunohistochemistry was performed in human femoral arteries containing atherosclerotic lesions from patients undergoing leg amputation (Figure 1A, top 5 panels; baseline characteristics in Table). Consecutive serial sections showed macrophages (CD68-positive cells) that enriched around the lipid core of atheroma expressed high levels of TLR4 as well as Src, in contrast to the negative staining for TLR4 and Src in normal internal thoracic arteries (Figure 1A, bottom 5 panels). Moreover, co-localization of TLR4, Src and CD68 in the atherosclerotic lesions was confirmed by immunofluorescence staining (Figure 1B).

**TLR4 Signaling Regulates Src Kinase Activity Upon oxLDL Stimulation**

Human primary macrophages and RAW264.7 cells were treated with different doses or varying time intervals of oxLDL. We found that oxLDL promoted phosphorylation of Src kinase at Y-418 in a time- and dose-dependent manner, which peaked at 50 μg/ml after incubation for 60 min (Figures 2A, B). (C) RAW264.7 cells transfected with a negative control or TLR4-specific small interference RNAs (siRNA) were incubated with or without oxLDL. Src phosphorylation and TLR4 expression were detected by Western blotting. (D) Densitometric quantifications are shown. Data are expressed as mean±SEM from 3 independent tests. **P<0.01 when compared with oxLDL (–) cells; ##P<0.01 when compared with a negative control (NC).

**Statistical Analysis**

All values are expressed as mean±SD. A Student’s paired t-test was performed for comparison of paired samples, and ANOVA was used for multiple group comparisons, followed by Friedman’s post-test. A probability (P) value <0.05 was considered significant.
TLR4/Src Signaling Regulates Lipid Accumulation in Macrophages

To investigate the role of Src in macrophage lipid accumulation, Src-specific siRNA (Figure 3) or the Src kinase inhibitor, PP2, was introduced to either reduce Src expression levels or to block its activity. PP3, an inactive PP2 analog, was also applied as a control. Intracellular lipid levels were analyzed both by ORO staining and total cholesterol quantification. We found enhanced lipid accumulation in response to oxLDL exposure was significantly reduced by Src knockdown and PP2 treatment (Figures 3A–C). Additionally, TLR4 knockdown markedly inhibited intracellular lipid accumulation in macrophages (Figure S1). Moreover, TLR4 or Src knockdown significantly attenuated the expression of CD36 but not LOX-1 (Figures 3E,F).

oxLDL Induces TLR4 Interaction With Src in Macrophages

Immunoprecipitation assays were then performed to better characterize mechanisms involved in the TLR4-Src signaling. We found that TLR4 associated with Src in RAW264.7 cells after oxLDL stimulation for 30 and 60 min (Figure 4A). Furthermore, co-localization of TLR4 and Src on the cell membrane upon oxLDL exposure was also detected by immunofluorescence (Figure 4B). In addition, this interaction was unaffected by TRAF6 knockdown (Figure 4C). No detectable interaction of TLR4 with Fyn and Lyn, other members in the Src Family Kinases (SFKs) group, was observed (Figure 4D).
c-Jun in response to LPS, thereby promoting the expression of pro-inflammatory genes. Thus, Src could alter the expression of a variety of genes involved in lipid influx and efflux to affect intracellular lipid contents.

Among various ligands for TLR4, LPS from Gram-negative bacteria has been well studied. The TLR4 signaling cascade in response to LPS is dependent on recruited adaptor proteins and can be broadly divided into myeloid differentiation factor 88 (MyD88)-dependent and independent pathways, both leading to the activation of the nuclear factor κB (NF-κB) pathway and expression of target pro-inflammatory genes.

CD14 has been considered mainly as a molecule that concentrates and delivers LPS to TLR4/MD-2, thereby facilitating TLR4 activation.

CD14 recruited Lyn, a member from the Src family kinases group, to participate in the signaling pathway of TLR4.

The function of translocation associated membrane protein 1 (TRAM) is to take part in LPS-TLR4 signaling.
to regulate the MyD88-independent pathway during the innate immune response to LPS. However, knockdown of MyD88 or CD14 or TRAM did not have an effect on the interaction of TLR4 and Src (Figure S2). TRAF6, a critical signaling element involved in both pathways, has been shown to physically interact with Src kinase via a proline-rich SH3 domain in its COOH-terminal. In this study, we found for the first time that TLR4 interacts with Src kinase via a proline-rich SH3 domain in its COOH-terminal. In contrast, oxLDL binds to CD36 to trigger assembly of a heterotrimeric complex composed of CD36-TLR4-TLR6; however, TLR4-TLR6 signaling is triggered by a proximal membrane event initiated by CD36 inter- action with Lyn kinase. Similarly, we observed that oxLDL induced CD36-TLR4-TLR6 co-localization on the cell membrane, but knockdown of TLR6 did not have an effect on Src activation (Figure S3). Hence, TLR4 and CD36 may share a common mechanism to regulate intracellular lipid levels by modulating Src activity. Moreover, knockdown of Src or TLR4 weakened CD36 expression (Figures 3E, F). These results highlight the importance of crosstalk between TLR4 and CD36 caused lipid disorder via Src in macrophages.

In summary, we for the first time demonstrated that TLR4/Src signaling is important for macrophage lipid accumulation and atherosclerotic progression. The clinical implication of this study is that deactivation of Src or dissociation of the TLR4/Src complex by synthetic chemicals could effectively attenuate the pathogenesis of atherosclerosis.

Acknowledgments

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References


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**Supplementary Files**

**Supplementary File 1**

*Figure S1.* (A) Boiled oxidized low-density lipoprotein (oxLDL) no longer promotes Src phosphorylation.

*Figure S2.* (A) The efficiency of myeloid differentiation factor 88 (MyD88) or translocation associated membrane protein 1 (TRAM) or CD14 knockdown was analyzed by Western blotting.

*Figure S3.* (A) RAW264.7 cells treated with oxidized low-density lipoprotein (oxLDL) for 60min. Please find supplementary file(s); http://dx.doi.org/10.1253/circj.CJ-15-0345