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Original Article

Toll-like receptor 4 is up-regulated by mTOR activation during THP-1 macrophage foam cells formation

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Macrophage foam cells formation is the most important process in atherosclerotic plaque formation and development. Toll-like receptor 4 (TLR4) is one of the important innate immune sensors of endogenous damage signals and crucial for regulating inflammation. Growing evidence indicates that TLR4 plays a very important role in macrophage foam cells formation. However, the underlying mechanisms regulating TLR4 expression in macrophage are not fully understood. In this study, we induced THP-1 macrophage foam cells formation with oxidative modified low-density lipoprotein (ox-LDL). We observed that TLR4 mRNA and protein expression were markedly up-regulated, and the phosphorylation of mammalian target of rapamycin (mTOR) and its downstream target p70S6K were promoted during foam cells formation. The mTOR inhibitor rapamycin blocked mTOR phosphorylation and inhibited TLR4 expression induced by ox-LDL. Silencing mTOR, rictor or raptor protein expression by small interfering RNA, also inhibited the up-regulation of TLR4 expression, respectively. Inhibition of mTOR with rapamycin reversed the down-regulation of cellular lipid efflux mediator ABCA1, which resulted from the activation of TLR4 by ligands. These data suggested that TRL4 expression was up-regulated by a mechanism dependent on mTOR signal pathway activation during THP-1 macrophage foam cells formation. Inhibition of ox-LDL induced mTOR activation reduced TLR4 expression, and improved the impaired lipid efflux.

Keywords foam cells; macrophage; toll-like receptor 4; atherosclerosis

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Introduction

Excessive lipid accumulation in macrophages, also known as foam cells formation, is an imbalance of intracellular lipid turnover, and a key early event in development of atherosclerosis [1–3]. Abundant foam cells formation leads to vascular inflammation, and the production of reactive oxygen species, which promotes low-density lipoprotein (LDL) oxidation, contributes in turn to further foam cells formation and atherosclerosis plaque development.

Toll-like receptor 4 (TLR4) is widely recognized as a key innate immune sensor. TLR4 plays a very important role in triggering innate immunity and initiating a cascade of pro-inflammatory events through binding pathogen-associated molecules that may drive either from bacteria (such as lipopolysaccharide, LPS), viruses or fungi [4–6]. Recently, evidence for TLR4 expression in endothelial cell, monocyte and macrophage has been shown [7–9]. Several studies indicated that TLR4 expression can be up-regulated by ox-LDL and minimally modified LDL (mm-LDL) in macrophage [10,11].

Experimental animal study involving Apo $E^{-/-}$ mice fed a high-cholesterol diet clearly indicated the relative importance of TLR4 to foam cells formation and atherosclerosis. When TLR4 expression was abrogated in these mice, lesion size, lipid content, and macrophage infiltration also decreased [12,13]. More importantly, recent studies have identified some pro-atherosclerosis factors, such as mm-LDL and its active components, polyoxygenated cholesteryl ester hydroperoxides, and C-reactive protein (CRP) are involved in endogenous activation of TLR4 [14,15]. It has been established that TLR4 plays a very important role in the formation of foam cells. One of the major mechanisms by which TLR4 enhances cellular lipid accumulation is the inhibition of liver X receptor (LXR) pathway and the down-regulation of LXRα target genes ATP-binding cassette transporters, ABCA1, which play a pivotal role in cholesterol efflux from macrophage [16–18].

TLR4 has therefore been recognized as an important pharmacological target for the prevention of foam cells formation and atherosclerosis. However, the signaling

pathways regulating TLR4 expression in macrophage are poorly understood.

The serine/threonine protein kinase mammalian target of rapamycin (mTOR) critically regulates cellular lipid metabolism, protein synthesis, and differentiation [19–21]. Two functionally and structurally distinct mTOR-containing protein complexes, mTORC1 and mTORC2, have been identified, both of which contain phosphorylated mTOR as their central component. The mTORC1 complex composed of mTOR and raptor can phosphorylates p70-S6 kinase 1 to activate protein synthesis. The mTORC2 complex composed of mTOR, rictor, G-protein β-subunit like protein, and mammalian stress-activated protein kinase interacting protein is regulated by growth factors, nutrient levels and other factors, and exerts effect on cellular metabolism and other functional regulations. There is growing evidence that mTOR special inhibitor rapamycin could reduce lipid accumulation in cells and prevent atherogenesis [22,23]; however, the underlying signals and mechanisms are not yet fully clear. In this study, mTOR activation in THP-1 macrophage was detected during foam cells formation, effect of mTOR activation on TLR4 expression and cholesterol efflux from THP-1 macrophage was analyzed.

Materials and Methods

Reagents

Monoclonal antibodies against TLR4, mTOR, phosphor-mTOR, p70S6K, phosphor-p70S6K, rictor, raptor, ABCA1, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Unless otherwise noted, all other chemicals were from Sigma-Aldrich (St Louis, USA).

LDL extraction and preparation of ox-LDL

Human LDL was isolated from ethylendiamine tetra-acetic acid (EDTA) plasma of healthy volunteers by sequential floating ultracentrifugation. Extensive oxidative modification of LDL was performed according to published protocols by dialyzing purified LDL fractions (1 mg protein/ml) against 5 μM $CuSO_4$ [24]. The degree of oxidation of ox-LDL was evaluated by measuring the concentration of thiobarbituric acid-reactive substances (TBARS). The ox-LDL contained 20–30 nM TBARS/mg protein. Modified lipoproteins were stored at 4 °C and used within a week.

THP-1 cell differentiation and foam cells formation

To obtain THP-1 macrophage, THP-1 cell obtained from the Cell Culture Center of Chinese Academy of Medical Sciences (Beijing, China) was plated in 35-mm dishes (1×10^7 cells/dish) and incubated with phorbol 12-myristate 13-acetate at a concentration of 160 nM for 48 h [25]. After being washed three times with phosphate-buffered saline (PBS), differentiated THP-1 cells, termed as THP-1 macrophages, were cultured in RPMI-1640 with 5% fetal bovine. Foam cells formation was induced by incubation of THP-1 macrophage for 48 h with ox-LDL (50 μg/ml).

Oil red O staining

The THP-1 macrophages were incubated with ox-LDL for different duration. Immediately after incubation, media were aspirated and cells were fixed in the same 12-well plates used for incubation, with 4% paraformaldehyde in water, for 2–4 min. Cells were stained with 0.2% oil red O in methanol for 1–3 min. Cells were observed via light microscope (Axiovert 25-Zeiss; Carl Zeiss, Jena, Germany) with ×100 magnification and then photographed using a Kodak DC 290 Zoom Digital Camera (Eastman Kodak, Rochester, USA).

Protein extraction and western blot analysis

The THP-1 macrophages and foam cells were washed with PBS, scrapped with lysis buffer (Tris–EDTA–ethylene glycol tetra-acetic acid + Complete protease inhibitor; Roche, Indianapolis, USA) and mechanically homogenized. Equal amounts of total proteins (50 μg) were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, fractionated by electrophoresis for 1.5 h at 100 V, and transferred to polyvinylidene fluoride membranes. The membrane was blocked for 4 h at 4°C in blocking solution [3% non-fat dry milk dissolved in 1× Tween20-tris-buffered saline (TTBS)] and then the primary antibodies (Santa Cruz Biotechnology, Inc., 1:1000) were used against TLR4, mTOR, phosphor-mTOR, p70S6K, phosphor-p70S6K, rictor, raptor, ABCA1 and β-actin. The five washes in TTBS were repeated, and then the immunoreactive protein was detected using enhanced chemiluminiscence western blotting detection reagent (GE Healthcare, Waukesha, USA) and film development in SRX-101A (Konica Minolta Holdings, Inc., Tokyo, Japan). As control, β-actin was detected using mouse anti-β-actin antibody.

Total RNA extraction and reverse transcription–polymerase chain reaction

Total RNA was extracted from cultured cells using RNeasy Mini kit (Qiagen, Valencia, USA) according to the manufacturer's protocol and was converted into complementary DNA with the iScript cDNA synthesis kit (Bio-Rad, Richmond, USA). The obtained complementary DNAs were then used as the templates for reverse transcription–polymerase chain reaction (RT–PCR). Forward and reverse primer pairs are listed as follows: *TLR4* sense, 5′-TGTG GCTCACAATCTTATCCA-3′; *TLR4* antisense, 5′-CTAAA TGTTGCCATCCGAAA-3′; *β-actin* sense, 5′-CGTGCGT GACATTAAGGAGA-3′; *β-actin* antisense, 5′-ATACTCC TGCTTGCTGATCCA-3′. The PCR amplification included an initial denaturation at 94 °C for 2 min, 30 cycles of

denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension for 2 min, and a final extension of 5 min at 72 °C. The PCR products were analyzed using an ethidium bromide-stained 1% agarose gel. The images were digitized and the optical density of each band was quantified with the Kodak Digital Science EDAS System (Kodak, Norwalk, USA). The data (optical density) were first normalized against the internal standard (β-actin) and then expressed as fold change from the control.

Transfection of small interfering RNA

One day before transfection, THP-1 macrophages were plated on a 35-mm culture dish in RPMI-1640 complete medium. After cells reached 50%–60% confluence, they were transfected with siRNA as previously described [26]. Briefly, cells were placed in 800 μl of RPMI-1640 medium along with 200 μl of transfection solution (Opti-MEM medium), mirus transfection reagent and chemically synthesized siRNA (100 nM, human mTOR: 5′-CCAAAGU GCUGCAGUACUAUU-3′, human rictor: 5′-ACUUGUG AAGAAUCGUAUC-dT.dT-3′, human raptor: 5′-GGAC AACGGCCACAAGUAC.dT.dT-3′, negative control siRNA: 5′-CGUACGCGGAAUACUUCGAdT.dT-3′). After 6 h of transfection, 1 ml of RPMI-1640 complete medium was added, and experiments were conducted 48 h after transfection. Protein levels of mTOR, rictor, and raptor were analyzed by western blot.

Cholesterol efflux from THP-1 macrophages

Radiolabeled cholesterol efflux assays were carried out as described previously [27]. Briefly, THP-1 macrophages (5×10^5 cells) were incubated for 48 h with 1 μCi/ml ^{3}H-cholesterol and 50 μg/ml ox-LDL in base medium in the presence or absence of rapamycin (10 nM) or TLR4 ligand lipid A (a bioactive center of LPS, 50 ng/ml) as described previously [17]. After 2 h equilibration with base medium, cholesterol efflux was induced with 10 μg/ml apoAI in base medium for 4 h. Cholesterol efflux was represented as the percentage of medium counts per minute (cpm) to total cpm (medium plus cellular cpm), with the subtraction of basal cholesterol efflux which is defined as the percent of cholesterol in the absence of apoAI.

Statistical analysis

Data were present as the means ± SD. Differences were analyzed by analysis of variance with appropriate *post hoc* analyses. $P < 0.05$ was considered significant.

Results

TLR4 expression was up-regulated in THP-1 macrophage during foam cells formation.

To investigate the expression change of *TLR4* during macrophage foam cells formation, human monocyte-like leukemic cell line THP-1, a frequently studied *in vitro* foam cell model system was used. The foam cell formation was induced with ox-LDL (50 ng/ml) and the time course change of *TLR4* expression was evaluated with RT–PCR and western blot. As shown in **Fig. 1(A)**, lipid-laden foam cells formation, as identified by oil red O staining, was apparent in THP-1 macrophages treated with ox-LDL for 24 and 48 h. We observed that *TLR4* mRNA expression was increased after 12 h of incubation [**Fig. 1(B,C)**]. TLR4 protein levels had a trend of increase and were similar to mRNA expression levels. Averaged densitometric quantification showed that TLR4 protein level reached a peak (increased about 3 folds) at 24 h of exposure to ox-LDL [**Fig. 1(D,E)**].

The mTOR inhibitor rapamycin down-regulated *TLR4* expression

To determine whether the above observed increase in *TLR4* mRNA and protein level was associated with mTOR signal pathway activation, we co-incubated the THP-1 macrophages using ox-LDL with or without rapamycin in a range of concentrations (5, 10, 20 nM) for a period of 24 h. *TLR4* mRNA and protein level was assessed by RT–PCR and western blot analysis. Results presented in **Fig. 2(A,C)** showed that rapamycin significantly reduced the expression levels of TLR4 mRNA and protein. Results from averaged densitometric quantification of western blot showed that rapamycin reduced protein level of TLR4 in a concentration-dependent manner with a minimum significant reduction at 10 nM [**Fig. 2(C)**].

mTOR signal pathway was activated during foam cells formation

There is evidence that rapamycin has multiple direct and indirect effects on cellular lipid metabolism. To determine whether the decreased TLR4 protein level in rapamycin-treated foam cells resulted from inhibition of mTOR signal pathway, we investigated the phosphorylation of mTOR at Ser-2448 using western blot. As shown in **Fig. 3(A,B)**, incubation of THP-1 macrophages with ox-LDL (50 ng/ml) led to significant increase of mTOR phosphorylation at Ser-2448. Averaged densitometric quantification showed that Ser-2448 phosphorylation peaked at 30 min and remained at a high level for 720 min. Rapamycin reduced the active effect of ox-LDL on Ser-2448 phosphorylation, but the total mTOR protein levels were not affected by rapamycin. To further confirm that mTOR signal pathway was activated by ox-LDL during foam cells formation, we detected the phosphorylation of p70S6K, a major direct downstream target of mTOR, using western blot. Results presented in **Fig. 3(C)** showed that incubation of THP-1

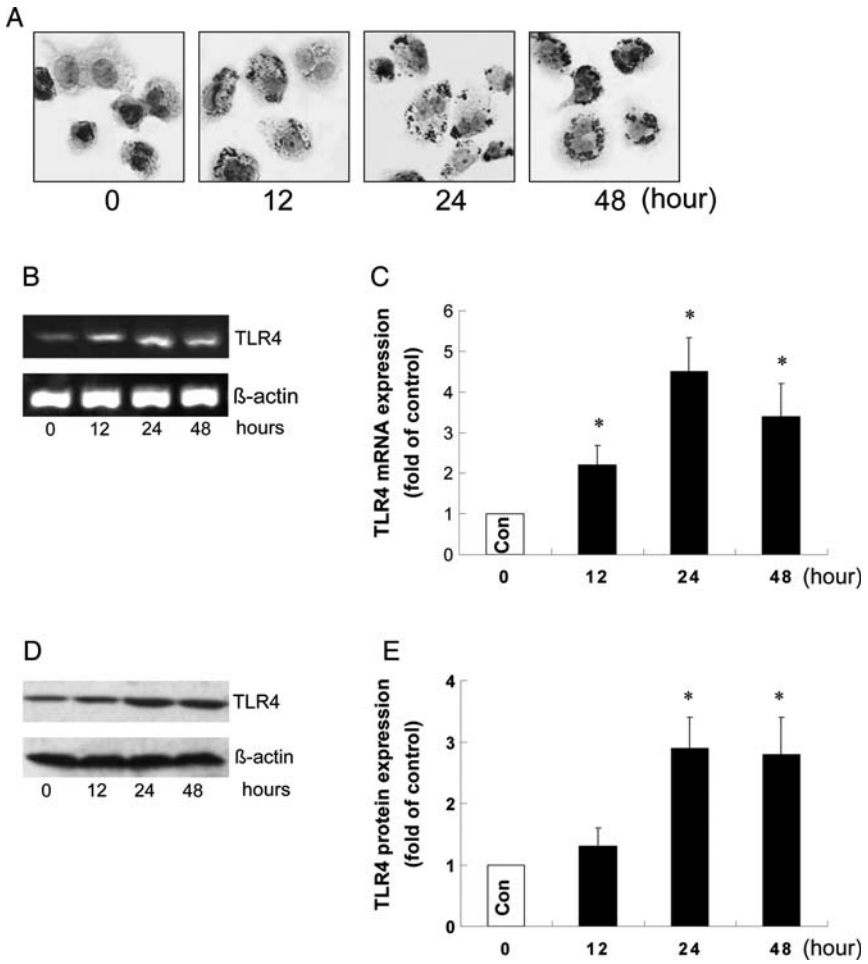

Figure 1 RT–PCR and western blot analysis of TLR4 level in THP-1 macrophages during foam cells formation (**A**) THP-1 macrophages were cultured in 24-well plates and treated with ox-LDL (50 ng/ml) for 12, 24, 48 h, respectively. The cells were fixed with 10% formalin and stained with oil-red O. The intracellular lipid droplets (intense black bodies in the stroma) were then detected under a light microscopy. (**B**) The total mRNA was isolated from the ox-LDL stimulated cells. *TLR4* mRNA expression was determined by RT–PCR and was normalized to β-actin. (**C**) Quantification of band intensity using ImageJ software. Data are expressed as fold change ± SEM from the controls (Con, incubation time 0; $n = 8–9$), $*P < 0.05$ vs. control, $n = 8–9$. (**D**) TLR4 protein level was determined by western blot using anti-TLR4 antibody. (**E**) Averaged densitometric quantification of TLR4 protein level. $*P < 0.05$ vs. control (Con, incubation time 0). Experiments were repeated in five times in duplicate.

macrophages with ox-LDL (50 ng/ml) for 30 min significantly increased p70S6K phosphorylation at Thr-389, which could be blocked by rapamycin.

The mTOR complex 1 and mTOR complex 2 were both needed for up-regulation of *TLR4* expression

Protein rictor and raptor play pivotal and distinct roles in mTOR-mediated signal transduction. The phosphorylated mTOR interacts with rictor to form mTOR complex1, and interacts with raptor to form mTOR complex2, respectively. To further address the potential role of mTOR in regulation of TLR4 expression, we transfected THP-1 macrophages with siRNA to inhibit mTOR, rictor, and raptor expression. To verify the silencing efficiency of this siRNA, we measured the protein expression of mTOR, rictor, and raptor in THP-1 macrophages transfected with siRNA. Western blot results showed that the silence efficiency of the siRNA was 80% higher than the control siRNA [**Fig. 4(A)**]. Western blot and averaged densitometric quantification results (presented as folds of the control) showed that transfection with mTOR, rictor, or raptor siRNA significantly attenuated the TLR4 protein level during foam cells formation compared with transfection with control siRNA [simTOR: 1.34 ± 0.33, siRictor: 1.47 ± 0.38, siRaptor: 1.65 ± 0.41 vs. control siRNA (siCon): 3.37 ± 0.52, $*P < 0.05$] [**Fig. 4(B,C)**]. The THP-1 macrophages transfected with siCon and non-treated with ox-LDL were used as control.

Inhibition of *TLR4* expression reversed the down-regulation of ABCA1 and cholesterol efflux

To investigate whether inhibition of *TLR4* expression through blocking mTOR signaling activity can reverse the inhibitory effect of TLR4 on ABCA1 protein levels and

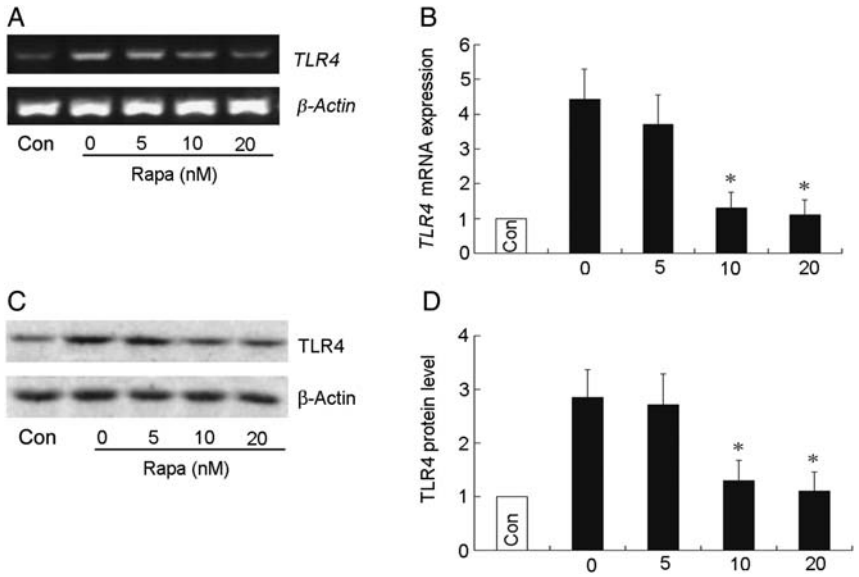

Figure 2 RT–PCR and western blot analysis of the effect of rapamycin on TLR4 level THP-1 macrophages were co-incubated with ox-LDL (50 ng/ml) and rapamycin (5, 10, 20 nM) for 24 h. (**A**) RT–PCR analysis of *TLR4* mRNA expression. Non-treated THP-1 macrophages were used as control (Con). (**B**) Quantification of band intensity using ImageJ software. Data were expressed as fold change ± SEM from the controls (Con; $n = 8–9$), $^*P < 0.05$ vs. Rapa 0 (0 nM Rapa), $n = 8–9$. (**C**) Western blot analysis of TLR4 protein level. Non-treated THP-1 macrophages were used as control (Con). (**D**) Averaged densitometric quantification of TLR4 protein level. $^*P < 0.05$ vs. Rapa 0. Experiments were repeated in five times in duplicate.

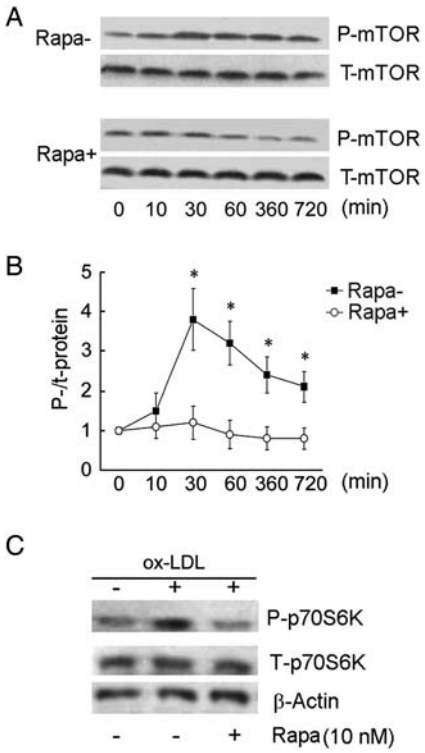

Figure 3 Western blot analysis of mTOR and p70S6K phosphorylation THP-1 macrophages were incubated using ox-LDL (50 ng/ml) without or with rapamycin (10 nM) for the indicated time. (**A**) Western blot analysis of mTOR phosphorylation (P-mTOR) at Ser-2448 and total mTOR (T-mTOR) levels in total THP-1 macrophages lysates. (**B**) Averaged densitometric quantification of P-mTOR relevant to T-mTOR protein. $^*P < 0.05$ vs. 0 min. Experiments were repeated in five times in duplicate. (**C**) Western blot analysis of p70S6K phosphorylation at Thr-389. P-p70S6K, phosphor-p70S6K; T-p70S6K, total p70S6K.

cellular cholesterol efflux, we incubated THP-1 macrophages with ox-LDL absence or in the presence of TLR4 ligand lipid A (50 ng/ml) to induce TLR4 activation, then we measured protein level of TLR4 and ABCA1. Western blot results showed that ABCA1 protein level was down-regulated by TLR4 ligand lipid A, and rapamycin significantly inhibited the level of TLR4 and abolished the inhibitory effect of lipid A on ABCA1 protein level [**Fig. 5(A)**]. To further determine the functional consequence of down-regulation of *TLR4* expression, we measured cholesterol efflux from THP-1 macrophages. Results showed that cholesterol efflux was markedly decreased from 6.27 ± 0.28% (non-treated) to 3.82 ± 0.47% by lipid A. However, co-incubation with rapamycin led to a significant increase in cholesterol efflux from THP-1 macrophages (5.95 ± 0.63%) [**Fig. 5(B)**].

Discussion

The present study demonstrated that *TLR4* mRNA and protein level was up-regulated in THP-1 macrophage during foam cells formation. This up-regulation was mediated by activating the mTOR signal pathway. Inhibition of mTOR activity reduced *TLR4* expression and improved cholesterol efflux from THP-1 macrophage.

Formation of lipid-laden foam cells is the key event in early atherogenesis, and is crucial for atherosclerosis plaque development. It is now well accepted that atherosclerosis initially develops as a pathological accumulation of modified LDL and other lipid in the vascular wall

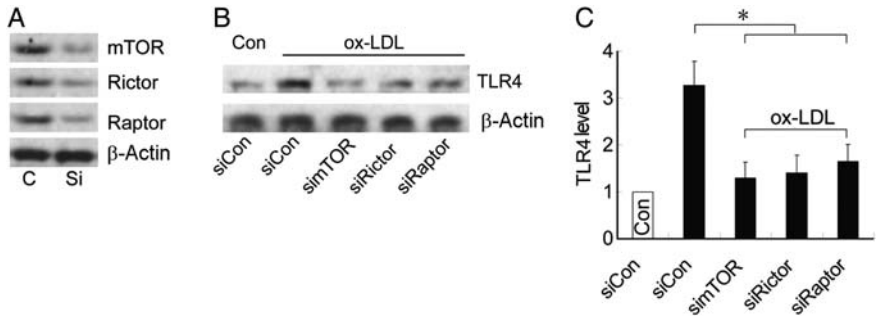

Figure 4 Silencing mTOR, rictor, or raptor protein expression by siRNA inhibited the up-regulation of *TLR4* expression (**A**) THP-1 macrophages were transfected with either control siRNA (C), mTOR, rictor. or raptor siRNA (Si) for 24 h, the expression of mTOR, rictor and raptor were determined using western blot, respectively. (**B**) THP-1 macrophages were transfected with siRNA, then the cells were incubated with ox-LDL (50 ng/ml) for 24 h, and TLR4 protein expression was determined using western blot. (**C**) Averaged densitometric quantification of western blot was normalized to the level of β-actin. THP-1 macrophages transfected with control siRNA and non-untreated with ox-LDL were used as control (Con). $*P < 0.05$ vs. group transfected with siCon and treated with ox-LDL. Experiments were repeated in four times in duplicate.

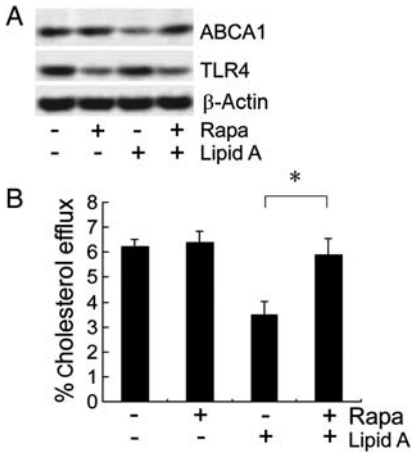

Figure 5 Analysis of ABCA1 level and cellular cholesterol efflux (**A**) Western blot analysis of TLR4 and ABCA1 protein level. (**B**) Radiolabeled cholesterol efflux assays were carried out, cellular cholesterol efflux was represented as the percentage of medium cpm to total cpm. $*P < 0.05$.

(foam cells formation), and then gradually progresses into a chronic immune inflammatory vascular disease.

The process of macrophage-derived foam cells formation involves retention of circulating LDL in the arterial intima and its modification, including oxidation, proteolytic, or lipolytic mechanisms [28,29], followed by uncontrolled uptake of modified LDL by macrophage via the scavenger receptor and macropinocytosis [30,31]. Excessive uptake of modified LDL combined with down-regulated efflux of cellular lipids stimulates the expression of inflammatory cytokines, antigen presentation, secretion of extracellular matrix degrading enzymes by macrophages, and often results in cell apoptosis or death, thereby promotes further lesion development and its eventual rupture causing angina, myocardial, and cerebral infarction.

In general, foam cells formation is thought to arise from an imbalance between lipid unregulated uptake and an inadequate efflux. Removal of cholesterol from the plasma membranes of cells is essential for maintenance of physiological intracellular cholesterol homeostasis. In fact, cellular cholesterol efflux occurs at all stages of foam cells formation and atherosclerosis and protects cells from free cholesterol and oxysterol-induced toxicity. This process is especially critical for macrophages, which lack the ability to control the influx of cholesterol, and therefore are easily transformed into cholesterol-loaded foam cells when the activity of cellular lipid efflux is inhibited [32–34].

The ATP-binding cassette transporter ABCA1, also known as cholesterol efflux regulatory protein, is responsible for the major part of macrophage cholesterol efflux to serum or apoAI, even though other less efficient pathways such as passive efflux are also involved. The expression of ABCA1 is regulated by LXR pathway; however, LXR pathway activity and ABCA1 expression are inhibited by TLR4 activation [17].

It is widely accepted that TLRs are pattern recognition receptors that sense the presence of numerous microbial components called pathogen-associated molecular patterns, initiate immunity and inflammation, and control infection processes via promotion of cytokine synthesis. The various TLRs exhibit different patterns of expression. TLR4 is most abundantly expressed in placenta and in myelomonocytic subpopulation of the leukocytes. However, recent studies conducted *in vitro* and *in vivo* have shown that TLR4 is expressed in non-immune cells, such as vascular endothelial cells, and is highly up-regulated in many disease situations. While TLR4 is originally postulated to recognize only exogenous pathogens, it is now increasingly documented to respond to endogenous molecules, such as CRP, mm-LDL, and its active components, polyoxygenated cholesteryl ester hydroperoxides.

In this study, we observed a time-dependent increase in *TLR4* mRNA and protein expression in THP-1 macrophages during foam cells formation, and ox-LDL-induced up-regulation of TLR4 via a mechanism involving mTOR

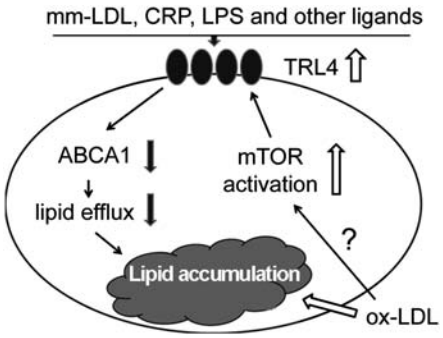

Figure 6 A schematic illustration of the role of mTOR activation in the control of TLR4 protein and lipid accumulation in THP-1 macrophage

signal pathway activation. The activation of mTOR is regulated by phosphorylation at Ser-2448 or autophosphorylation at Ser-2481 [35,36]. In present study, ox-LDL appeared to stimulate mTOR signaling by inducing a rapid and sustained high-level phosphorylation of mTOR at Ser-2448, but not Ser-2481 (data not shown). The activation of mTOR was also confirmed using western blot of the phosphorylation of p70S6K, a major direct downstream target of activated mTOR. The co-incubation of THP-1 macrophage with rapamycin inhibited mTOR-Ser2448 phosphorylation and significantly attenuated *TLR4* mRNA and protein level. The inhibition of mTOR, rictor or raptor protein expression with siRNA significantly reduced TLR4 level. These results indicated that up-regulated expression of *TLR4* mRNA and protein level in THP-1 macrophage was mTOR signal pathway dependent, meanwhile mTOR complex, mTOR-rictor, and mTOR-raptor contributed to the enhanced expression of *TLR4*.

ABCA1 is a key membrane-associated protein involved in reverse cholesterol transport and is usually atheroprotective [37,38]; its down-regulation may contribute to a propensity toward atherogenesis as a result of TLR4 activation. To further determine the functional effect of inhibition of *TLR4* expression by blocking mTOR pathway activation, the change of ABCA1 expression was detected. As results showed that inhibition of mTOR activation with rapamycin enhanced ABCA1 expression, more importantly, promoted cholesterol efflux from THP-1 macrophage incubated with ox-LDL and TLR4 ligand lipid A.

Recently, it was reported that mm-LDL (an early form of oxidized LDL) induced TLR4 activation recruited Syk to a TLR4 signaling complex and stimulated activation of signaling components of cytoskeleton regulation, leading to dramatic actin-dependent membrane ruffling. In addition, mm-LDL induced TLR4-independent activation of PI3K, which in combination with Rho activity facilitates ruffles closure into endosomes [39]. All these TRL4 mediating cellular events result in macropinocytosis and also facilitate macrophage foam cell formation.

Now, it is well established that TLR4 plays a very important role in macrophage foam cells formation through multiple mechanisms. Regulation of TLR4 level in macrophage is crucial for the prevention of foam cells formation and the atherosclerosis plaque development. We think these results can provide more information concerning the mechanisms of macrophage foam cells formation, and are beneficial for paving the way for development of therapeutic tools based on novel targets. It is not clear, however, how the upstream signaling pathways regulate mTOR activation in macrophage during foam cells formation. Further studies are needed to clarify the underlying mechanisms.

In summary, our data demonstrated that *TLR4* mRNA and protein level was up-regulated in THP-1 macrophage by a mechanism dependent on mTOR signal pathway activation (**Fig. 6**), blocking mTOR activity reduced *TLR4* expression, and reversed the down-regulation of ABCA1 and cholesterol efflux from THP-1 macrophage. Ox-LDL-induced mTOR activation might play an important role in up-regulating *TLR4* expression during macrophage foam cells formation.

Funding

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