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*Original Article*

# Lipoxin A4 promotes ABCA1 expression and cholesterol efflux through the LXRα signaling pathway in THP-1 macrophage-derived foam cells

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**Abstract:** Adenosine triphosphate-binding cassette transporter A1 (ABCA1) is a crucial cholesterol transporter and plays a central role in the high density lipoproteins (HDL) cholesterol metabolism and lipid clearance from the foam cell. Lipoxin A4 (LXA4) is an endogenous lipid mediator that requires cell-cell interaction or cell-platelet interaction for its synthesis. The roles of LXA4 on inflammatory responses are well described, while its effects on mediating ABCA1 and underlying mechanisms remain unclear. In this study, we showed that LXA4 significantly increases expression of ABCA1 and LXRα in a dose-dependent manner in THP-1 macrophage-derived foam cells. Cellular cholesterol content was decreased while cholesterol efflux was increased by LXA4 treatment. However, after short interfering RNA of LXRα, the effects of LXA4 on ABCA1 expression and cholesterol metabolism were significantly abolished. These results provide evidence that LXA4 increases ABCA1 expression and promotes cholesterol efflux through LXRα pathway in THP-1 macrophage-derived foam cells.

**Keywords:** LXA4, LXRα, ABCA1, cholesterol efflux, THP-1 macrophage-derived foam cells

## Introduction

Cardiovascular diseases are a major health problem and the leading cause of death in the Western societies [1]. Atherosclerosis, the major form of cardiovascular disease, is a disorder of lipid metabolism [2]. It is initiated when circulating monocytes adhere to the vascular wall, enter the intima and engulf lipoprotein particles to differentiate into large macrophage-derived foam cells [3]. To prevent atherosclerosis progression, HDL facilitates the removal of excess free cholesterol from vascular cells. Reverse cholesterol transport (RCT), a process by which excess cholesterol is transported from macrophages to the liver for ultimate fecal excretion, is a mechanism critical to the anti-atherogenic property of HDL [4]. During this process, ABCA1 plays a central role in producing HDL levels through the efflux of free cholesterol to lipid-poor apoA1 [5]. ABCA1 is a member of a large family of transporters to transport various substrates including lipids, ions and metabolites [6]. Mutation in ABCA1 causes Tangier disease, resulting in a loss of ABCA1 function and near absent HDL levels [7]. ABCA1 knockout mice, similar to the patients with TD, show nearly absent plasma HDL levels [7, 8]. In contrast, overexpressing ABCA1 in mice increases plasma HDL levels by promoting cholesterol efflux from macrophages [9]. Thus, ABCA1 has become a promising therapeutic target for the treatment of atherosclerosis.

LXRs are members of the nuclear receptor superfamily of ligand-activated transcription factors and exist as two isoforms, LXRα and LXRβ [10]. LXRs promote cholesterol efflux by stimulating the mobilization of cholesterol from intracellular pools to the plasma membrane [11, 12]. LXRα deficient mice fed a high-cholesterol diet developed massive hepatic accumulation of cholesterol, whereas wild-type mice were highly resistant to cholesterol feeding

[13]. Mesangial cells treating with a specific LXRα agonist TO-901317 significantly enhance the promoter activity of ABCA1 to promote basal and apoA1-mediated cholesterol efflux [14]. In addition, Qiu *et al.* showed that atorvastatin inhibits ABCA1 expression through an LXRα-dependent pathway in a dose-dependent manner [15]. Thus, The LXRα-ABCA1 pathway represents a powerful means to stimulate cholesterol efflux from macrophages and therefore strongly influence the progression of atherosclerotic plaque development.

Lipoxins are generated in humans from arachidonic acid via LOX enzymes and comprise two distinct regioisomers, lipoxin A4 (LXA4) and LXB4 [16]. Lipoxins are the first class of lipid mediators and function as "braking signals" in inflammation [17]. Serhan *et al.* showed that administration of LXA4 can reduce polymorphonuclear leukocytes (PMN) transmigration, adhesion receptor expression, pro-inflammatory cytokine generation and excessive PMN infiltration into inflamed tissues [18]. Patients with cystic fibrosis have a serious defect in lipoxin-mediated anti-inflammatory activity [19]. In addition, LXA4 can reduce inflammatory responses induced by lipopolysacchride and significantly inhibit the apoptosis stimulated by staurosporine in macrophages [20, 21]. Ho *et al.* reported that lipoxins may have the potential to prevent atherosclerosis by confering a protective phenotypic switch in VSMCs [22].

However, correlation between Lipoxin A4 administration and expression of ABCA1 in THP-1 macrophage-derived foam cells remains unclear. Moreover, the role of the LXRα pathway in this process has not yet been explored. In this study, we demonstrated that LXA4 could markedly up-regulate ABCA1 expression and promote cholesterol efflux through enhancing LXRα expression in THP-1 macrophage-derived foam cells.

## Materials and methods

### *Reagents*

Lipoxin A4 was purchased from Sigma Chemical Company (St. Louis, MO, USA). The PrimeScript RT Reagent kit (Perfect Real Time; catalog no. DRR037A) and the SYBR Premix Ex TaqTM II kit (Tli RNaseH Plus; catalog no. DRR820A) were obtained from TaKaRa Bio, Inc. (Shiga, Japan). All other chemicals were of pharmaceutical grade and purchased from commercial suppliers.

### *Preparation of Ox-LDL*

Native LDL was purchased from Sigma-Aldrich (St.Louis, MO, USA). Ox-LDL was prepared as previously described [23].

### *Cell culture*

THP-1 macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute (RPIM) 1640 medium supplemented with 10% fetal calf serum under standard culture conditions (5% $CO_2$, 37°C). The cells were differentiated into macrophages by the addition of 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 72 h. Macrophages were transformed into foam cells by incubation with 50 μg/ml of Ox-LDL in serum-free RPMI 1640 medium containing 0.3% bovine serum albumin (BSA) for 48 h. Cells were seeded in 6-or 12-well plates or 60-mm dishes and grown to 60%-80% confluence before use.

### *RNA isolation and real-time quantitative polymerase chain reaction analysis*

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. RT-qPCR with SYBR Green detection chemistry was performed on an ABI7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Melt curve analysis determined that all RT-PCR products produced a single DNA duplex. All samples were measured in triplicate, and the mean value was considered for comparative analysis. Quantitative measurements were determined using the $^{\Delta\Delta}$Ct method and glyceraldehyde 3-phosphate dehydrogenase expression was used as the internal control.

### *Western blot analysis*

Proteins were extracted from cultured cells using RIPA buffer (Biocolor Ltd., Belfast, Northern Ireland, UK), quantified using the BCA protein assay kit (KeyGen Biotechnologies, Nanjing, China), and then subjected to Western blot analysis (10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 50 μg protein per lane) using rabbit polyclonal anti-ABCA1 (Novus Biologicals, Littleton, CO, USA), using

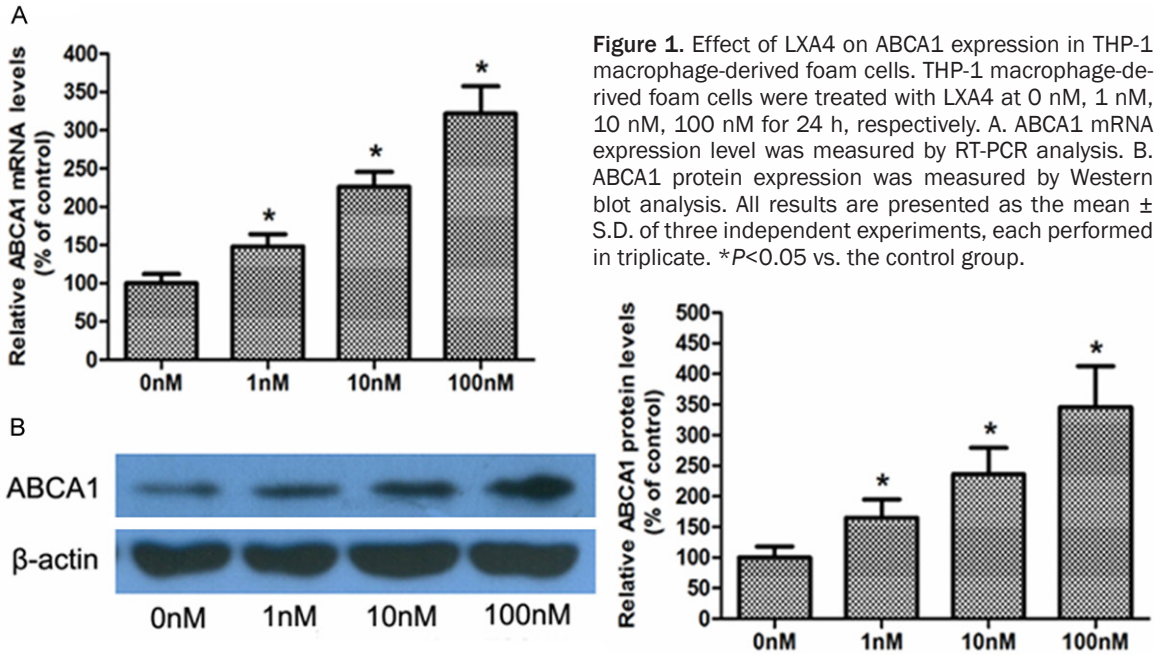


**Figure 1.** Effect of LXA4 on ABCA1 expression in THP-1 macrophage-derived foam cells. THP-1 macrophage-derived foam cells were treated with LXA4 at 0 nM, 1 nM, 10 nM, 100 nM for 24 h, respectively. A. ABCA1 mRNA expression level was measured by RT-PCR analysis. B. ABCA1 protein expression was measured by Western blot analysis. All results are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. *$P$<0.05 vs. the control group.

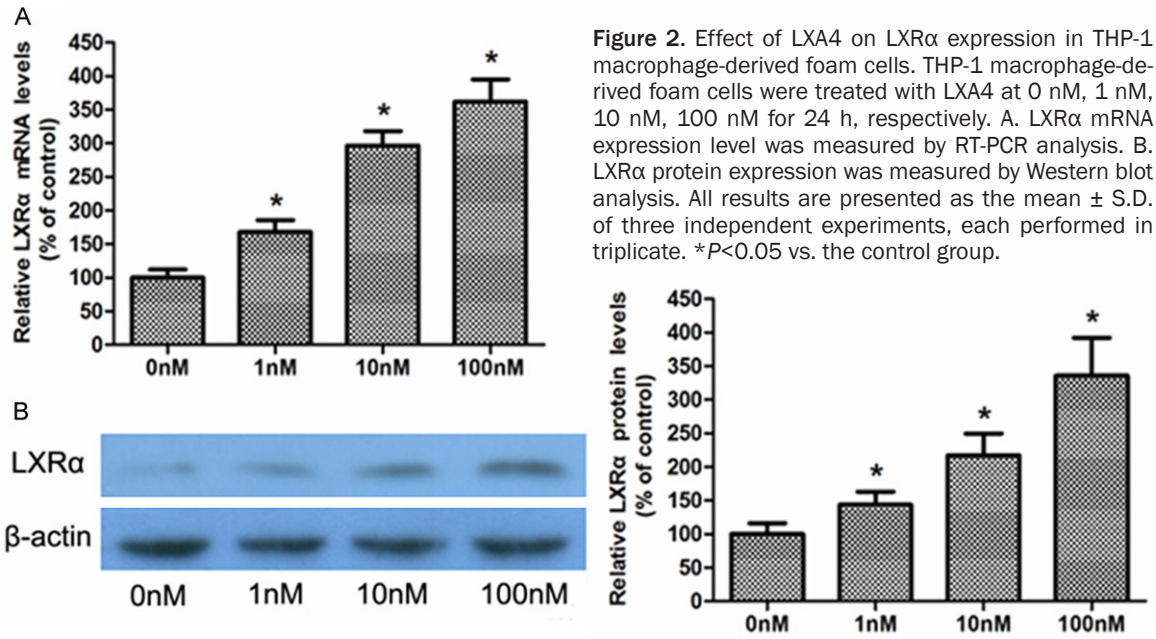


**Figure 2.** Effect of LXA4 on LXRα expression in THP-1 macrophage-derived foam cells. THP-1 macrophage-derived foam cells were treated with LXA4 at 0 nM, 1 nM, 10 nM, 100 nM for 24 h, respectively. A. LXRα mRNA expression level was measured by RT-PCR analysis. B. LXRα protein expression was measured by Western blot analysis. All results are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. *$P$<0.05 vs. the control group.

rabbit polyclonal anti-LXRα (Proteintech Group, Inc., Chicago, IL, USA). The proteins were visualized using a chemiluminescence method (ECL Plus Western Blot Detection System; Amerisham Biosciences, Foster City, CA, USA).

*Transfection with small interfering RNA*

The small interfering RNAs (siRNAs) against LXRα and an irrelevant 21-nucleotide siRNA, as a negative control, were purchased from Ribo Biotech Co.Ltd. (Guangzhou, Guangdong, China). THP-1 macrophage-derived foam cells (2 × $10^6$ cells/well) were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) for 48 h according to the manufacturer's instructions. After 48 h of transfection, real-time RT-PCR and western blotting were performed.

*Cellular cholesterol efflux experiments*

Cells were labeled with 0.2 μ Ci/ml [$^3$H] cholesterol for 72 h. Then cells were washed with PBS

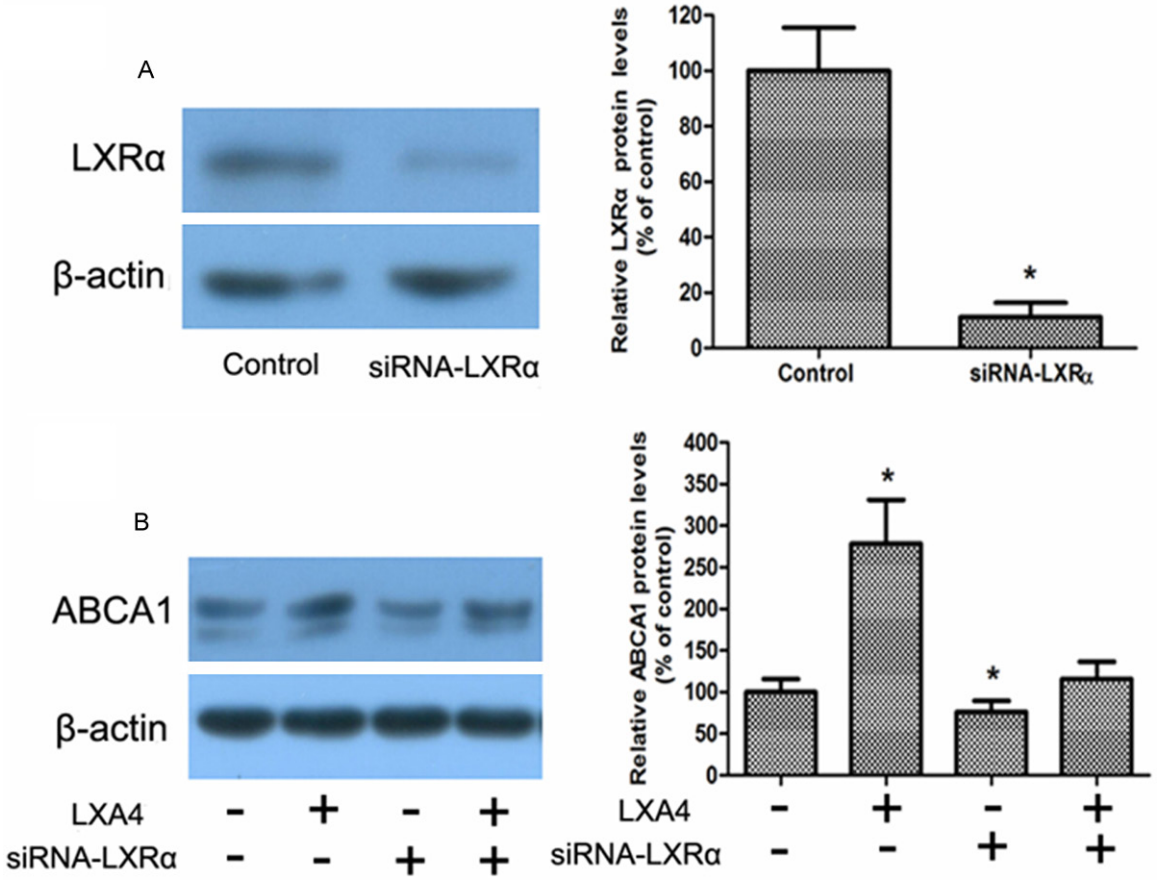


**Figure 3.** LXRα is involved in the regulation of ABCA1 induced by LXA4 in THP-1 macrophage-derived foam cells. A. THP-1 macrophage-derived foam cells were transfected with negative control or LXRα siRNA. And then LXRα protein expression was measured by Western blot analysis. B. Cells were transfected with control or LXRα siRNA, incubating with or without LXA4 (100 nM) for 24 h. Then ABCA1 protein expression was measured by western blot analysis. All results are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. **P*<0.05 vs. the control group.

and incubated with RPMI 1640 medium containing 0.1% (wt/vol) BSA overnight to allow equilibration of [$^3$H] cholesterol in all cellular pools. Cells were washed with PBS and incubated in 2 ml efflux medium containing RPMI 1640 and 0.1% BSA with or without 25 μg/ml of human plasma apoA1 for 12 h. Medium and cell-associated [$^3$H] cholesterol were then measured by liquid scintillation counting. Percent efflux was calculated using the following equation: [total media counts/(total cellular counts+total media counts)] × 100%.

*High-performance liquid chromatography analysis of cellular cholesterol levels*

High-performance liquid chromatography (HPLC) analysis was conducted as described previously [24]. Sterol analyses were performed using a HPLC system (model 2790, controlled with Empower Pro software; Waters Corp, Milford, MA, USA). Data were analyzed with Total Chrom software from PerkinElmer.

*Statistical analyses*

Data are expressed as means ± standard deviations (S.D.). Results were analyzed by one-way analysis of variance followed by the Student's *t*-test, using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical significance was obtained when *P* values were less than 0.05.

## Results

*LXA4 up-regulates ABCA1 expression in THP-1 macrophage-derived foam cells*

ABCA1 is a crucial cholesterol transporter, which is widely expressed and is abundant in macrophages. Increasing ABCA1 activity can

**Table 1.** Effect of LXA4 and LXRα on cholesterol content in THP-1 macrophage-derived foam cells

| | TC (μg/mg cell protein) | FC (μg/mg cell protein) | CE (μg/mg cell protein) | CE/TC (%) |
|---|---|---|---|---|
| Control | 518±21 | 203±13 | 315±19 | 60.8 |
| LXA4 | 339±12* | 142±10* | 197±17* | 58.1 |
| LXRα-siRNA | 569±25 | 218±16 | 351±23 | 61.7 |
| LXA4+LXRα-siRNA | 515±21 | 204±15 | 311±19 | 60.3 |

THP-1 cells were differentiated for 72 h with 100 nM PMA and then macrophages were transformed into foam cells by incubation in the presence of 50 μg/ml of Ox-LDL for 48 h. THP-1 macrophage-derived foam cells were divided into four groups as indicated. Cellular TC, FC, and CE were determined by HPLC. The results are expressed as mean ± S.D. from three independent experiments, each performed in triplicate. *$P$<0.05 vs. the control group.

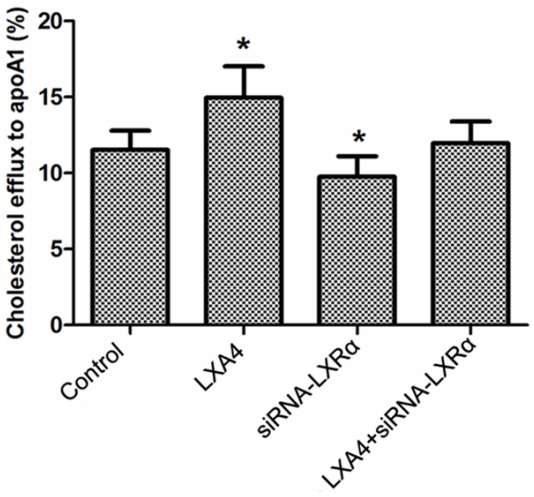


**Figure 4.** LXA4 contributes to cholesterol efflux in THP-1 macrophage-derived foam cells. THP-1 macrophage-derived foam cells were transfected with control or LXRα siRNA, incubating with or without LXA4 (100 nM) for 24 h. ApoA1-specific cholesterol efflux was analyzed by liquid scintillation counting assays as shown above. All results are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. *$P$<0.05 vs. the control group.

elevate plasma HDL levels. The effect of LXA4 on ABCA1 remains unclear. Thus, we first examined the effect of LXA4 on ABCA1 expression in THP-1 macrophage-derived foam cells by real-time quantitative PCR and Western blot assays. As shown, LXA4 obviously increased ABCA1 mRNA (**Figure 1A**) and protein expression (**Figure 1B**) in a dose-dependent manner.

*LXRα is enhanced by LXA4 in THP-1 macrophage-derived foam cells*

LXRα is an important regulator of cholesterol, free fatty acid, and glucose metabolism [25]. Our group also suggested that LXRα plays a critical role in cholesterol metabolism and homeostasis [26] Moreover, a specific LXRα agonist TO-901317 significantly enhances the promoter activity of ABCA1 in Mesangial cells [14]. And here we showed LXA4 can increase ABCA1 expression. Thus we next explored whether LXRα expression can be affected by LXA4 in THP-1 macrophage-derived foam cells by real-time quantitative PCR and western blot analysis. As shown, LXA4 markedly increased LXRα expression at both transcriptional levels (**Figure 2A**) and translational levels (**Figure 2B**) in a dose-dependent manner.

*LXRα is involved in LXA4-induced ABCA1 increase in THP-1 macrophage-derived foam cells*

The previous study has proved that LXR agonists stimulate ABCA1 gene expression in human macrophages [27]. Moreover, our group previously reported that Dihydrocapsaicin could increase expression levels of ABCA1 in macrophages through the LXRα pathway in THP-1 macrophage-derived foam cells [28]. Furthermore, here we showed that LXA4 could increase ABCA1 and LXRα expression in THP-1 macrophage-derived foam cells. Thus we speculated that LXA4 may upregulate ABCA1 expression through the LXRα pathway in THP-1 macrophage-derived foam cells. We then examined the effect of LXRα siRNA on regulation of ABCA1 with and without LXA4 treatment. As shown in **Figure 3A**, in comparison to the control siRNA, treatment with siRNA targeting LXRα decreased LXRα protein expression by 88% in THP-1 macrophage-derived foam cells. After LXRα siRNA treatment, the basal expression of ABCA1 is decreased (**Figure 3B**), which indicated LXRα can mediate ABCA1 expression. In addition, the up-regulation of ABCA1 expression by LXA4 treatment was markedly reversed by treating with LXRα siRNA (**Figure 3B**).

*LXA4 contributes to cellular cholesterol content and cholesterol efflux*

ABCA1 promotes free cholesterol efflux from macrophages into apoA1 to form HDL, which is central to prevent atherosclerosis progression. And here we indicated that expression levels of

ABCA1 could be increased by LXA4 and completely abolished by siRNA-targeted silencing of LXRα. Therefore, we next examined the effect of LXA4 on celluar cholesterol content and apoA1-specific cholesterol efflux and whether the LXRα pathway is involved in this process in THP-1 macrophage-derived foam cells. As shown, cellular cholesterol content (**Table 1**) was decreased while cholesterol efflux (**Figure 4**) was increased when cells were treated with LXA4. After LXRα siRNA treatment, inhibition of cellular cholesterol content (**Table 1**) and promotion of cholesterol efflux (**Figure 4**) by LXA4 treatment were markedly abolished.

## Discussion

Cholesterol homeostasis in macrophages is of critical importance because these cells have functional significance for the development of atherosclerotic lesions [29]. Cholesterol efflux from macrophages is the first and potentially most critical step in macrophage RCT, a process by which excess cholesterol is transported to the liver for excretion. Accordingly, the macrophage ABCA1 has been identified as a key regulator for this process [4]. In addition, recent studies indicated that peripheral vascular disease patients have a deficiency to generate 15-epi-LXA4 [22], which indicated that LXA4 may be associated with atherosclerosis protective effects. Here, we provide evidence that LXA4 increases the ABCA1 expression through induction of LXRα signaling pathway in THP-1 macrophage-derived foam cells.

Lipoxins are arachidonic acid metabolites formed during inflammation via transcellular metabolic traffic. The effects of LXA4 with potent anti-inflammatory actions are well documented [20, 30]. To the best of our knowledge, there is less concern about the impact of LXA4 on cholesterol efflux and potential mechanisms. Cellular cholesterol content in macrophages is partly determined by efflux of cholesterol. ABCA1 promotes free cholesterol efflux from macrophages into apoA1 to form HDL. Here we examined the effect of LXA4 on cholesterol content and cholesterol efflux in THP-1 macrophage-derived foam cells by HPLC and liquid scintillation counting assays, respectively. The results showed that LXA4-treated macrophages exhibited a decrease in cellular cholesterol content and a significant increase in cholesterol efflux, which is consistent with an increase expression of ABCA1 induced by treating with LXA4. Our observation reinforce the idea that LXA4 may play a critical role in the cholesterol homeostasis and keep consistent with the reported anti-atherogenic property of LXA4.

LXRα is an important regulator of cholesterol, free fatty acid, and glucose metabolism. Results from Wong *et al.* and our previous study proved that LXRα pathway is necessary for increase of ABCA1 [28, 31]. It indicates that elevating LXRα-ABCA1 pathway would probably inhibit the formation of foam cells and the development of atherosclerosis. Here our data showed that the basal expression of ABCA1 is decreased after LXRα siRNA treatment. Moreover, the up-regulation of ABCA1 expression and promotion of cholesterol efflux by LXA4 administration was markedly reversed by treating with LXRα siRNA. Our results proved that LXRα-ABCA1 is involved in LXA4-induced cholesterol efflux increase in THP-1 macrophage-derived foam cells.

A series of studies showed that lipoxin A4 was associated with cardiovascular protective effects, including inhibiting inflammatory responses in macrophages and confering a protective phenotypic switch in VSMCs [20, 22]. Moreover, LXA4 increases plasma nitric oxide (NO), which is correlated with a reduction in inflammation [32] and stimulates the endothelial production of prostacyclin (PGI2), which is anti-thrombotic mediator [33]. In this study we addressed the missing link between LXA4 treatment and increasing cholesterol efflux in THP-1 macrophage-derived foam cells by providing evidence that LXA4 administration was positively associated with expression of ABCA1, possibly by LXRα signaling pathway. These observations provide a possible explanation for a new mechanism of LXA4 on promoting cholesterol efflux in macrophages.

In summary, our studies illustrate that LXA4 promotes ABCA1 expression and cholesterol efflux while decreasing cellular cholesterol content in a LXRα-dependent manner in THP-1 macrophage-derived foam cells. These findings indicate that LXA4 is a critical molecular for cholesterol efflux and potentially is an effective strategy for atherosclerosis therapy.

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## Disclosure of conflict of interest

None.

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