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## Oxidized LDL upregulated ATP binding cassette transporter-1 in THP-1 macrophages<sup>1</sup>

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**KEY WORDS** ATP-binding cassette transporters; apolipoprotein A-I; hydroxycholesterols; LDL lipoproteins; oxidation-reduction; macrophages

### ABSTRACT

**AIM:** To study the effect of oxidized low density lipoprotein (ox-LDL) on ATP binding cassette transporter A1 (ABCA1) in THP-1 macrophages. **METHODS:** After exposing the cultured THP-1 macrophages to ox-LDL for different periods, cholesterol efflux was determined by FJ-2107P type liquid scintillator. ABCA1 mRNA and protein level were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot, respectively. The cholesterol level in THP-1 macrophage foam cells was detected by high performance liquid chromatography. **RESULTS:** ox-LDL elevated ABCA1 in both protein and mRNA levels and increased apolipoprotein (apo) A-I-mediated cholesterol efflux in a time- and dose-dependent manner. 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid did significantly increase cholesterol efflux in THP-1 macrophage foam cells ( $P < 0.05$ ), respectively. Both of them further promoted cholesterol efflux ( $P < 0.01$ ). As expected, liver X receptor (LXR) agonist decreased content of esterified cholesterol in the macrophage foam cells compared with control, whereas only a slight decrease of free cholesterol was observed. LXR activity was slightly increased by oxidized LDL by 12 % at 12 h compared with 6 h. However, LXR activity was increased about 1.8 times at 24 h, and oxidized LDL further increased LXR activity by about 2.6 times at 48 h. **CONCLUSION:** ABCA1 gene expression was markedly increased in cholesterol-loaded cells as a result of activation of LXR/RXR. ABCA1 plays an important role in the homeostasis of cholesterol in the macrophages.

### INTRODUCTION

Oxidized lipid signaling in macrophages is central to the pathogenesis of atherosclerosis<sup>[1]</sup>. Exposure of

macrophages and other vascular cells to oxidized low-density lipoprotein (ox-LDL) leads to complex changes in gene expression that are collectively thought to influence the development of the atherosclerotic lesion<sup>[2]</sup>. Using two-dimensional gel electrophoresis, the overall protein map in U937 control cells and U937 foam cells was obtained. Compared with U937 cells, 37 spots changed in the foam cells, among which the expression levels in 28 spots increased and those in 9 spots decreased<sup>[3]</sup>. Mounting evidence suggest that nuclear receptor signaling pathways mediate many of the effects of oxidized lipids on cellular gene expression<sup>[4]</sup>.

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High-density lipoproteins (HDLs) play a role in transporting cholesterol from peripheral tissues to the liver. Two hallmarks of cardiovascular disease are the presence of cholesterol-laden macrophages in the artery wall and reduced plasma HDL levels. ATP binding cassette transporter A1 (ABCA1) mediates the cholesterol efflux from cells into the HDL. Mutations in ABCA1 cause Tangier disease, a severe HDL deficiency syndrome characterized by accumulation of cholesterol in tissue macrophages and prevalent atherosclerosis<sup>[5]</sup>.

Intracellular cholesterol homeostasis is exquisitely regulated and depends on the balance between cholesterol synthesis, influx, and degradation; cholesterol ester formation; and translocation of cholesterol to the plasma membrane for efflux<sup>[6,7]</sup>. The free cholesterol (FC) content of cells cultured in normal media containing lipoprotein appears to be determined mainly by a balance between the uptake of FC and the efflux of FC. This response is directly linked to the function of ABCA1. In this study we investigated the expression, regulation, and role of ABCA1 in the efflux of cholesterol in THP-1 macrophages.

## MATERIALS AND METHODS

**Cell culture** The human monocytes line THP-1 was purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences. THP-1 cells were maintained in RPMI-1640 containing 10 % fetal bovine serum in a humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air. Differentiation of THP-1 monocytes into macrophages was induced by culturing the cells at a density of  $1.0 \times 10^6$  cells/well in a six-well plate in the presence of phorbol 12-myristate 13-acetate (PMA) 160 nmol/L for 24 h.

**LDL isolation and oxidization**<sup>[8]</sup> LDL was isolated from nonfrozen human plasma. The remaining sample present in the lower section after isolation of VLDL/IDL was divided equally between two new Quick-Seal tubes. The volume and solution density in each tube were adjusted to 35 mL and 1.063 kg/L with 38 % NaBr, NaCl 0.15 mol/L, and a digital balance, respectively. Upon being sealed, each tube was centrifuged at 129 400×g and 8 °C for 20 h. About 5 μL sample was isolated in the upper section. The isolated LDL was oxidized with CuSO<sub>4</sub> 10 μmol/L for 18 h at 37 °C. Oxidation of LDL was measured by the thiobarbituric acid-reactive substances assay.

**Assessment of cholesterol efflux**<sup>[9]</sup> THP-1 mac-

rophages were labeled by incubation in growth medium containing [<sup>3</sup>H]cholesterol 7.4 MBq/L (0.2 mCi/L) for 48 h and were cholesterol-loaded using various concentrations of ox-LDL. In some experiments, cells were simultaneously labeled with [<sup>3</sup>H]cholesterol and cholesterol-loaded using ox-LDL (50 mg/L) for various periods. After being labeled with [<sup>3</sup>H]cholesterol, cells were washed and incubated for an additional 24 h in serum-free media containing bovine serum albumin 2 g/L to allow for equilibration of [<sup>3</sup>H]cholesterol with intracellular cholesterol. Cholesterol efflux was initiated by adding the indicated amount of apoA-I, usually 10 mg/L, in serum-free medium. After 12 h, media were harvested, and cells were dissolved in HEPES 1 mmol/L, pH 7.5 containing 0.5 % Triton X-100. Media were briefly centrifuged to remove nonadherent cells, and then aliquots of both supernatants and dissolved cells were subjected to FJ-2107P type liquid scintillator to determine radioactivity. Cholesterol efflux is expressed as a percentage, calculated as [<sup>3</sup>H]cholesterol in medium / ([<sup>3</sup>H]cholesterol in medium + [<sup>3</sup>H]cholesterol in cells) × 100 %.

**High performance liquid chromatography (HPLC)**<sup>[10]</sup> Briefly, cells were washed three times with PBS and the appropriate volume (usually 1 mL) of 0.5 % sodium chloride was added to each well to obtain a solution containing cellular protein 0.05 to 0.2 g/L. Cells were sonified for 2 min using an ultrasonic processor. Two 100-μL aliquots of the solution (5-20 μg cell protein) were taken for the measurement of free and total cholesterol and the rest of the sample could be used to measure protein with BCA kit. Free cholesterol was dissolved in isopropanol (1 g/L) and stored at -20 °C as stock standard solutions. Calibration curves were obtained by diluting these standards in the same medium ranging from 0 to 40 mg/L. Then, 0.1 mL of each sample (either standard or cell solution containing 0.005 to 0.02 mg cell protein) was supplemented with 10 μL of a reaction mixture containing (MgCl<sub>2</sub> 500 mmol/L, Tris buffer 500 mmol/L, pH 7.4, dithiothreitol 10 mmol/L, and 5 % sodium chloride). Enzymes (either cholesterol oxidase 0.4 kU/L for free cholesterol determination or cholesterol oxidase supplemented with cholesterol esterase 0.4 kU/L for total cholesterol measurement) were added to each tube in 10 μL of 0.5 % sodium chloride. The tubes were incubated at 37 °C for 30 min and then 100 μL of methanol-ethanol 1:1 was added to stop the reaction. Samples were kept cold for 30 min to allow protein precipitation and subsequently centrifuged for 10 min. Fifty microliters of

the supernatant was injected in the HPLC system. Samples were analyzed using a System Chromatographer (PerkinElmer Inc) consisting of a PerkinElmer series 200 vacuum degasser, a PerkinElmer series 200 pump, a PerkinElmer series 600 LINK, and a PerkinElmer series 200 UV/vis detector, equipped with a Discovery C-18 HPLC column (25 cm×4.6 mm, 5 μm internal diameter) (Supelco Inc). Isopropanol: *n*-heptane: acetonitrile (35:13:52) was used as eluent at a flow rate of 1 mL/min for 8 min. Detection was achieved by monitoring absorbance at 216 nm in a PerkinElmer series 200 UV/vis detector. Data were analyzed with TotalChrom software from PerkinElmer.

**Reverse transcription-polymerase chain reaction (RT-PCR)** Total RNA was isolated using Trizol reagent. Total RNA content was determined by measuring the optical absorbance ratio at 260/280 nm after the sample was dissolved in diethylpyrocarbonate-treated water. RNA was then stored at -70 °C before two-step RT-PCR protocol using 2 μg of total RNA. RNA was treated with DNase I, reverse transcribed, and amplified for ABCA1, LXR, and GAPDH using PCR enzymes and reagents according to the following conditions: 10 min 95 °C, then 34 cycles of 1 min 95 °C, 1 min 60 °C, and 1 min 72 °C, and then a final annealing step at 72 °C for 10 min. All thermocycling was performed on a Perkin Elmer Gene Amp PCR System 5700. PCR amplification was performed using ABCA1 (306 bp) primers (forward: 5'-GCTGCTGAAGCCAGGGCATGGG-3', and reverse: 5'-GTGGGGCAGTGGCCATACTCC-3') or LXRα (965 bp) primers (forward: 5'-GGGGCCA-GCCCCCAAATGCTG-3' and reverse: 5'-GCATCCG-TGGGAACATCAGTCG-3') and GAPDH (697 bp) primers (forward: 5'-TCACCATCTTCCAGGAGCGAG-3', reverse: 5'-TGTCGCTGTTGAAGTCAGAG-3'). PCR products were separated on 1.5 % agarose gel containing ethidium bromide. Densitometric quantitation of the intensity of GAPDH and ABCA1 or LXRα products was determined using the labwords analysis software. The relative abundance of ABCA1 or LXRα was expressed as the ratio of ABCA1 or LXRα to GAPDH product.

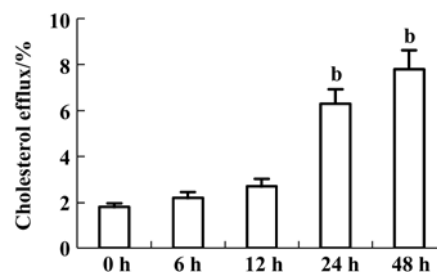
**Western blotting** Cells were lysed by gentle trituration in HEPES 20 mmol/L, KCl 5 mmol/L, MgCl<sub>2</sub> 5 mmol/L, 0.5 % (v/v) Triton X-100, and complete protease inhibitor. Cell debris was removed by centrifugation at 4470×g at 4 °C for 4 min. Tissues were homogenized in an ice-cold buffer containing HEPES 20 mmol/L, KCl 5 mmol/L, MgCl<sub>2</sub> 5 mmol/L, 0.5 % (v/v) Triton X-100, and complete protease inhibitor. Homogenates

were sonicated once for 10 s followed by centrifugation at 25750×g at 4 °C for 5 min. The protein concentration in cellular supernatants was determined by the BCA assay. Equal amounts of protein (typically 80 μg) were separated on 6.0 % SDS-PAGE gels and electrophoretically transferred to PVDF membrane. Membranes were probed with either ABCA1 antibody or LXRα antibody (Santa Cruz Biotechnology, Inc) for equal loading. Immunoreactivity was detected by ECL. Protein abundance was calculated by densitometry using labwords analysis software.

**Statistics** Quantitative data were expressed as mean±SD. Statistical significance of the data was evaluated by analysis of variance and *q* test. *P*<0.05 were considered significant. For nonquantitative data, results represent at least 3 independent experiments.

## RESULTS

**Effect of ox-LDL on cholesterol efflux** Ox-LDL 50 mg/L promoted cholesterol efflux in macrophages in a time-dependant manner. It increased cholesterol efflux in THP-1 macrophages at 24 h and 48 h (*P*<0.05), but did not promote cholesterol efflux at 6 h and 12 h (*P*>0.05, Fig 1).



**Fig 1. Oxidized LDL 50 mg/L increased cholesterol efflux in a time-dependent manner in the presence of apoA-I 10 mg/L. *n*=3 independent experiments and each performed in triplicate. Mean±SD. <sup>b</sup>*P*<0.05 vs 0 h.**

Oxidized LDL promotes cholesterol efflux in macrophages in a concentration-dependant manner (*P*<0.05, Fig 2).

**LXR/RXR agonist promoted cholesterol efflux in macrophage foam cells** Treatment with 22(*R*)-hydroxycholesterol 10 μmol/L and 9-*cis*-retinoic acid 10 μmol/L for 24 h increased cholesterol efflux in THP-1 macrophage foam cells (*P*<0.05). Cholesterol efflux was elevated further if treatment with both of them (*P*<0.01, Fig 3).

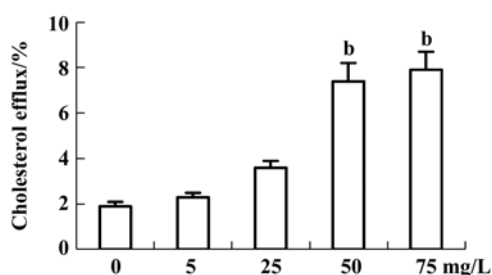


Fig 2. Effect of different concentrations of oxidized LDL on cholesterol efflux from THP-1 macrophages in the presence of apoA-I 10 mg/L. *n*=4 wells. Mean±SD. <sup>b</sup>*P*<0.05 vs 0 mg/L.

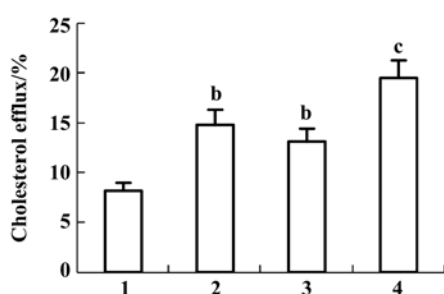


Fig 3. Effect of LXR/ RXR agonist on cholesterol efflux in THP-1 macrophage foam cells in the presence of apoA-I 10 mg/L. *n*=3 independent experiments. Mean±SD. (1) Control; (2) 22(*R*)-hydroxycholesterol 10 μmol/L; (3) 9-*cis*-retinoic acid 10 μmol/L; (4) 22(*R*)-hydroxycholesterol 10 μmol/L+9-*cis*-retinoic acid 10 μmol/L. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control.

**Effect of LXR/RXR agonist on cholesterol level in macrophage foam cells** 22(*R*)-hydroxycholesterol 10 μmol/L and 9-*cis*-retinoic acid 10 μmol/L decreased the content of cholesterol ester in macrophage foam cells compared with control, whereas only a slight decrease of free cholesterol was observed (Tab 1). Chromatography picture of cholesterol and cholesterol ester in THP-1 macrophage-derived foam cells was identified by HPLC (Fig 4).

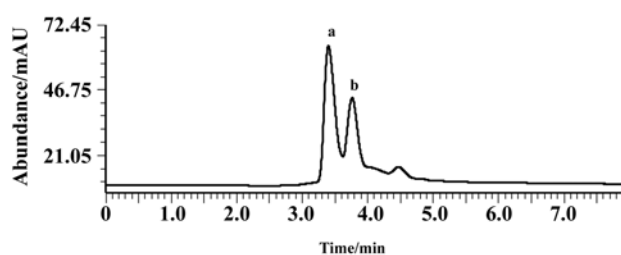


Fig 4. Chromatography of cholesterol and cholesterol ester in THP-1 macrophage-derived foam cells by high performance liquid chromatography. a: cholesterol peak; b: cholesterol ester peak.

**Effect of ox-LDL on ABCA1 mRNA and protein expression in THP-1 macrophages** ABCA1 mRNA began to increase after THP-1 macrophages were exposed to ox-LDL 50 mg/L for 12 h, and increased further at 24 h and 48 h (*P*<0.05, Fig 5).

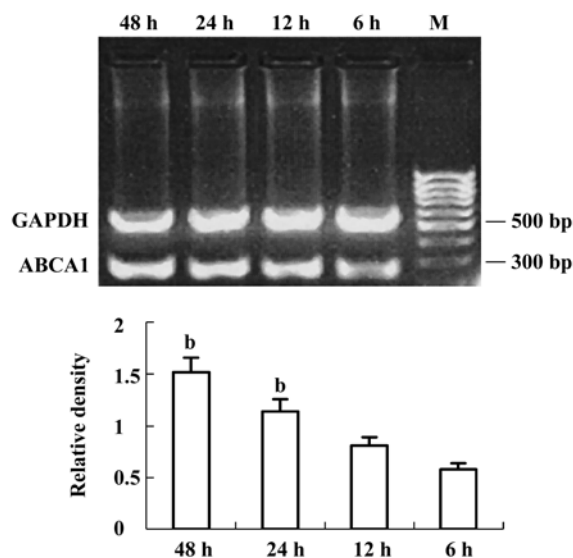


Fig 5. Effect of oxidized LDL 50 mg/L on ABCA1 mRNA level in THP-1 macrophages for various periods. *n*=3 independent experiments. Mean±SD. <sup>b</sup>*P*<0.05 vs 6 h.

Tab 1. The level of total, free and cholesterol ester (mg/g) in cells. *n*=4. Mean±SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs foam cell group.

Index	Foam cell group	22( <i>R</i> )-HCh group	9-CRA group	22( <i>R</i> )-HCh+ 9-CRA group
Total cholesterol (TC)	618±29	425±34 <sup>b</sup>	437±32 <sup>b</sup>	291±25 <sup>c</sup>
Free cholesterol (FC)	227±19	187±13 <sup>b</sup>	192±16 <sup>b</sup>	167±11 <sup>c</sup>
Cholesterol ester (CE)	391±27	238±21 <sup>b</sup>	245±22 <sup>b</sup>	124±8 <sup>c</sup>
CE/TC (%)	63.31	56.0	56.1	42.6

22(*R*)-HCh; 22(*R*)-hydroxycholesterol, 9-CRA: 9-*cis*-retinoic acid.

ABCA1 protein expression in THP-1 macrophages was up-regulated by ox-LDL in a time-dependent way (Fig 6).

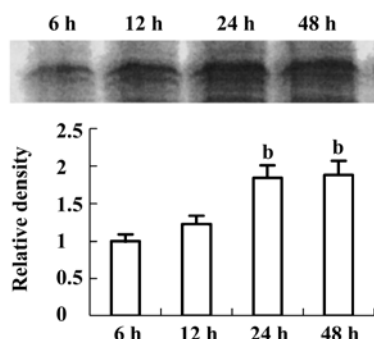


Fig 6. Effect of oxidized LDL 50 mg/L on ABCA1 protein level in THP-1 macrophages at various time points.  $n=3$  independent experiments. Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs 6 h.

**Ox-LDL activates the LXR in THP-1 macrophages** LXR activity was slightly increased by ox-LDL 50 mg/L by 12 % at 12 h compared with 6 h. However, LXR activity was increased 1.8 times at 24 h, and further increased about 2.6 times at 48 h (Fig 7).

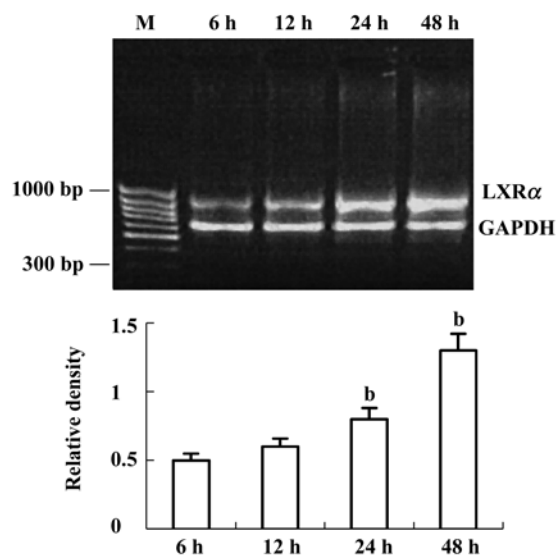


Fig 7. Effect of oxidized LDL 50 mg/L on LXR activation in THP-1 macrophages.  $n=3$  independent experiments. Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs 6 h.

## DISCUSSION

ABCA1 plays a major role in cholesterol homeostasis and HDL metabolism. ABCA1 mediates cellular cholesterol and phospholipid efflux to lipid-poor

apolipoproteins, and upregulation of ABCA1 activity is antiatherogenic. Hypercholesterolemia is a risk factor for the development of atherosclerosis. Both lipoproteins and the macrophages play important roles in the development of atherosclerosis. ABCA1 is expressed in a variety of human tissues such as placenta, liver, lung, adrenal glands, and fetal tissues<sup>[11]</sup> and various cell lines<sup>[12]</sup>, and within atherosclerotic tissues<sup>[13]</sup>. In the present study, we investigated ABCA1 expression, regulation, and role in cholesterol efflux in THP-1 macrophages. Our findings demonstrated that ABCA1 was expressed in THP-1 macrophages and elevated by ox-LDL; ox-LDL increased apoA-I-mediated cholesterol efflux in THP-1 macrophages; ox-LDL activated LXR activity. We concluded that ABCA1 was up-regulated by ox-LDL in THP-1 macrophages and played an important role in cholesterol trafficking in THP-1 macrophages. Such a finding may have broad implications for cholesterol homeostasis.

Our data demonstrated the presence of ABCA1 mRNA and its protein in THP-1 macrophages and ox-LDL up-regulated ABCA1 in THP-1 macrophages. Because cells have few LDL receptors, such as quiescent macrophages cultured in the presence of serum, cholesterol trafficking appears to be controlled by the mechanism of its efflux<sup>[7]</sup>. Cholesterol efflux responded to free cholesterol levels in cells. Thus, free cholesterol burdening by ox-LDL, which increases cholesterol content in the cells, may cause an adaptive response to maintain cholesterol homeostasis. We previously reported that incubation of THP-1 macrophages with ox-LDL increased cellular free cholesterol content and cholesterol ester level<sup>[14]</sup>. Elevated contents of cellular cholesterol may consequently upregulate ABCA1 expression, which in turn increases free cholesterol efflux in macrophages. Fielding's group<sup>[15]</sup> reported smooth muscle and endothelial cells *in vivo* were quiescent yet after they were exposed to high levels of lipoprotein lipids. Phospholipid and free cholesterol efflux maintain homeostasis. Smooth muscle cells expressed high levels of ABC-1 transporter mRNA. Umbilical vein and aortic endothelial cells expressed little ABC-1 mRNA, nor did these cells promote either phospholipid or free cholesterol efflux in response to apoA-I. When we performed cholesterol efflux assays under low-serum conditions (2 % FBS), ox-LDL pre-exposure increased free cholesterol efflux. Thus, other ABCA1-independent mechanisms may be also involved in intracellular cholesterol efflux<sup>[16]</sup>. Wang's group<sup>[17]</sup> reports that the dose

responses of the apoA-I effect on ABCA1 levels and on binding and cholesterol efflux indicated that the effects of apoA-I could be mediated either by increased binding or by cholesterol or phospholipid efflux. The caveolae and caveolin seem to be involved in cholesterol efflux to lipidated HDL<sup>[18,19]</sup>.

Because cholesterol homeostasis may have an essential role in macrophages, we examined ABCA1 expression and function in THP-1 macrophages. The ABCA1 mRNA and protein levels detected in THP-1 macrophages were increased markedly after exposure to ox-LDL in a time- and dose-dependent manner. Koldamova's group<sup>[20]</sup> reported that 22*R*-hydroxycholesterol and 9-*cis*-retinoic acid induced ATP-binding cassette transporter A1 expression and cholesterol efflux in brain cells. Our data demonstrated that LXR agonist 22(*R*)-hydroxycholesterol and RXR agonist 9-*cis*-retinoic acid promoted apoA-I-mediated cholesterol efflux in THP-1 macrophage foam cells. These ligands decreased the content of cholesterol ester in macrophage foam cells, whereas only a slight decrease of free cholesterol was observed. These effects of 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid may provide a novel strategy to increase cholesterol efflux and consequently reduce the cholesterol burden in the macrophage foam cells.

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