

# Curcumin affects ox-LDL-induced IL-6, TNF- $\alpha$ , MCP-1 secretion and cholesterol efflux in THP-1 cells by suppressing the TLR4/NF- $\kappa$ B/miR33a signaling pathway

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**Abstract.** The aim of the present study was to study the molecular mechanism of how curcumin decreases the formation of ox-LDL induced human monocyte macrophage foam cells, promotes the efflux of cholesterol and reduces the secretion of inflammatory cytokines. *In vitro* cultured THP-1 cells were induced to become macrophages using phorbol-12-myristate-13-acetate. The cells were then pre-treated with curcumin before inducing the foam cell model by addition of oxidized low-density lipoprotein (ox-LDL). Western blot assays were used to detect expression levels of toll-like receptor (TLR)4, nuclear factor  $\kappa$ B (NF- $\kappa$ B), NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ), phosphorylated-I $\kappa$ B $\alpha$  and ATP binding cassette transporter (ABC)A1. Reverse transcription-quantitative PCR was employed to examine mRNA levels of TLR4, microRNA (miR)33a and ABCA1. ELISAs were used to detect inflammatory factors, including tumor necrosis factor (TNF)- $\alpha$ , monocyte chemotactic protein (MCP)-1 and interleukin (IL)-6. ox-LDL successfully induced the foam cell model, promoted phosphorylation of I $\kappa$ B $\alpha$ , promoted nuclear translocation of NF- $\kappa$ B, promoted the expression of TLR4 and miR33a, and promoted the secretion of TNF- $\alpha$ , MCP-1 and IL-6. Additionally, ox-LDL reduced the expression of ABCA1 and cholesterol efflux. However, pretreatment with curcumin increased the expression of ABCA1 and cholesterol efflux and suppressed secretion of TNF- $\alpha$ , MCP-1 and IL-6. TLR4 antibodies, the NF- $\kappa$ B blocker, PDTC, and the

miR33a inhibitor also reduced the abnormal transformations induced by ox-LDL. Curcumin promoted cholesterol efflux by suppressing the TLR4/NF- $\kappa$ B/miR33a signaling pathway, and reduced the formation of foam cells and the secretion of inflammatory factors.

## Introduction

Cardiovascular disease is the leading cause of death in western countries (1). According to data from 2013, >17.3 million (or 31.5%) of all deaths worldwide every year (2-4). The American Heart Association estimated that >25 million people will die of cardiovascular disease every year by 2020 (5). Coronary atherosclerotic heart disease is a common cardiovascular disease, therefore, preventing the development of atherosclerosis (AS) will be a major task to help with the prevention of cardiovascular diseases.

AS is a chronic inflammatory disease (6). It has been confirmed by numerous studies that various cytokines play important roles in the progression of AS and plaque instability (7-9). Interleukin (IL)-6 is a cytokine that regulates the inflammatory response produced by leukocytes and other cells, and is also considered to be a biomarker of inflammation (8). Tumor necrosis factor (TNF)- $\alpha$  is considered to be an effective pro-inflammatory mediator, which promotes the expression of other inflammatory cytokines and adhesion molecules as well as increasing the apoptosis of vascular smooth muscle cells, thus promoting AS and plaque instability (9). Chemotactic factors are small molecule proteins that recruit leukocytes from circulation in the blood to inflammatory injury sites (10). In chronic inflammatory diseases such as AS, the binding of monocyte chemotactic protein (MCP)-1 and its receptor, C-C motif chemokine receptor-2, induces monocyte chemotaxis to the inflammatory site, leading to aggravation of the inflammatory response (11).

Toll-like receptors (TLRs) are pattern recognition receptors present on macrophage surfaces, which are extensively expressed on macrophages in lipid-rich atherosclerotic plaques in humans and mice and play an essential role in the host defense response (12). It has been shown by Zhou *et al* (13)

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that TLR2 and TLR4 are highly expressed in human umbilical vein endothelial cells and in the human acute monocytic leukemia cell line, THP-1 epidermal cells. The expression of TLR2 and TLR4 is induced by oxidized (ox)-low-density lipoprotein (LDL), and in TLR2 or TLR4 deficient cells, the formation of foam cells decreases significantly (14). Thus, TLR4 has the potential to enhance ox-LDL intake and/or impair the reverse transportation of cholesterol.

MicroRNAs (miRNAs), are single-stranded, non-coding nucleotides, 21-24 base pairs (bp) in length, which were first found in nematodes (15). miRNAs are involved in genomic expression and regulation by binding to the target site of the mRNA 3'-untranslated region (3'-UTR), leading to the suppression of transcription and/or affecting mRNA instability (16). miRNA (miR)33 is localized in the sterol-regulatory element-binding factor (SREBP) intron (17). A previous study (16) reported that there are 3 highly conserved miRNA binding sites in the 3'-UTR of ATP binding cassette transporter (ABC)A1. Therefore, the role of miR33 in the regulation of cholesterol efflux and the biosynthesis of high-density lipoprotein (HDL) may be through the downregulation ABCA1 and ABCG1.

Curcumin is a polyphenolic compound found primarily in the rhizomes of the ginger plant, and is believed to be one of the most biologically active natural products. It has been shown that curcumin has pharmacological effects in a wide variety of chronic diseases (18,19). Dong *et al* (20) speculated that curcumin or food rich in curcumin, have the potential to be a novel therapy for decreasing the risk of AS by increasing the expression levels of ABCA1 and increasing the cholesterol efflux in mouse adipocytes by the peroxisome proliferator activated receptor  $\gamma$ /liver X receptor  $\alpha$  signaling pathway.

Lin *et al* (18) found that curcumin can inhibit ox-LDL-induced MCP-1 expression of VSMCs via the mitogen activated protein kinases (MAPK) and nuclear transcription factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway. Although a number of studies investigated the mechanisms behind the pharmacological activity of curcumin (18-20), the exact mechanism behind its pharmacological effects still remains to be elucidated.

Overall, the mechanism of action for curcumin, TLR4, NF- $\kappa$ B and miR33a in the transfer of cholesterol and the secretion of TNF- $\alpha$ , MCP-1 and IL-6 is still remains unclear. It has been hypothesized that curcumin promotes cholesterol efflux and reduces the secretion of TNF- $\alpha$ , IL-6 and MCP-1 through the TLR4/NF- $\kappa$ B/miR33a signaling pathway.

## Materials and methods

**Reagents.** THP-1 cells were purchased from the American Type Culture Collection. FBS, v1640 medium, myllicin and trypsin were purchased from Gibco; Thermo Fisher Scientific, Inc. Human ox-LDL was purchased from Anhui Yiyuan Biotechnology Co., Ltd. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. Free cholesterol, CE and Triglycerides assay kits were purchased from Nanjing Jiancheng Biotechnology Co., Ltd. Curcumin, phorbol-12-myristate-13-acetate (PMA) and Ammonium pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma-Aldrich; Merck KGaA. Antibodies targeting TLR4 (mouse monoclonal antibody raised against TLR4 of human origin; cat. nos. 14358), TLR4 non-related isotype (cat. no. 2985)

(isotype Ab), NF- $\kappa$ B p65 (cat. no. 8242), NF- $\kappa$ B inhibitor  $\alpha$  (cat. no. 4814) (I $\kappa$ B $\alpha$ ), phosphorylated (p)-I $\kappa$ B $\alpha$  (cat. no. 2859), GAPDH (cat. no. 5174), ABCA1 (cat. no. 96292) and histone H1 (cat. no. 41318) were purchased from Cell Signaling Technology, Inc. Horseradish peroxidase (HRP) goat anti-mouse IgG (cat. no. 074-1506) used as secondary antibodies and was purchased from Kirkegaard & Perry Laboratories, Inc. Chemiluminescence (ECL) test kit and RIPA lysis buffer were purchased from ASPEN Biotechnology Co., Ltd. Image Laboratory Software 4.0 was used (Bio-Rad Laboratories, Inc.). Lipofectamine™ 2000 and M-MLV Reverse Transcriptase (cat. no. 28025013) was purchased from Invitrogen; Thermo Fisher Scientific, Inc. PrimeScript™ RT reagent kit with gDNA Eraser (cat. no. RR047Q) and SYBR® Premix Ex Taq™ (cat. no. DRR041A) were purchased from Takara Biomedical Technology Co., Ltd. The EpiQuik Nuclear Extraction kit was purchased from AmyJet Scientific Inc. The Cholesterol Efflux Fluorometric assay kit was purchased from BioVision, Inc. The IL-6 (cat. no. E-EL-H0102)/TNF- $\alpha$  (cat. no. E-EL-M0049)/MCP-1 (cat. no. E-EL-H6005) ELISA kit was purchased from Elabscience Biotechnology Co., Ltd. All were used according to the manufacturers protocol.

**THP-1 cell culture and foam cell model establishment.** THP-1 cells were cultured in RPMI-1640 medium which contained 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO<sub>2</sub> incubator. Culture medium was replaced every 2 days, cells were subcultured for a period of 5 days. To obtain THP-1 macrophages, medium containing 160 nmol/l PMA was used to induce culture for 48 h in an incubator at 37°C and 5% CO<sub>2</sub>. The cell culture medium was replaced with serum-free RPMI-1640 culture medium containing 50  $\mu$ g/ml ox-LDL. Cells were then incubated in the incubator for another 48 h in an incubator at 37°C and 5% CO<sub>2</sub>. An intracellular cholesterol ester (CE)/total cholesterol (TC) ratio >50% was used as the standard for successful replication of foam cell models (14).

**Cell viability assay.** THP-1 cells at the logarithmic growth phase were prepared using a complete medium containing 160 nmol/l of PMA. THP-1 cells (1 $\times$ 10<sup>4</sup> cells) were then seeded into 96-well plates and cultured for 48 h in an incubator at 37°C and 5% CO<sub>2</sub>. The cells were treated with various concentrations (0, 5, 10, 20, 40 and 80 mol/l) of curcumin for 24 h in an incubator at 37°C and 5% CO<sub>2</sub>, and subjected to the CCK-8 assay. The absorbance was measured at a wavelength of 490 nm and was used as an indicator of cell viability.

**Oil Red O staining and foam cell formation rate.** According to the aforementioned method, THP-1 macrophages were adjusted to a cell density of 4 $\times$ 10<sup>5</sup> cells/ml and 1 $\times$ 10<sup>6</sup> cells/well were seeded into 6-well plates containing pre-implanted sterile coverslips in an incubator at 37°C and 5% CO<sub>2</sub>. Subsequently, cells on the coverslips were washed with PBS 3 times, 5 min each time, and then fixed with 4% paraformaldehyde for 30 min at room temperature. Oil red O staining was performed for 10 min and the cells were counterstained for 5 min with hematoxylin at room temperature. Under the optical microscope, the number of larger red dye particles in the cell >5 can be used as the standard for foam cell formation. Ten fields were randomly observed to calculate the number of foam cells and the total

Table I. Primer sequences used for quantitative PCR.

Gene		Primer sequence
H-GAPDH	Forward	5'-GGTCGGAGTCAACGGATTTG-3'
	Reverse	5'-GGAAGATGGTGATGGGATTTC-3'
H-ABCA1	Forward	5'-AGGAAACCCAATCCCAGATACCC-3'
	Reverse	5'-GCTCGGAGGAAGTGCTTGAGAAT-3'
H-TLR4	Forward	5'-GGATGAGGACTGGGTAAGGAAT-3'
	Reverse	5'-AATGAAGATGATACCAGCACGAC-3'
U6	Looped RT primer	5'-AACGCTTCACGAATTTGCGT-3'
	Forward	5'-AACGCTTCACGAATTTGCGT-3'
	Reverse	5'-AACGCTTCA CGAATTTGCGT-3'
miR-33a-5p	Looped RT primer	5'-CTCAACTGGTGT CGTGGAGTCGGCAATT CAGTTGAGGCAATGCA-3'
	Forward	5'- GGTGCATTGTAGTTGCATTGC-3'
	Reverse	5'-GCGACGAGCAAAAAGCTTGT-3'

ABCA1, ATP binding cassette transporter A1; H, human; miR, microRNA; RT, reverse transfer; TLR, toll-like receptor.

number of cells at magnification, x200. The ratio of foam cells to total cells was deemed the foam cell formation rate.

**Measurement of intracellular TC, free cholesterol (FC), CE and triglycerides (TGs).** THP-1 cell suspension stimulated by ox-LDL was centrifuged at 200 x g at room temperature for 10 min. Cell samples were collected and cell homogenates were obtained using ultrasonic disruption at a low temperature (-4°C). TC and FC were detected by FC, CE assay kit. The CE levels were calculated by subtracting the FC from the TC. Cells were treated as aforementioned, and the TG content was determined using the TG assay kit.

**Western blot assays.** The cells were collected and the total protein was extracted using RIPA buffer at a low temperature (-4°C) NF-κB p65 is located in the nucleus of the cell; therefore, the EpiQuik Nuclear Extraction kit was used to extract the nuclear protein. Protein concentrations were determined using BCA assays. A 10% separation gel and a 5% spacer gel were prepared based on instructions provided in the SDS-polyacrylamide gel electrophoresis preparation kit, which were then transferred to PVDF membranes. After being blocked with 5% skim milk at room temperature for 1 h, the membranes were incubated with primary antibodies against GAPDH (1:1,000), histone H1 (1:500), ABCA1 (1:500), NF-κB p65 (1:2,000), IκBα (1:1,000), p-IκBα (1:1,000), TLR4 and TLR4 non-related isotype Ab (1:1,000) overnight at 4°C, followed by an incubation with HRP-Goat anti-mouse IgG secondary antibodies for 90 min. The optical densities of bands were detected using an ECL test kit and quantified using Image Lab software 4.0 (National Institutes of Health). GAPDH was used as the endogenous control.

**RNA extraction and reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from cells using TRIzol™ reagent (Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using M-MLV Reverse Transcriptase kit

at 4°C. Primers were designed and synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Table I). The thermocycling conditions for qPCR were as follows: Pre-denaturation at 95°C for 1 min followed by 3 steps of 40 cycles. Each cycle consisted of denaturation at 95°C for 15 sec, annealing at 58°C for 20 sec and extension 72°C for 20 sec. miRNA molecules are a class of nucleotides with a short length of ~21-24 bp, which cannot be amplified using conventional RT-qPCR. miR33a pre-treatment was performed with Stem-loop RT. U6 and GAPDH served as the endogenous controls for miRNA and mRNA. Results were presented as the level of mRNA relative to endogenous and calculated using the  $2^{-\Delta\Delta C_q}$  method (21).

**miR33a inhibitor and control sequence transfection.** Cells were transfected with 60 nM miR33a inhibitor or an equal concentration of miR-control using Lipofectamine™ 2000. In order to ensure successful transfections were achieved, RT-qPCR was used to detect the expression levels of miR33a mRNA after 6 h in an incubator at 37°C and 5% CO<sub>2</sub>. Cells were then used for further experimentation.

**ELISA.** Cells were seeded in 6-well plates at a density of 4x10<sup>5</sup>/ml, and treated according to the different experimental conditions. The cells were collected by centrifugation at 1,000 x g for 20 min and the supernatant were stored at -20°C prior to further experiments. The content of IL-6, TNFα and MCP-1 was measured by ELISA assays according to the manufacturers protocol. Standard concentration curves were drawn using standard diluents and the actual content of IL-6, TNF-α and MCP-1 was calculated.

**Cholesterol efflux assays.** Cholesterol efflux was measured based on instructions provided in Cholesterol Efflux Fluorometric assay kit (cell-Based). Macrophages derived from THP-1 cells were inoculated into the 96-well plates at a density of 1x10<sup>5</sup>/ml. Cholesterol was pre-labeled with 50 μl fluorescent labeling agent and balanced buffer and cultured in

the incubator overnight (>16 h) in an incubator at 37°C and 5% CO<sub>2</sub>. After different experimental treatments, the fluorescence levels (Ex/Em=482/515) of the supernatant, as well as supernatant containing dissolved cell and cellular debris, obtained using 100  $\mu$ l RIPA lysis buffer were measured. The ratio of fluorescence intensity of the media to the total fluorescence intensity of the cell lysate and media, x100 was the percentage cholesterol efflux.

**Statistical analysis.** All values are presented as the means  $\pm$  SD of three independent experiments. Differences between the groups were analyzed using the one-way ANOVA followed by Dunnett's or Tukey's post hoc tests, as applicable, with SPSS software (version 17.0; SPSS, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Detection of curcumin toxicity by CCK-8.** Curcumin of various concentrations (0, 5, 10, 20, 40 and 80  $\mu$ M) was incubated with THP-1 derived macrophages for 24 h. The toxicity of curcumin was detected using CCK-8 assays. Curcumin of various concentrations was found to have an effect on cell viability (Fig. 1). Further analysis found that there was no significant difference between the effects of curcumin at the concentrations of 0–40  $\mu$ M. However, when the concentration of curcumin was increased to 80  $\mu$ M, the viability of THP-1 derived macrophages decreased as a result of curcumin toxicity.

**Effect of curcumin on the intracellular lipid content and foam cell formation of THP-1 cells induced by ox-LDL.** THP-1 macrophages were stimulated with 50  $\mu$ g/ml ox-LDL, and then treated with curcumin at a safe concentration (40  $\mu$ M) for 24 h. When observed at x200 and x400 using light microscopy, it was found that numerous intracellular red stained particles were assembled following ox-LDL treatment, while in the safe concentration curcumin group, the number of intracellular red stained particles and the clustering of foam cells (Fig. 2A b,e) was less than that of the model group (Fig. 2A a,d). Detection of TC, FC, CE and TG content using the TC, FC and TG kits showed that the intracellular lipid content in THP-1 macrophages increased due to ox-LDL induction. Due to the accumulation of lipids in cells, the ratio of CE/TC increased up to 65.09%, the model met the criterion (22) to be considered a macrophage model (Table II). Curcumin significantly reduced the accumulation of intracellular cholesterol and triglyceride, consistent with the previous research in the current report. It was demonstrated that ox-LDL significantly promoted the formation of foam cells, while curcumin reduced this formation (Fig. 2B). However, the specific mechanism of action needs further investigation.

**Effect of curcumin on the cholesterol efflux rate of THP-1 induced by ox-LDL.** Ox-Ldl promoted lipid inflow, and the effect of intervention conditions on cholesterol outflow rate was subsequently examined (20). It was discovered that curcumin of various concentration (10–40  $\mu$ M) increased the cholesterol efflux rate compared with the foam cell model group (Fig. 3). The effect was associated with curcumin concentration, but the

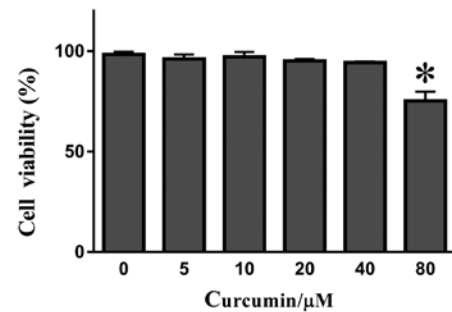


Figure 1. Effect of curcumin on cell viability (n=3 per group). \* $P < 0.05$  vs. the control group.

curcumin with the lowest analyzed concentration (5  $\mu$ M) was unable to significantly affect the cholesterol efflux rate. This observation helps explain how curcumin reduced the intracellular lipid content and formation of foam cells by promoting cholesterol efflux in THP-1 macrophages. Curcumin was then used at 40  $\mu$ M to stimulate THP-1 macrophages for various time periods, and it was found that the cholesterol efflux was increased between 6–24 h. However, when the stimulation of curcumin lasted 48 h, the cholesterol efflux did not increase significantly compared with that at 24 h. Thus, curcumin stimulation for 24 h resulted in peak cholesterol efflux (Fig. 4). To ensure the validity of the experiment and to exclude inaccuracy, THP-1 macrophages were incubated with 40  $\mu$ M curcumin for 24 h for subsequent experiments.

**Curcumin promotes the expression of ABCA1 and reduce the secretion of IL-6, TNF- $\alpha$  and MCP-1.** ox-LDL (50  $\mu$ g/ml) was used to stimulate cells, which significantly reduced the expression levels of ABCA1 mRNA and protein, while pre-treatment with 40  $\mu$ M curcumin significantly aggravated the expression of ABCA1 mRNA and protein (Fig. 5). A previous study (9,11) confirmed that ox-LDL can promote the expression of IL-6, TNF- $\alpha$  and MCP-1, which has also been verified by the current experiments. Secretion of IL-6, TNF- $\alpha$  and MCP-1 were increased by ox-LDL (Fig. 6), which were decreased by curcumin. Thus, curcumin had the ability to promote the expression of ABCA1 mRNA and protein in ox-LDL stimulated macrophages *in vitro*, and curcumin also reduced the secretion of IL-6, TNF- $\alpha$  and MCP-1.

**Curcumin and miR33a.** Successful transfections of miR33a inhibitors were firstly confirmed (Fig. S1). Using 50  $\mu$ g/ml ox-LDL-stimulated cells, detection of miR33a expression levels showed that ox-LDL significantly promoted the expression of miR33a, while the expression of miR33a mRNA was significantly decreased in curcumin pre-treated cells (Fig. 7). Therefore, it was concluded that curcumin had the capacity to regulate the expression of miR33a. Further detection demonstrated that ox-LDL had the ability to significantly reduce the expression levels of ABCA1 at the mRNA and protein level, as well as promote the secretion of IL-6, TNF- $\alpha$  and MCP-1. Both curcumin and the miR33a inhibitor increased the expression of ABCA1, as well as the cholesterol efflux rate (Figs. 8–10). However, there was no significant difference between the foam cell model group and the miRNA control consequence group. Moreover, it was discovered that the use of both curcumin and

Table II. Effect of curcumin on the contents of TC, FC and CE in cells. (n=3 per group, mean  $\pm$  SD).

Treatment groups	TC, mmol·g <sup>-1</sup>	FC, mmol·g <sup>-1</sup>	CE, mmol·g <sup>-1</sup>	TG, mmol·g <sup>-1</sup>	CE:TC, %
Control	5.64 $\pm$ 0.15	3.56 $\pm$ 0.16	2.08 $\pm$ 0.06	3.83 $\pm$ 0.15	36.83 $\pm$ 1.41
Foam cell model	18.78 $\pm$ 0.18 <sup>a</sup>	6.55 $\pm$ 0.18 <sup>a</sup>	12.22 $\pm$ 0.36 <sup>a</sup>	20.29 $\pm$ 0.47 <sup>a</sup>	65.09 $\pm$ 1.30 <sup>a</sup>
Curcumin (40 $\mu$ M)	11.97 $\pm$ 0.07 <sup>b</sup>	5.94 $\pm$ 0.17 <sup>b</sup>	6.02 $\pm$ 0.21 <sup>b</sup>	13.34 $\pm$ 0.41 <sup>b</sup>	50.33 $\pm$ 1.53 <sup>b</sup>

CE, cholesterol ester; FC, free cholesterol; TC, total cholesterol; TG, triglyceride. <sup>a</sup>P<0.05 vs. control group; <sup>b</sup>P<0.05 vs. Foam cell model group.

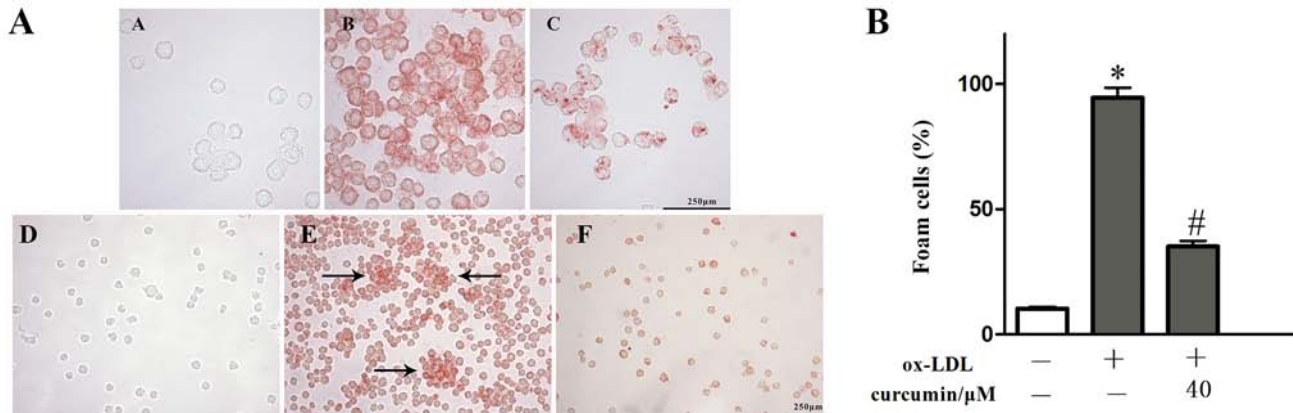


Figure 2. Effect of curcumin on (A) lipid accumulation and (B) formation of foam cells (n=3 per group). Cells in the (a) control, (b) ox-LDL (50  $\mu$ g/ml) and (c) curcumin groups observed under a light microscope (magnification, x400). Cells in the (d) control, (e) ox-LDL (50  $\mu$ g/ml) and (f) curcumin groups observed under a light microscope (magnification, x200). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group. ox-LDL, oxidized low-density lipoprotein; black arrow, clustering of foam cells.

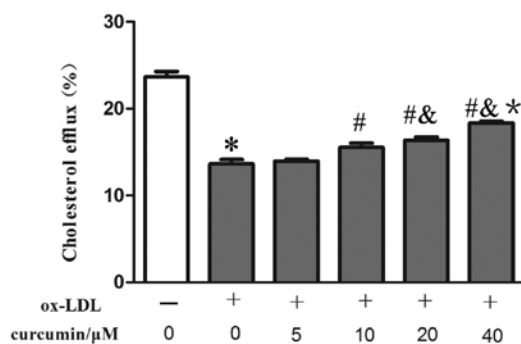


Figure 3. Effects of various concentrations of curcumin on the cholesterol efflux rate of THP-1 macrophages (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; &P<0.05 vs. the curcumin (10  $\mu$ M) group; \*P<0.05 vs. the curcumin (20  $\mu$ M) group. ox-LDL, oxidized low-density lipoprotein.

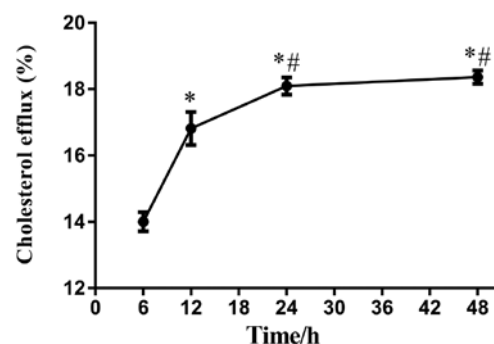


Figure 4. Effects of different stimulation time of curcumin on the cholesterol efflux rate of THP-1 macrophages (n=3 per group). \*P<0.05 vs. the 6 h group; #P<0.05 vs. the 12 h group.

miR33a inhibitors further significantly increased the expression of ABCA1 at the mRNA and protein levels as well as further increasing the cholesterol efflux rate. The combined treatment also further significantly reduced the secretion of IL-6, TNF- $\alpha$  and MCP-1. While no difference was indicated between the curcumin-only group and the curcumin combined with the control consequence co-group. Therefore, curcumin had the capacity to promote the expression of ABCA1 and increase the cholesterol efflux rate in THP-1 derived macrophages. Curcumin also reduced the secretion of IL-6, TNF- $\alpha$  and MCP-1 through regulating miR44.

**Relationship between curcumin and NF- $\kappa$ B/miR33a.** An inhibitor of the NF- $\kappa$ B signaling pathway, PDTC (50  $\mu$ M), was utilized in an incubator at 37°C and 5% CO<sub>2</sub> for 1 h, and was used to block the activation of NF- $\kappa$ B and detect the expression of miR33a, nuclear NF- $\kappa$ B p65 and cytoplasmic I $\kappa$ B $\alpha$  and p-I $\kappa$ B $\alpha$ . The data showed that ox-LDL significantly increased the expression of miR33a and the phosphorylation of I $\kappa$ B $\alpha$ , and also led to an increase in nuclear NF- $\kappa$ B p65 and cytoplasmic p-I $\kappa$ B $\alpha$  and a decrease in cytoplasmic I $\kappa$ B $\alpha$  (Figs. 11 and 12). Expression of miR33a in cells pre-treated with curcumin and PDTC was significantly decreased. Cytoplasmic p-I $\kappa$ B $\alpha$  and nuclear NF- $\kappa$ B p65 expression levels were also reduced significantly, while the cytoplasmic I $\kappa$ B $\alpha$  levels increased.

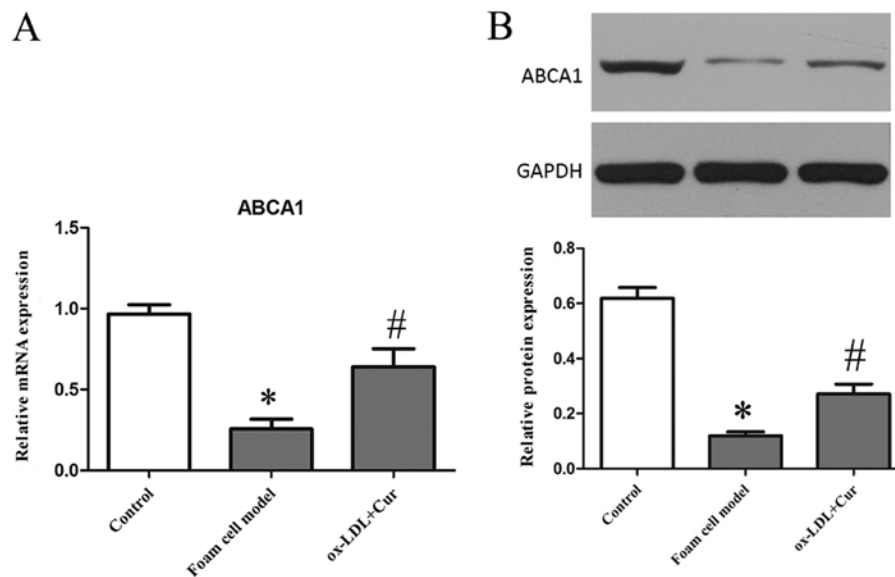


Figure 5. Effects of curcumin on the (A) mRNA and (B) protein expression levels of ABCA1 in THP-1 macrophages (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group. ABCA1, ATP binding cassette transporter A1; ox-LDL, oxidized low-density lipoprotein.

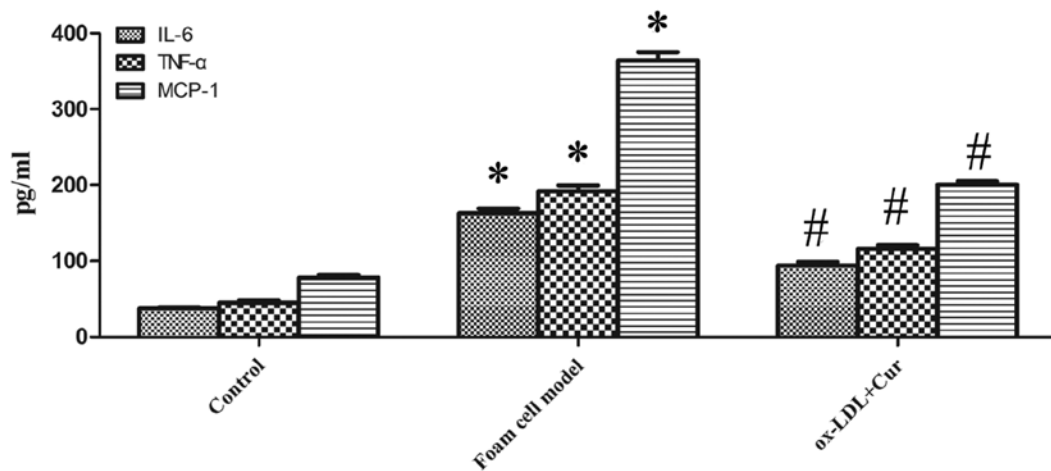


Figure 6. Effects of curcumin on the secretion of IL-6, TNF- $\alpha$  and MCP-1 in THP-1 macrophages (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group. IL, interleukin; MCP, monocyte chemotactic protein; ox-LDL, oxidized low-density lipoprotein; TNF, tumor necrosis factor.

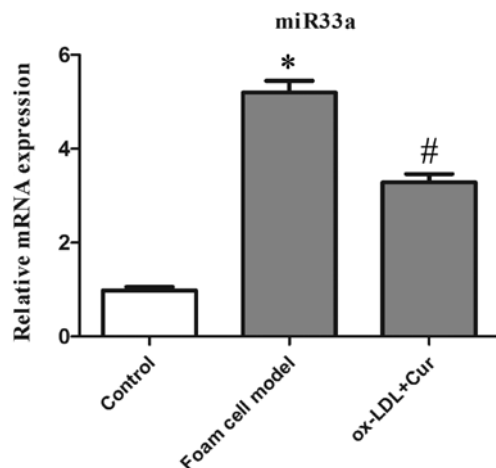


Figure 7. Effect of curcumin on the miR33a expression levels in THP-1 macrophages (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group. miR, microRNA; ox-LDL, oxidized low-density lipoprotein.

*Relation of NF- $\kappa$ B/miR33a with ABCA1 expression, the cholesterol efflux rate and secretion of IL-6, TNF- $\alpha$  and MCP-1 in THP-1 macrophages.* To study the mechanism of action behind the effect of miR33a on the development of AS and pharmacological activity of curcumin, miR33a inhibitors or control sequences were transfected into THP-1 macrophages. The results indicated that ox-LDL dramatically inhibited the expression of ABCA1 both at the mRNA and protein levels, and boosted the secretion of IL-6, TNF- $\alpha$  and MCP-1 (Figs. 13 and 14). While curcumin, NF- $\kappa$ B blocker and miR33a inhibitor promoted the expression of ABCA1. Secretions of IL-6, TNF- $\alpha$  and MCP-1 were also decreased by these treatments. However, the miRNA control sequence had no significant effect.

*Relation of curcumin with TLR4.* Cells were stimulated with 50  $\mu$ g/ml ox-LDL and the expression of TLR4 was detected. ox-LDL significantly increased the expression levels of TLR4

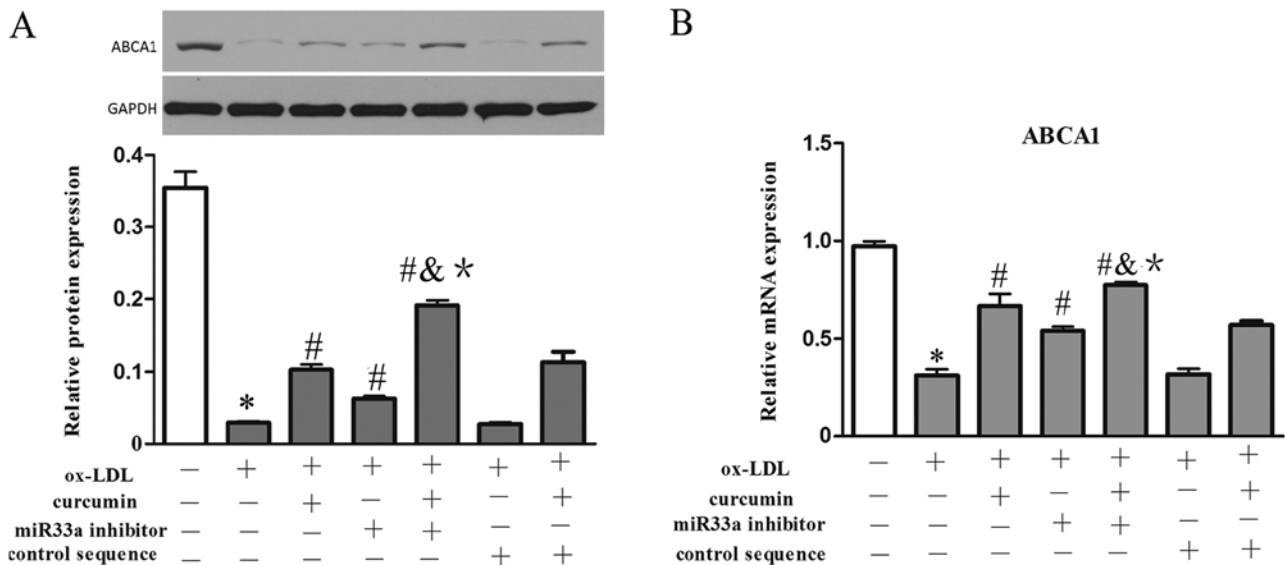


Figure 8. Effect of curcumin and the miR33a inhibitor on ABCA1 (A) protein and (B) mRNA expression levels (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; &P<0.05 vs. the curcumin group; \*P<0.05 vs. the miR33a inhibitor group. ABCA1, ATP binding cassette transporter A1; miR, microRNA; ox-LDL, oxidized low-density lipoprotein.

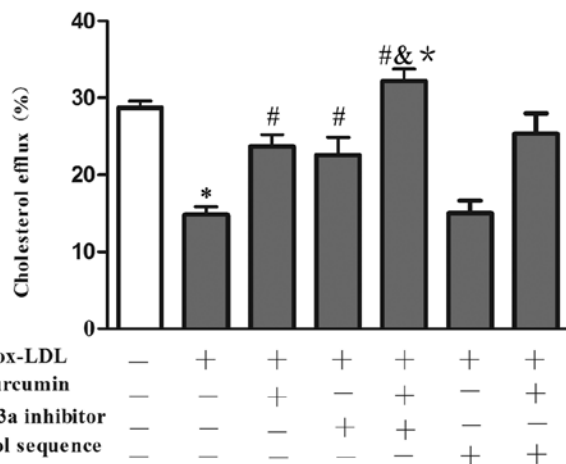


Figure 9. Effects of curcumin and the miR33a inhibitor on the cholesterol efflux rate (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; \*P<0.05 vs. the curcumin group; &P<0.05 vs. the miR33a inhibitor group. miR, microRNA; ox-LDL, oxidized low-density lipoprotein.

both at the mRNA and protein level, which was reduced by curcumin (Fig. 15). As such, it was concluded that curcumin inhibited the expression of TLR4 and the TLR4 signaling pathway.

**Relation of curcumin with TLR4/NF- $\kappa$ B/miR33a.** To further study the relationship between TLR4 and NF- $\kappa$ B/miR33a, THP-1 macrophages were pretreated with TLR4 antibodies or TLR4 isotype Ab antibodies at a concentration of 10  $\mu$ g/ml in an incubator at 37°C and 5% CO<sub>2</sub> for 1 h. The foam cell model was established following the same protocol. The data showed that ox-LDL made the expression of miR33a increase significantly at the mRNA level (Fig. 16). Simultaneously, nuclear NF- $\kappa$ B p65 and cytoplasmic p-I $\kappa$ B $\alpha$  were increased as well, while the protein content of I $\kappa$ B $\alpha$  was reduced (Fig. 17).

Curcumin and TLR4 antibodies reduced the expression levels of miR33a at the mRNA level as well as the content of nuclear NF- $\kappa$ B p65 and cytoplasmic p-I $\kappa$ B $\alpha$ . The levels of I $\kappa$ B $\alpha$  were also increased. The TLR4 isotype Ab group had no obvious difference with that of the foam cell model group. Additionally, it was discovered that there was a synergistic effect gained from curcumin and the TLR4 antibodies. However, there was no significant difference between the effects when combining TLR4 homologous isotype Ab and curcumin with that of curcumin used alone. Therefore, it is believed that both the TLR4 antibodies and curcumin have the capacity to suppress the expression of TLR4 at the mRNA and protein levels. The expression levels of NF- $\kappa$ B and miR33a were downregulated by inhibiting TLR4. Therefore, TLR4 may be involved in regulating the NF- $\kappa$ B/miR33a signaling pathway and curcumin may block the NF- $\kappa$ B/miR33a signaling pathway by blocking the expression of TLR4.

**Relation of TLR4/NF- $\kappa$ B/miR33a with the expression of ABCA1, cholesterol efflux rate and secretion of IL-6, TNF- $\alpha$  and MCP-1 in THP-1 macrophages.** To validate the effect of curcumin and the TLR4/NF- $\kappa$ B/miR33a signaling pathway on the expression of ABCA1; cholesterol efflux rate; and secretion of IL-6, TNF- $\alpha$  and MCP-1, these parameters were investigated using the aforementioned treatment groups. The data showed that the expression of ABCA1 at the mRNA and protein levels was decreased by ox-LDL treatment, which also promoted the secretion of IL-6, TNF- $\alpha$  and MCP-1 (Figs. 18 and 19). Curcumin and the TLR4 antibodies were able to boost the expression of ABCA1 at the mRNA and protein level and boost the cholesterol efflux rate, as well as reduce the secretion of IL-6, TNF- $\alpha$  and MCP-1. While there was no difference between the TLR4 isotype Ab group and the foam cell model group. In addition, a synergistic affect was gained from curcumin and TLR4 antibodies, which was enhanced compared to that of curcumin or TLR4 antibodies alone. There

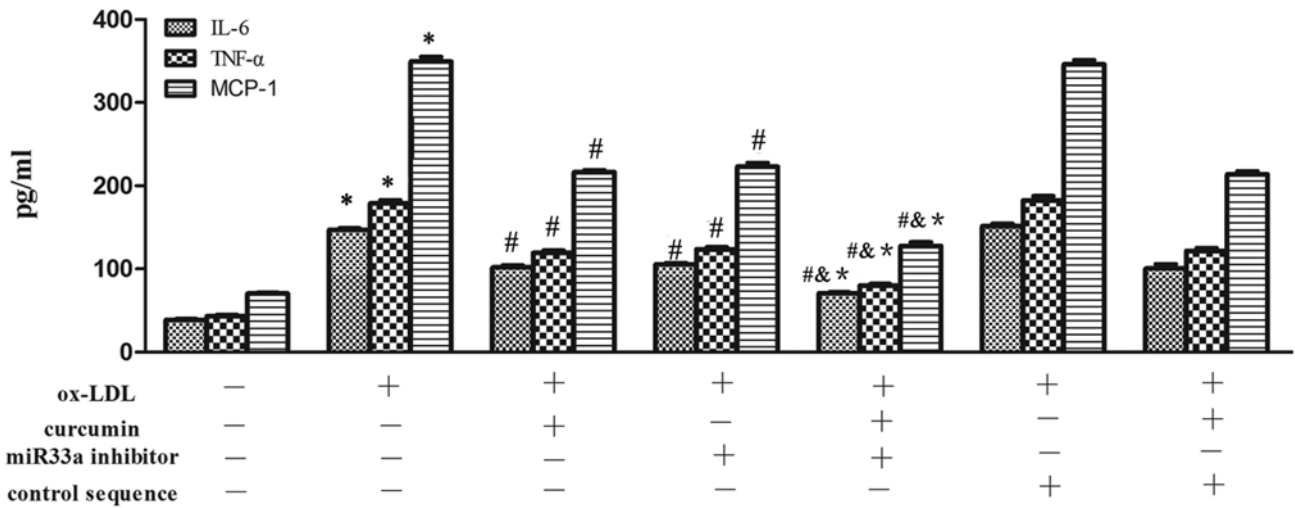


Figure 10. Effects of curcumin and the miR33a inhibitor on the secretion of IL-6, TNF- $\alpha$  and MCP-1 (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; &P<0.05 vs. the curcumin group; \*P<0.05 vs. the miR33a inhibitor group. IL, interleukin; MCP-1, monocyte chemotactic protein-1; miR, microRNA; ox-LDL, oxidized low-density lipoprotein; TNF, tumor necrosis factor.

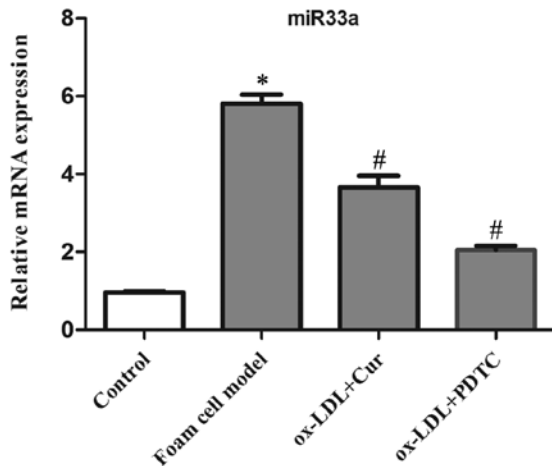


Figure 11. Effects of curcumin and PDTC on miR33a expression levels (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group. Cur, curcumin; miR, microRNA; ox-LDL, oxidized low-density lipoprotein; PDTC, pyrolidone dithiocarbamate.

was no difference between the effect of the combination of TLR4 isotype Ab and curcumin with that of curcumin used alone. Therefore, the TLR4/NF- $\kappa$ B/miR33a pathway may be involved in the regulation of ABCA1 expression; cholesterol efflux; and secretion of IL-6, TNF- $\alpha$  and MCP-1. Curcumin promoted the expression of ABCA1 in THP-1 macrophages and reduced secretions of IL-6, TNF- $\alpha$  and MCP-1 through the TLR4/NF- $\kappa$ B/miR33a signaling pathway.

## Discussion

A large number of cardiovascular diseases begin with the development of AS, which can be characterized by formation of foam cells, accumulation of lipids and inflammation (23). Most natural products are active ingredients in plants. It has been shown that lipid metabolic disorders can be regulated by natural products (active ingredients within plants), which may also affect the intake and efflux of cholesterol, stabilize the

atherosclerotic plaques, reduce the cholesterol content in the blood, inhibit or downregulate the activity of pro-inflammatory cytokines, as well as their related mediators, and reduce the inflammatory response (24,25). Thus, natural products have the potential to provide a therapy for preventing and treating AS (26,27).

Curcumin is a natural polyphenol active substance in turmeric, which has been widely used in the food industry as a spice and food pigment (20). Studies have shown that curcumin has pharmacological effects such as anti-inflammation, anti-oxidation, antitumor and cardiovascular protection (28,29). The effect may be attributed to the ability of curcumin to regulate various molecular targets. Zhong *et al* (30) reported that curcumin inhibited the overexpression of vascular smooth muscle cell (VSMC) pro-inflammatory factor induced by ox-LDL and suppressed the activation of inflammatory signaling pathways. Moreover, *in vivo* experiments demonstrated that 20 mg/kg curcumin consumed orally, daily could alleviate the development of AS and systemic inflammatory responses in apoE<sup>-/-</sup> mice, as well as reduce serum cholesterol and triglyceride levels and increase HDL levels. The results indicated that curcumin regulates esters and has an anti-inflammatory effect, which can inhibit the formation of foam cells and prevent AS. However, the specific effect of curcumin requires further investigation.

LDLs are regarded as a marker of AS pathogenesis, and high concentrations of LDLs in serum are usually considered a major risk factor for Coronary atherosclerotic disease (CAD). Steinberg (31) revealed that the main mechanism behind macrophage formation stems from disorders of ox-LDL intake and lipid efflux. Additionally, ox-LDL is toxic to cells and induces inflammatory gene expression, thus promoting the formation of foam cells. The present results indicated that ox-LDL boosted the accumulation of intracellular lipids and increased the amount of large Oil Red O stained lipid particles deposited within cells. Further examination also showed that the amount of intracellular TC, CE and TG increased significantly and the rate of foam cell formation increased as

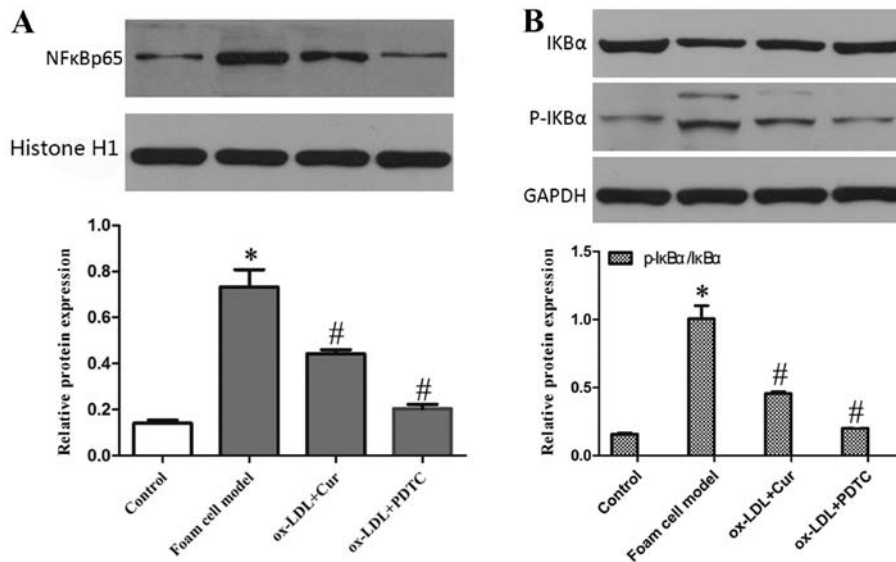


Figure 12. Effects of curcumin and PDTC on the (A) nuclear expression levels of NF-κB p65 as well as the (B) cytosolic expression levels and the ratio of p-IκBα and IκBα (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group. IκBα, NF-κB inhibitor α; NF-κB, nuclear factor κB; p, phosphorylated; PDTC, pynolidine dithiocarbamate.

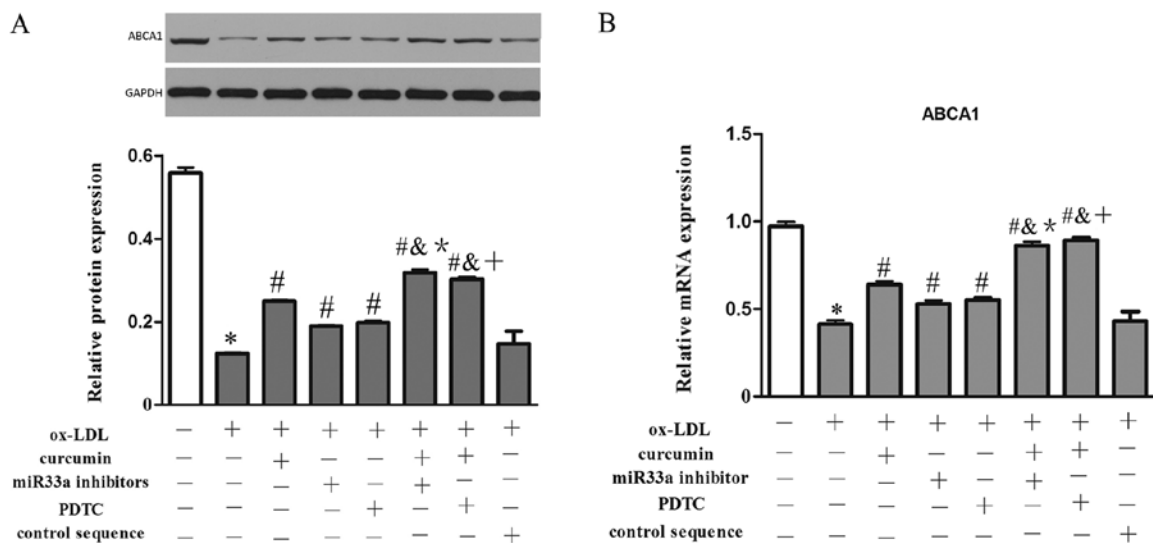


Figure 13. Effects of curcumin, PDTC and the miR33a inhibitor on the expression levels of ABCA1 (A) protein and (B) mRNA (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; &P<0.05 vs. the curcumin group; \*P<0.05 vs. the miR33a inhibitor group; +P<0.05 vs. the PDTC group. ABCA1, ATP binding cassette transporter A1; miR, microRNA; ox-LDL, oxidized low-density lipoprotein; PDTC, pynolidine dithiocarbamate.

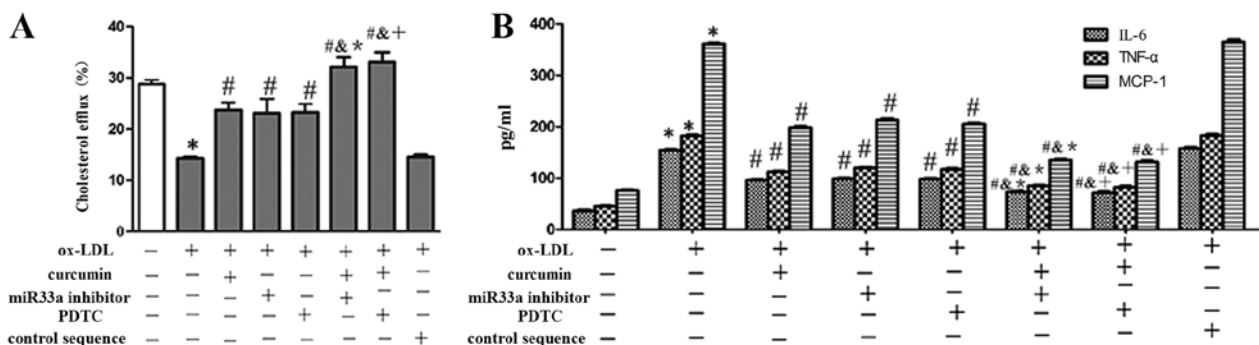


Figure 14. Effects of curcumin, PDTC and the miR33a inhibitor on (A) the cholesterol efflux rate and (B) IL-6, TNF-α and MCP-1 (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; &P<0.05 vs. the curcumin group; \*P<0.05 vs. the miR33a inhibitor group; +P<0.05 vs. the PDTC group. IL, interleukin; MCP, monocyte chemotactic protein; miR, microRNA; ox-LDL, oxidized low-density lipoprotein; TNF, tumor necrosis factor; PDTC, pynolidine dithiocarbamate.

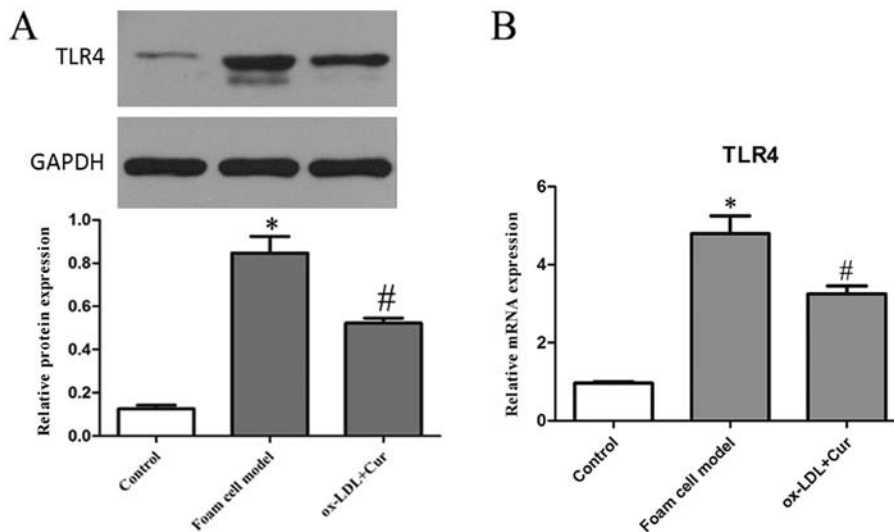


Figure 15. Effect of curcumin on the expression levels of TLR4 (A) protein and (B) mRNA (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group. ox-LDL, oxidized low-density lipoprotein; TLR, toll-like receptor.

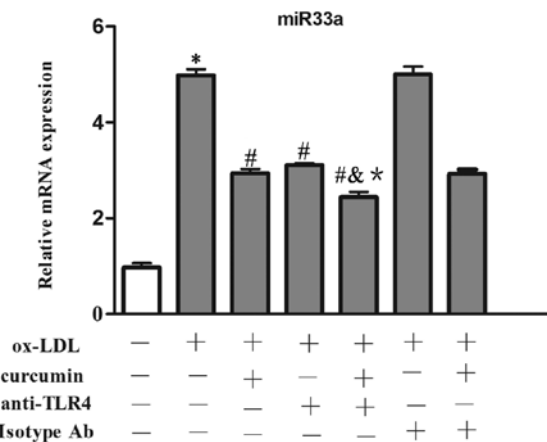


Figure 16. Effects of curcumin and TLR4 antibodies on miR33a mRNA expression levels (n=3 per group). \*P<0.05 versus control group; #P<0.05 vs. foam cell model group; &P<0.05 vs. curcumin group; \*P<0.05 vs. TLR4 antibody group. miR, microRNA; ox-LDL, oxidized low-density lipoprotein; TLR, toll-like receptor.

well. Compared with the control group, the CE/TC ratio was up to  $65.09 \pm 1.30$ , demonstrating the formation of foam cells and that ox-LDL had successfully induced the establishment of the foam cell model.

Determination of the rate of cholesterol efflux usually requires the advanced employment of ox-LDL to promote the intake of lipid in macrophages, then various interventions are used for further study (14,26). By adopting this method, the present study discovered that curcumin could increase the cholesterol efflux rate, and in the safe concentration range (10-40  $\mu$ M), the potency of curcumin acted in a dose-dependent manner. The cholesterol efflux rate was positively associated with the concentration of curcumin, while the lower concentration (5  $\mu$ M) of curcumin did not significantly increase cholesterol efflux. Moreover, it was also found that the effects of curcumin increased with time. When the treatment time lasted 24 h, the effect of curcumin on cholesterol efflux rate reached its peak. Therefore, 40  $\mu$ M for

24 h was chosen as a suitable treatment condition for further experiments.

ABCA1 plays a vital role in the metabolism of cholesterol, which mediates the release of cellular FC and phospholipids to extracellular receptor apolipoprotein I and finally forms the nascent HDL (20,26). Data from the present study revealed that ox-LDL reduced the expression of ABCA1 at the mRNA and protein levels, while curcumin promoted its expression, thus providing a pathway for cholesterol efflux. The rate of cholesterol efflux was indeed increased in these experiments. From these results, it was speculated that curcumin may promote cholesterol efflux by regulating the expression of ABCA1 at both the mRNA and protein levels.

Curcumin has been reported to have the capacity of boosting secretions of TNF- $\alpha$  in human umbilical vein endothelial cells and slow down the development of AS (25). According to the present study, ox-LDL significantly aggravated the secretion of TNF- $\alpha$ , while curcumin could remarkably reduce the secretion of TNF- $\alpha$ , IL-6 and MCP-1 in THP-1 macrophages induced by ox-LDL. As a limiting factor for HDL assembly, ABCA1 is also regulated post-transcriptionally (32,33). It has been previously shown decreases in ABCA1 expression are attributed to the stimulation of inflammation, for example, through the activation of IL-1 $\beta$ , TNF- $\alpha$ , interferon  $\gamma$  and NF- $\kappa$ B (34). Hence, inhibition of inflammatory pathways could alleviate the effect of inflammation through the expression of ABCA1. Therefore, curcumin exhibits anti-inflammatory effect by increasing the expression of ABCA1 and inhibiting the secretion of IL-6, TNF- $\alpha$  and MCP-1.

There are two subtypes of miR33 in humans, miR33a and miR33b. miR33a is located at the 16th intron of the SREBP-2 gene on chromosome 22 and is related to cholesterol efflux (35), while miR33b is located on intron 17 of the SREBP-1 gene of chromosome 17 and may be associated with the synthesis of fatty acids and TGs (36). There has been a focus on how miR33 regulates its possible target genes; however, little is known of the mechanism of action behind how miR33 regulates other targets within cells (17,26). It has been previously found that when the cholesterol load in mouse peritoneal macrophages

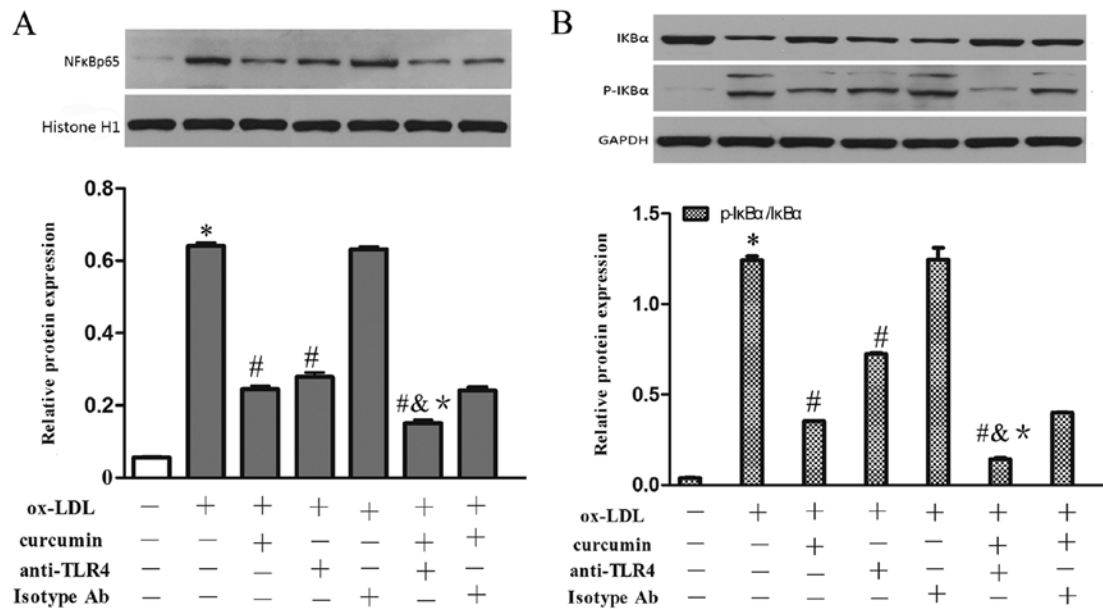


Figure 17. The effect of curcumin and TLR4 antibody on (A) nuclear expression levels of NF-κB p65, as well as the (B) cytosolic expression levels of IκBα and p-IκBα (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; &P<0.05 vs. IκBα, NF-κB inhibitor α; NF-κB, nuclear factor κB; ox-LDL, oxidized low-density lipoprotein; p, phosphorylated; TLR, toll-like receptor.

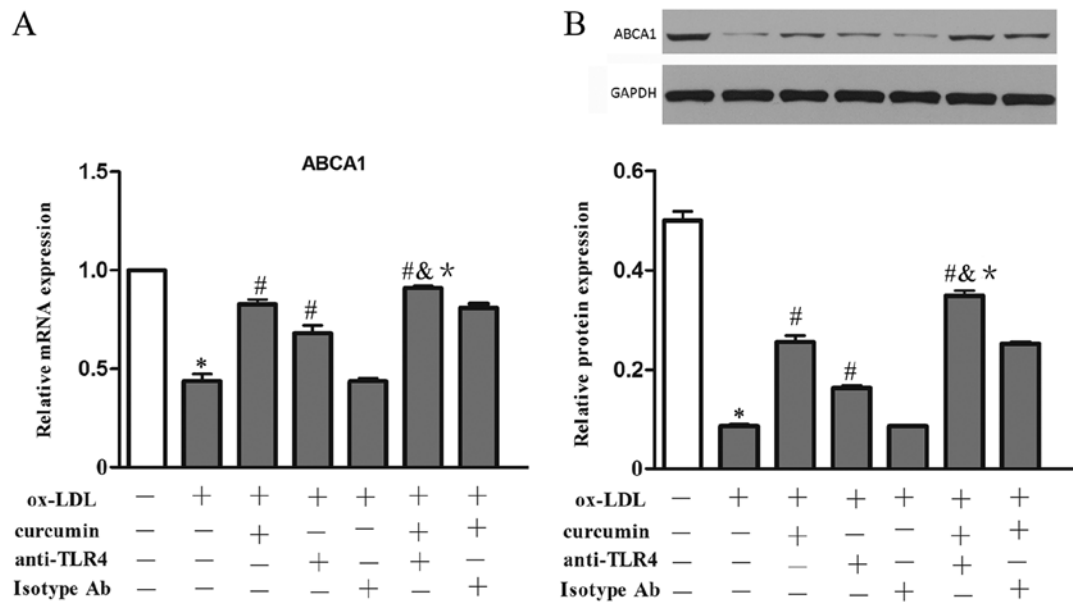


Figure 18. Effects of curcumin and TLR4 antibodies on the expression levels of ABCA1 (A) protein and (B) mRNA (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; &P<0.05 vs. the curcumin group; \*P<0.05 vs. the TLR4 antibody group. ABCA1, ATP binding cassette transporter A1; TLR, toll-like receptor.

was increased, the expression levels of miR33a and SREBP-2 are downregulated, indicating that molecules that regulate SREBP-2 may also regulate the production of miR33a (34).

According to Rakcheev *et al* (37), curcumin suppresses mRNA and protein expression of SREBP-2 and Niemann-Pick C1-Like 1 in mice fed with a high-fat diet, which plays a key part in preventing the formation of gallstones and reducing blood lipid and bile acid cholesterol content. Hence, it was hypothesized that miR33a, as an intron of the SREBP-2 gene, was involved in the regulation of curcumin in cholesterol homeostasis. Therefore, the miR33a inhibitor was transfected

into THP-1 macrophages in the present study, aiming to bind with miR33a and blocking its expression. In addition, miR33a control sequences were simultaneously transfected into cells using the same protocol, acting as a control and excluding non-sequence specific interactions. ox-LDL significantly promoted the expression of miR33a at the mRNA level, which was significantly inhibited by curcumin.

A previous study reported that 3'-UTR of ABCA1 contains 3 highly conserved binding sites for miR33a (38). By binding with these sites, miR33a suppresses the expression of ABCA1. According to the present data, compared with the foam cell

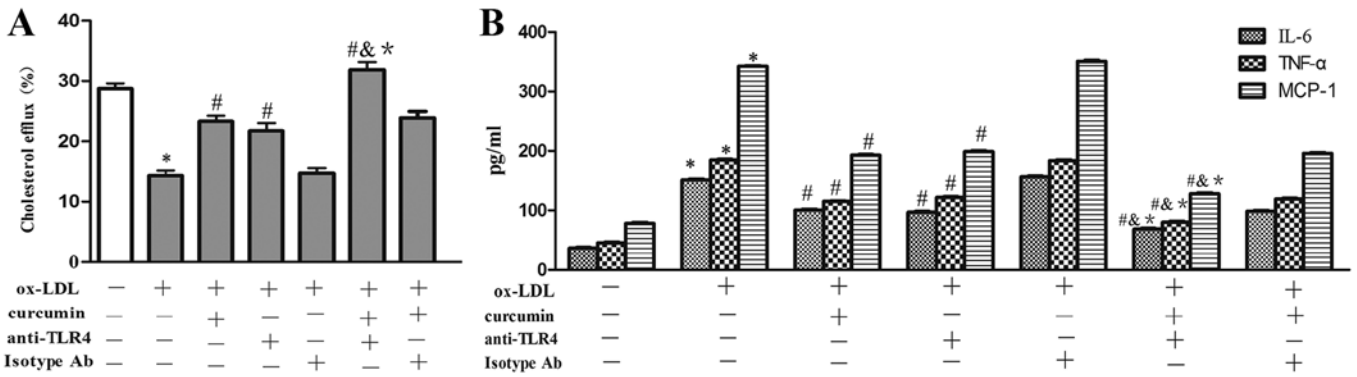


Figure 19. Effects of curcumin and TLR4 antibodies on (A) the cholesterol efflux rate and (B) the expression levels of IL-6, TNF- $\alpha$  and MCP-1 (n=3 per group). \*P<0.05 vs. the control group; #P<0.05 vs. the foam cell model group; &P<0.05 vs. the curcumin group; \*P<0.05 vs. the TLR4 antibody group. IL, interleukin; MCP, monocyte chemotactic protein; ox-LDL, oxidized low-density lipoprotein; TNF, tumor necrosis factor; TLR, toll-like receptor.

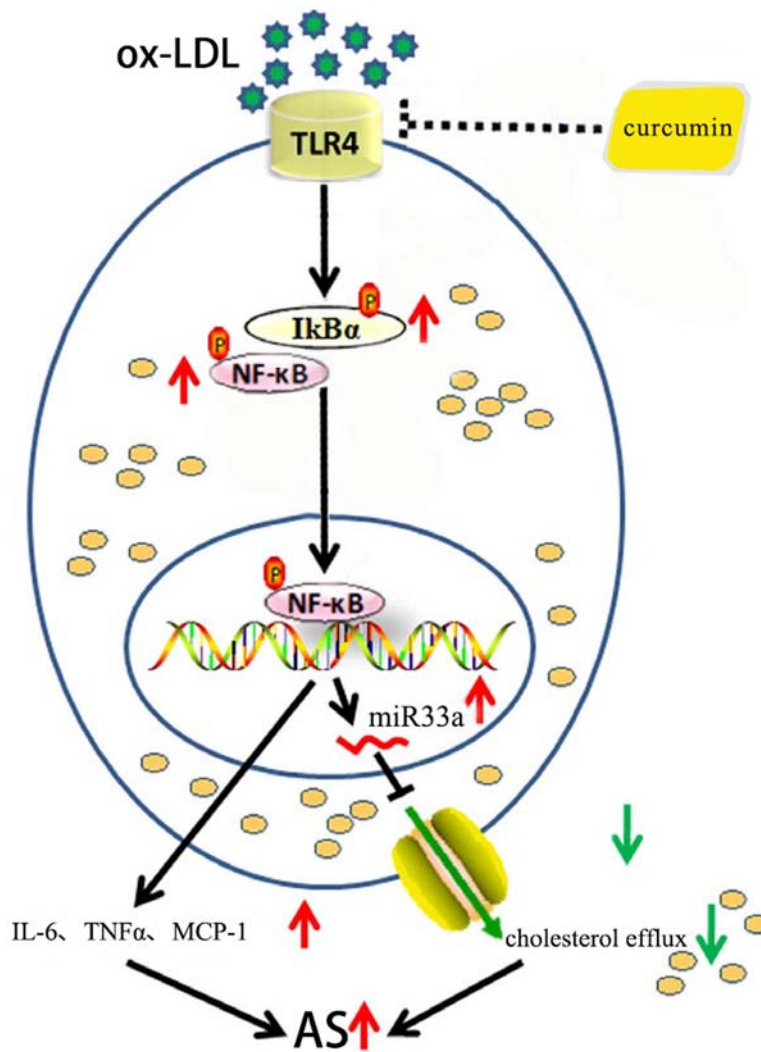


Figure 20. Effect of curcumin on the TLR4/NF- $\kappa$ B/miR33a signaling pathway in THP-1 macrophages; IL, interleukin; MCP, monocyte chemotactic protein; miR, microRNA; ox-LDL, oxidized low-density lipoprotein; p, phosphorylated; TNF, tumor necrosis factor; TLR, toll-like receptor; NF, nuclear factor.

model group, curcumin or the miR33a inhibitor increased the expression of ABCA1 at both the mRNA and protein levels, and promoted the cholesterol efflux, while the control sequence of miR33a had no obvious effects. Additionally, the combination of curcumin and miR33a inhibitor had a stronger

effect than that of curcumin or miR33a inhibitor used independently. The combinatory utilization of curcumin and miR33a control sequence had no remarkable difference with that of curcumin used alone. Thus, it was conjectured that miR33a participated in regulating the expression of ABCA1 at the

mRNA and protein levels and has an impact on the cholesterol efflux rate. Curcumin may affect the expression of ABCA1 by regulating miR33a.

Disorders of lipid metabolism are often accompanied by inflammation. It has been demonstrated that inflammation can boost the accumulation of lipids by promoting the intake and synthesis of cholesterol; however, the mechanisms remain to be discovered (34). A previous study has shown that the inflammatory factors IL-6 and TNF- $\alpha$  promote the expression of miR-33a-5p and SREBP2 and also to downregulate cholesterol transfer of ABCA1/G1, contributing to accumulation of intracellular lipids (39). Additionally, Rayner *et al* (40) showed that a high fat diet could boost the generation of receptor-interacting protein 140 (RIP140), TNF- $\alpha$  and IL-1 $\beta$  and reduce the content of miR33a simultaneously. Therefore, miR33a may act as the bridge between inflammation and lipid metabolism. The current data showed that curcumin or miR33a inhibitor could significantly reduce the expression of IL-6, TNF- $\alpha$  and MCP-1, while there was no remarkable difference between the effect of control sequence of miR33a and that of the foam cell group. Furthermore, it was also discovered that the effects of combining of miR33 and curcumin was stronger than that of curcumin or miR33a utilized independently. There was no clear difference between the effect of combining treatments of curcumin and the control sequence group and that of curcumin applied alone. Hence, curcumin may be involved in regulating the secretion of IL-6, TNF- $\alpha$  and MCP-1 and alleviating the inflammatory response by suppressing miR33a expression. Recently, Rayner *et al* (40) reported that the anti-inflammatory cytokines released from injured macrophages were increased in anti-miR33 treated mice, and pro-inflammatory cytokines also decreased. There was also a reduction in the concentration of inflammatory macrophages. The results of the present study were consistent with the results from the experiments of Rayner *et al* (40). Thus, miR33 may be a pro-inflammatory miRNA. Interestingly, Ho *et al* (41) reported that agonists of miR33 mimic could markedly reduce the content of RIP140, the NF- $\kappa$ B coactivator, both at the mRNA and protein levels, leading to inhibition of TNF- $\alpha$  and IL-1 $\beta$  in macrophages. This indicated that miR33 may be an anti-inflammatory miRNA in macrophages, opposing the current results. de Beer *et al* (42) demonstrated that ABCA1/G1 affected the expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 through the Janus activated kinase signal transducer 2/activator of transcription 3 signaling pathway. From the previous results, it was hypothesized that the effect of miR33 is related to the state of macrophages; however, miRNA33 downregulated the expression of ABCA1, which affects the expression of inflammatory cytokines. The contradictory results may be a result of the complex interaction between the two molecules. To summarize, it is hypothesized that miR33 may exert a pro-inflammatory role in the early stage of AS; therefore, inhibition of miR33a may be effective in preventing the inflammatory response in the early phase of AS.

NF- $\kappa$ B binds with I $\kappa$ B $\alpha$  in the cytoplasm, forming a complex. Once I $\kappa$ B $\alpha$  is phosphorylated and degraded by kinase dependent phosphorylation, the free NF- $\kappa$ B will translocate into the nucleus and bind to the DNA sequences of pro-inflammatory factors, promoting the expression of their DNA sequences. Curcumin may inhibit various inflammatory

responses that are induced by these pro-inflammatory factors, such as ox-LDL. Its inhibitory potency is associated with the inhibition of ox-LDL induced phosphorylation and degradation of I $\kappa$ B $\alpha$ ; phosphorylation and nuclear translocation of P65; and transcription of the target gene, NF- $\kappa$ B (43). The present data showed that ox-LDL could boost the levels of nuclear NF- $\kappa$ B p65 and cytoplasmic p-I $\kappa$ B $\alpha$  as well as reduce the content of cytoplasmic I $\kappa$ B $\alpha$ . This provided a strong case for the hypothesis that ox-LDL induces the degradation of I $\kappa$ B $\alpha$  and nuclear translocation of p65. Curcumin significantly reduced the expression levels of nuclear NF- $\kappa$ B p65 and cytoplasmic p-I $\kappa$ B $\alpha$ , while the content in cytoplasm was raised by curcumin compared to that of the foam cell model group. The results revealed that curcumin may suppress the activation of the NF- $\kappa$ B pathway effectively and that curcumin can reliably inhibit NF- $\kappa$ B.

Li *et al* (44) discovered that ox-LDL could upregulate mir146a expression by activating the NF- $\kappa$ B pathway, while over expressed mir146a can reverse regulate macrophage maturation by inhibiting the production of CD86 and CD80. As such, there may be a relationship between miRNA and NF- $\kappa$ B. According to the present data, ox-LDL may promote the expression of miR33a, while curcumin reduces its expression. The results showed that curcumin is indirectly involved in regulating the expression of miR33a by blocking the activation of the NF- $\kappa$ B signaling pathway. A previous study (39) confirmed that there is a connection between miR33a and the cholesterol efflux. Accordingly, it was speculated that there existed an association between NF- $\kappa$ B and miR33a with proteins involved in the transfer of cholesterol and in the cholesterol efflux. The present results revealed that the NF- $\kappa$ B inhibitor, PDTC, and miR33a inhibitors promoted cholesterol efflux and expression of ABCA1 at both the mRNA and protein levels. Similarly, the effect of the combination of curcumin with NF- $\kappa$ B inhibitor or miR33a inhibitor, was more significant than that of curcumin, NF- $\kappa$ B inhibitor or miR33a inhibitor used alone. ELISAs demonstrated that PDTC and miR33a inhibitor could both reduce the content of IL-6, TNF- $\alpha$  and MCP-1, and the synergistic effect of curcumin with PDTC or miR33a inhibitor is more stronger than that of curcumin, NF- $\kappa$ B inhibitor or miR33a inhibitor used independently. The results indicated that the NF- $\kappa$ B/miR33a pathway participated in the expression of IL-6, TNF- $\alpha$  and MCP-1. Curcumin may inhibit the secretion of IL-6, TNF- $\alpha$  and MCP-1 through the NF- $\kappa$ B/miR33a pathway.

TLR4 is a transmembrane, non-catalytic protein, which plays a key role in the initiation and progression of inflammation (45). It was discovered by Edfeldt *et al* (46) that there is a significantly raised expression of TLR4 in atherosclerotic plaques in human arteries. Michelsen *et al* (47) reported that knockdown of TLR4 can effectively reduce the area of atherosclerotic plaques and promote the stability of the plaques. Accordingly, inhibition of TLR4 favors prevention and reversal of AS. The present study showed that ox-LDL significantly promoted the expression of TLR4 at both the mRNA and protein levels, while curcumin significantly reduced its expression. There was no evident difference between the TLR4 isotype Ab group and the model group, thus non-specific interference was excluded. Curcumin suppressed the TLR4 pathway and inhibited the signal transduction in cells. According

to Baker *et al* (48), the NF- $\kappa$ B signaling pathway is downstream of the TLR4-mediated signaling pathway and various external stimuli can regulate the NF- $\kappa$ B pathway through the TLR4 pathway. This leads to the upregulation of a variety of pro-inflammatory cytokines and enhancing the inflammatory response. The current study indicated that curcumin regulates the NF- $\kappa$ B/miR33a signaling pathway, therefore it was speculated that curcumin may increase the expression of ABCA1 and enhance cholesterol efflux, thereby inhibiting the expression of inflammatory factors and reducing the secretion of IL-6, TNF- $\alpha$  and MCP-1 through the TLR4/NF- $\kappa$ B/miR33a signaling pathway. The present data revealed that curcumin or TLR4 specific antibodies significantly reduced the content of miR33a mRNA, nuclear NF- $\kappa$ B p65 and cytoplasmic p-I $\kappa$ B $\alpha$  that was stimulated by ox-LDL. Meanwhile, the content of cytoplasmic I $\kappa$ B $\alpha$  was increased, as well as the expression of ABCA1 and cholesterol efflux. Secretion of IL-6, TNF- $\alpha$  and MCP-1 was decreased. It was also discovered that the effect of combining curcumin and TLR4 was stronger than that of curcumin or TLR4-specific antibodies used independently. Finally, the results confirmed that the TLR4/NF- $\kappa$ B/miR33a signaling pathway is related to the expression of IL-6, TNF- $\alpha$  and MCP-1 and that curcumin increases the expression of ABCA1 and the cholesterol efflux rate, whilst also reducing secretion of IL-6, TNF- $\alpha$  and MCP-1.

In conclusion, it was confirmed that curcumin could increase the expression of ABCA1, a protein associated with cholesterol transfer, and promote cholesterol efflux, as well as reduce secretion of IL-6, TNF- $\alpha$  and MCP-1. The mechanism behind these responses is associated with the TLR4/NF- $\kappa$ B/miR33a signaling pathway (Fig. 20). Curcumin was shown to be an effective regulator of lipid metabolism and an anti-inflammatory agent, hence curcumin may be a promising new drug for the treatment of various chronic reactions, including AS.

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## Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

## Authors' contributions

YZ and CL designed all the experiments and carried out the experiments. JF performed the statistical analysis, created the figures, and wrote the manuscript. JFL and ZCF helped with designing the experiments and assisted in manuscript writing.

All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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