

## Original Article

# Triptolide suppresses secretion of inflammatory factors and promotes expression of ABCA1

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**Abstract:** Purpose: The aim of the present study was to explore the roles and molecular mechanisms of triptolide in the progression of atherosclerosis. Method: Genotypes of APOE knock out genes and corresponding wild type genes were tested via PCR assay. Levels of total cholesterol and total triglycerides were detected according to kit protocol. Fat tissues in livers were stained and observed by oil O staining. APOE<sup>-/-</sup> mice were selected by PCR assay, oil O staining, and H&E staining. ApoE mice were randomly divided into three groups: ApoE<sup>-/-</sup> mice, wild type mice, and ApoE<sup>-/-</sup> mice treated with 5 µg/kg TPL and 50 µg/kg of triptolide twice every day. Concentrations of inflammatory cytokines were determined by ELISA assay. Expression of ABCA1 was detected by Western blotting. Results: Body weight showed no statistical differences between the triptolide treated group and ApoE<sup>-/-</sup> group. Triptolide decreased levels of total cholesterol and total triglycerides in ApoE<sup>-/-</sup> mice. Secretion of inflammatory cytokines in macrophages was significantly decreased in 50 µg/mL of triptolide treated ApoE<sup>-/-</sup> mice. Triptolide increased expression of ABCA1 in macrophages. Conclusion: Triptolide treatment decreased secretion of inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 and promoted expression of ABCA1 in macrophages. Thus, triptolide may be a new agent for clinical therapy of atherosclerosis.

**Keywords:** Triptolide, inflammatory cytokines, ApoE, ABCA1

## Introduction

Atherosclerosis is an important risk factor of many cardiovascular diseases, such as coronary heart disease, cerebral infarction, and myocardial infarction [1]. The main feature of cardiovascular and cerebrovascular diseases is atherosclerosis, characterized by lipid deposition and inflammatory infiltration [2]. In China, dietary habits of residents have changed from carbohydrate-based to high-fat and high-protein, leading to high incidence of atherosclerosis [3]. Until now, cardiovascular and cerebrovascular diseases have occupied an important position in China's lethal diseases [4]. Relevant pathological basis and treatments of atherosclerosis have become urgent issues to be clarified. Clarification would be beneficial in the field of medicine at home and abroad for the prevention and treatment of related cardiovascular and cerebrovascular diseases.

Occurrence of atherosclerosis is not only a simple dyslipidemia, but it is caused by a combina-

tion of various factors. Known risk factors are hypertension, hyperlipidemia, eating habits, age, stress, diabetes, infections, and smoking. It has been reported that occurrence and progression of atherosclerosis is accompanied by occurrence of inflammatory reactions and disorders of lipid metabolism [5]. Chronic inflammation normally breaks the balance of related lipid metabolism, reduces the biological activity of the blood vessel wall, and finally causes arterial atherosclerosis [6]. Specifically, inflammatory cells often secrete inflammation-related chemokines and various cytokines (such as TNF-α, IL-1β and IL-6 etc.), which promote peripheral blood mononuclear cells differentiating into macrophages and migrating to the arterial intima, inducing progression of atherosclerosis [7]. Lipids are swallowed by the macrophages to form foam cells. Accumulation of foam cells forms the lipid core, which constitutes an atherosclerotic plaque, together with the fibrous cap and inflammatory factors [8].

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In mammals, all nucleated cells can synthesize cholesterol, but they do not have the ability to degrade cholesterol. Excess cholesterol will be stored in or released from the cytoplasm. Generally, to maintain the balance of cholesterol, excessive cholesterol in peripheral cells can be eliminated by reverse cholesterol transport (RCT) pathways by transferring protein to the liver, eventually excreting it in the liver with bile. ATP-binding cassette subfamily A, member 1 (ABCA1), is involved in reverse cholesterol transport (RCT) and required for apoA-I-mediated cholesterol efflux. ABCA1 is predominantly expressed at the plasma membrane and widely expressed in almost all tissues, with higher expression in the liver, intestine, adrenal glands, lungs, brain, and macro-saline cells. ABCA1 is normally combined with apolipoprotein A-I and other apolipoproteins, such as ApoE, in the initial stages of RCT. Thus, the present study aimed to detect whether treatment with anti-inflammatory agents would promote RCT in macrophages of ApoE<sup>-/-</sup> mice.

Triptolide is an epoxy diterpene lactone compound, extracted from roots, leaves, flowers, and fruits of the genus *Tripterygium*. It has been reported that triptolide possesses potent anti-inflammatory effects [9, 10]. The Zhou group found that inflammatory response was attenuated by triptolide, partly by inhibiting NF- $\kappa$ B signaling pathways [11]. Inhibition of inflammatory reactions of triptolide by inhibiting NF- $\kappa$ B signaling pathways was also found in LPS-induced acute lung injuries in a murine model [12, 13]. Moreover, in an adjuvant-induced arthritis (AA) murine model of rheumatoid arthritis (RA), triptolide effectively alleviated RA by inhibiting neutrophil inflammatory function [14]. Results suggest that triptolide might work as a potential therapeutic agent for anti-inflammatory response. However, the roles and molecular mechanisms of triptolide in the progression of atherosclerosis have not been clearly clarified. The present study used an ApoE<sup>-/-</sup> murine model of atherosclerosis, exploring the roles and molecular mechanisms of triptolide *in vivo* in the progression of atherosclerosis.

### Materials and methods

#### *Animals and mice grouping*

In this study, 6-8-week-old apolipoprotein E knockout mice (n=6) and corresponding back-

ground mice (n=6) were housed in specific pathogen-free conditions. The 8-week-old male wild type C57BL/6 mice with the same genetic background as ApoE<sup>-/-</sup> mice were used as negative controls. The mice were divided into four groups, including ApoE<sup>-/-</sup> mice treated with 5  $\mu$ g/kg of triptolide and 50  $\mu$ g/kg of triptolide, ApoE<sup>-/-</sup> mice, and negative control mice. ApoE<sup>-/-</sup> mice were fed with 5  $\mu$ g/kg triptolide or 50  $\mu$ g/kg of triptolide twice every day, respectively. Each group contained 6 mice. All mice were free to eat and drink. The ambient temperature was kept at (22 $\pm$ 2) $^{\circ}$ C, with a humidity of about 50% to 60%.

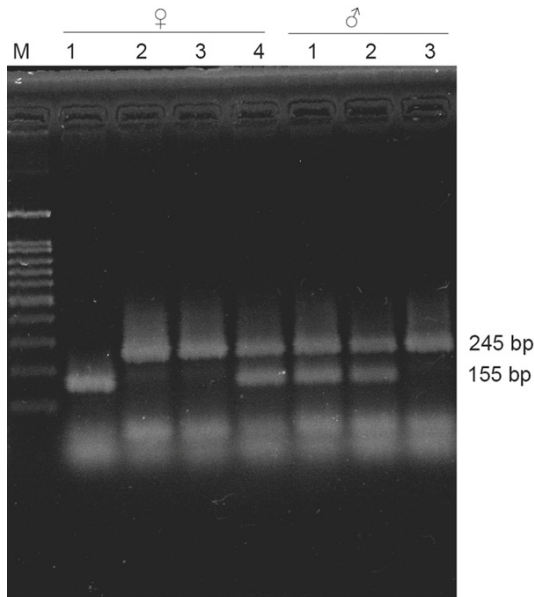
#### *PCR assay*

The mice were numbered when they were caged, including four female mice and three male mice. DNA samples were extracted with a DNA extraction kit (Biotek, Beijing, China) in ApoE<sup>-/-</sup> mice with the genetic background of C57BL/6 and the same background with C57BL/6 mice. DNA was taken from blood in the tail tip of each mouse. The mouse genotype was identified by PCR assay. Primer sequences used were as follows: Primer 1: 5'-GCCTAGCCG-AGGGAGAGCCG-3', Primer 2: 5'-TGTGACTTGGG-AGCTCTGCAGC-3', Primer 3: 5'-GCCGCCCGAC-TGCATCT-3'. Thermal cycling conditions were 95 $^{\circ}$ C for 10 minutes, followed by 30 cycles of 30 seconds at 95 $^{\circ}$ C, 30 seconds at 65 $^{\circ}$ C, and 30 seconds at 72 $^{\circ}$ C, then 10 minutes at 72 $^{\circ}$ C for elongation. The length of DNA fragments was 155 bp/155 bp for wild type mice, 245 bp/245 bp for ApoE<sup>-/-</sup> mice, and 155 bp/245 bp for heterozygous type mice.

#### *ELISA assay*

Kits for total cholesterol (Cat. No. YG-elisa-01-138) and total triglycerides (Cat. No. YG-elisa-01273) were both obtained from Yinggongbiotec Corporation (Shanghai, China). Total cholesterol and total triglycerides in plasma were determined according to kit protocol. Briefly, peritoneal macrophages were obtained in ApoE<sup>-/-</sup> mice treated with 5  $\mu$ g/kg triptolide, 50  $\mu$ g/kg of triptolide, ApoE<sup>-/-</sup> mice, and negative control mice. The single cell suspension was prepared and cell density was adjusted into 5 $\times$ 10<sup>4</sup> cells/mL and treated with 12.5  $\mu$ g/mL of ox-LDL for 48 hours. Next, cell supernatant was harvested and concentrations of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 were determined with ELISA kits (Neobio-

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**Figure 1.** APOE<sup>-/-</sup> mice are used as animal model and the genotype is identified by PCR assay. Four female mice and three male mice with the C57BL/6 genetic background were selected to detect the genotype of ApoE by PCR assay. Specifically, blood samples from the tails of the mice were collected and DNA samples were extracted, as described in Materials and methods. Three primers were used to detect the genotype of ApoE gene with WT allele of 155 bp and ApoE allele of 245 bp and the heterozygote with WT allele and ApoE allele of a mixture (155 bp and 245 bp).

science, Beijing, China), according to manufacturer instructions, using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA). The experiment was performed and repeated twice in triplicate. Total cholesterol and total triglycerides in plasma were detected using the corresponding ELISA kit. Specifically, the mice were divided into 4 groups, including ApoE<sup>-/-</sup> mice, 5 µg/kg triptolide-treated ApoE<sup>-/-</sup> mice, 50 µg/kg of triptolide-treated ApoE<sup>-/-</sup> mice, and C57BL/6 mice. At the 20<sup>th</sup> week, blood samples were collected after 12-16 hours of fasting by cutting a piece of the tail of each mouse. Total cholesterol and total triglycerides in plasma were determined by specific kits according to kit protocol.

### HE staining and Oil O red staining

Liver tissues were obtained from ApoE<sup>-/-</sup> mice and control mice. They were fixed in 4% paraformaldehyde for 2 hours, then transferred to 20% sucrose for dehydration. Paraffin-embedding was then carried out for HE staining and Oil O red staining, as previously described [15-17].

### Western blot

The 5 µg/kg of TPL and 50 µg/kg of TPL-treated ApoE<sup>-/-</sup> mice, as well as the control group, including ApoE<sup>-/-</sup> mice and control mice with the same background, were sacrificed at the 20<sup>th</sup> week. Peritoneal macrophage cells were collected and cultured at a density of  $3 \times 10^5$  cells/mL. Eight hours later, the cells were treated with 12.5 µg/ul oxLDL for 24 hours. Levels of ABCA1 were detected by Western blotting, as previously described [18-20]. Antibodies used in the experiment were as follows: Anti-ABCA1 (NB400-105) antibody was purchased from Novus Biologicals. Anti-β-Actin antibody (cat. no. sc-47778) is a mouse monoclonal antibody obtained from Santa Cruz Biotechnology, Inc.

### Statistical analysis

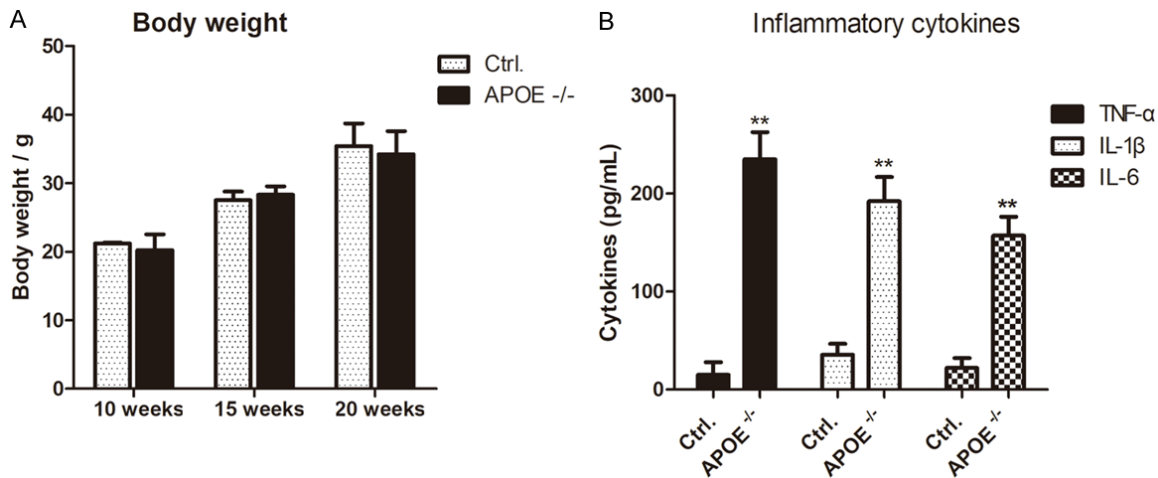
Data were analyzed with SPSS software. Data are shown as the mean ± standard error of the mean. Data between the two groups were compared with t-test and multiple comparisons were analyzed using one-way ANOVA and Tukey's test. Expression levels of ABCA1 in macrophage-derived foam cells were repeated three times. \*P<0.05, \*\*P<0.01, compared with control group.

## Results

### APOE<sup>-/-</sup> mice used as an animal model and genotypes identified by PCR assay

Apolipoprotein E (ApoE) gene-deficient mice are currently the most commonly used animal models in atherosclerosis research. The current study chose ApoE<sup>-/-</sup> mice as animal models to test the roles of triptolide in the progression of atherosclerosis. First, this study tested the genotype of each mouse by PCR assay, as described in Materials and methods. As shown in **Figure 1**, ApoE<sup>-/-</sup> mice had the allele of 245 bp and wild type mice had the allele of 155 bp. The ApoE gene in female mouse No. 1 was wild type and the ApoE gene in female mouse No. 2 and 3 was ApoE knockout type. No. 4 of the female mice had a mixture allele of ApoE knockout and wild type. It was the mixture of 155 bp and 245 bp. For the male mice, No. 1 male mouse and No. 2 male mouse had the mixture of 155 bp and 245 bp, while the genotype of No. 3 male mouse was ApoE genetic defective type. Thus, the mice used in the experiment were the offspring of the No. 3 male mouse and No. 2 and

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**Figure 2.** The major indicators are compared between ApoE<sup>-/-</sup> mice and its corresponding mice. A. Body weight shows no statistical differences between ApoE<sup>-/-</sup> mice and its corresponding mice. B. Inflammatory cytokines were determined by ELISA assay in ApoE<sup>-/-</sup> mice and its corresponding wild type mice. \*\* $P < 0.01$ , compared with control group.

No. 3 female mice. Additionally, corresponding wild type mice were collected with the same age. They were used as negative controls, as they had the same genetic background with ApoE<sup>-/-</sup> mice.

*Body weight shows no statistical differences between ApoE<sup>-/-</sup> mice and corresponding mice*

Body weight is an important indicator in the animal experiment. Here, ApoE<sup>-/-</sup> mice and corresponding mice were free to eat and drink in the cages. They were fed for 20 weeks. The body weight of each mouse was measured and recorded every two days. Body weight was compared between ApoE<sup>-/-</sup> mice and wild type mice at the 10<sup>th</sup> week, 15<sup>th</sup> week, and 20<sup>th</sup> week. As shown in **Figure 2**, there were no statistical differences between the ApoE<sup>-/-</sup> group and corresponding group.

*Secretion of inflammatory cytokines in macrophages determined by ELISA assay*

Chronic inflammation promotes the progression of atherosclerosis. Thus, the current study detected inflammatory cytokine production in ApoE<sup>-/-</sup> mice and wild type mice. Macrophages were collected from ApoE<sup>-/-</sup> mice and wild type mice, then treated with 20  $\mu\text{g}/\mu\text{l}$  of oxLDL for 24 hours. Levels of inflammatory cytokines were tested by ELISA assay. As shown in **Figure 2B**, levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were significantly increased in macrophages of ApoE<sup>-/-</sup>

mice, compared with those of wild type mice (\*\* $P < 0.01$ ). As inflammatory cytokines are important inducers in initiating atherosclerosis, ApoE<sup>-/-</sup> mice produced more inflammatory cytokines and were more prone to producing atherosclerosis.

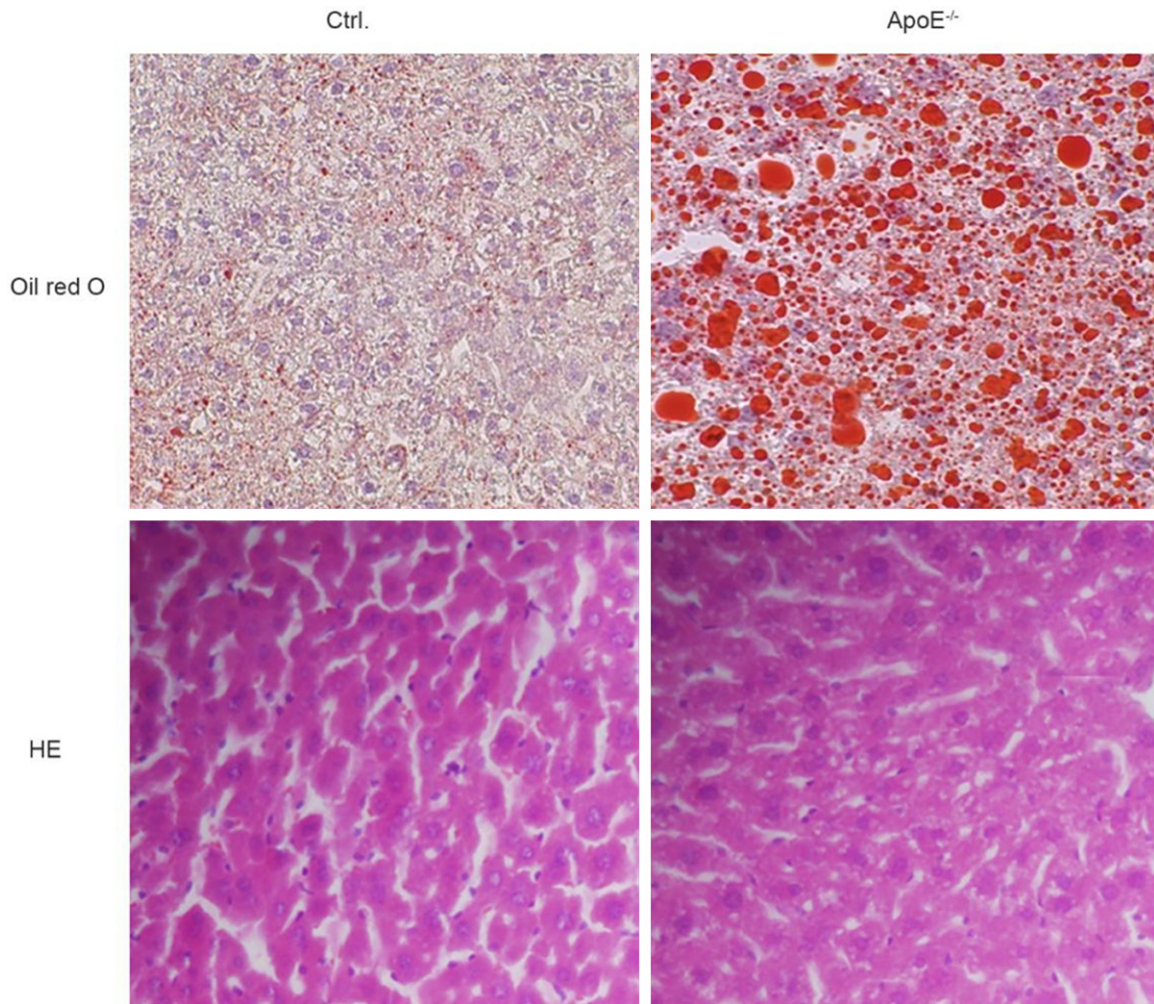
*Lipid droplets are more and larger in livers of ApoE<sup>-/-</sup> mice than that of wild type mice*

To observe whether the morphology of liver cells was intact, H&E staining of livers in ApoE<sup>-/-</sup> mice and mice with the same corresponding background was conducted. As shown in **Figure 3**, H&E staining results showed that the morphology of liver cells was intact, the nucleus was not obviously various, and the hepatic lobule was intact. Moreover, oil red O staining was performed to detect fat in liver tissues. Oil red O staining results showed that fat lipid droplets were more and larger in livers of ApoE<sup>-/-</sup> mice than in wild type mice, suggesting that fat spontaneously accumulated in ApoE<sup>-/-</sup> mice (**Figure 3**).

*Triptolide does not affect the body weight of ApoE<sup>-/-</sup> mice and decreases total cholesterol and total triglycerides in ApoE<sup>-/-</sup> mice*

Body weight is an important indicator reflecting whether triptolide is toxic or not. Thus, this study measured the body weights of ApoE<sup>-/-</sup> mice and control mice every two days. As shown in **Figure 4A**, body weight was gradually and

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**Figure 3.** Lipid droplets are more and larger in livers of ApoE<sup>-/-</sup> mice than in wild type mice. Oil red O staining and H&E staining of livers in ApoE<sup>-/-</sup> mice and mice with the same corresponding background.

stably increased in the three groups of mice. There were no statistical differences between ApoE<sup>-/-</sup> mice and 50 µg/kg of triptolide-treated ApoE<sup>-/-</sup> mice ( $P > 0.05$ ). Data revealed that 50 µg/kg of triptolide caused no toxicity to the mice. Thus, it was an appropriate drug dose in the experiment.

### *Triptolide treatment decreases levels of total cholesterol and total triglycerides in ApoE<sup>-/-</sup> mice*

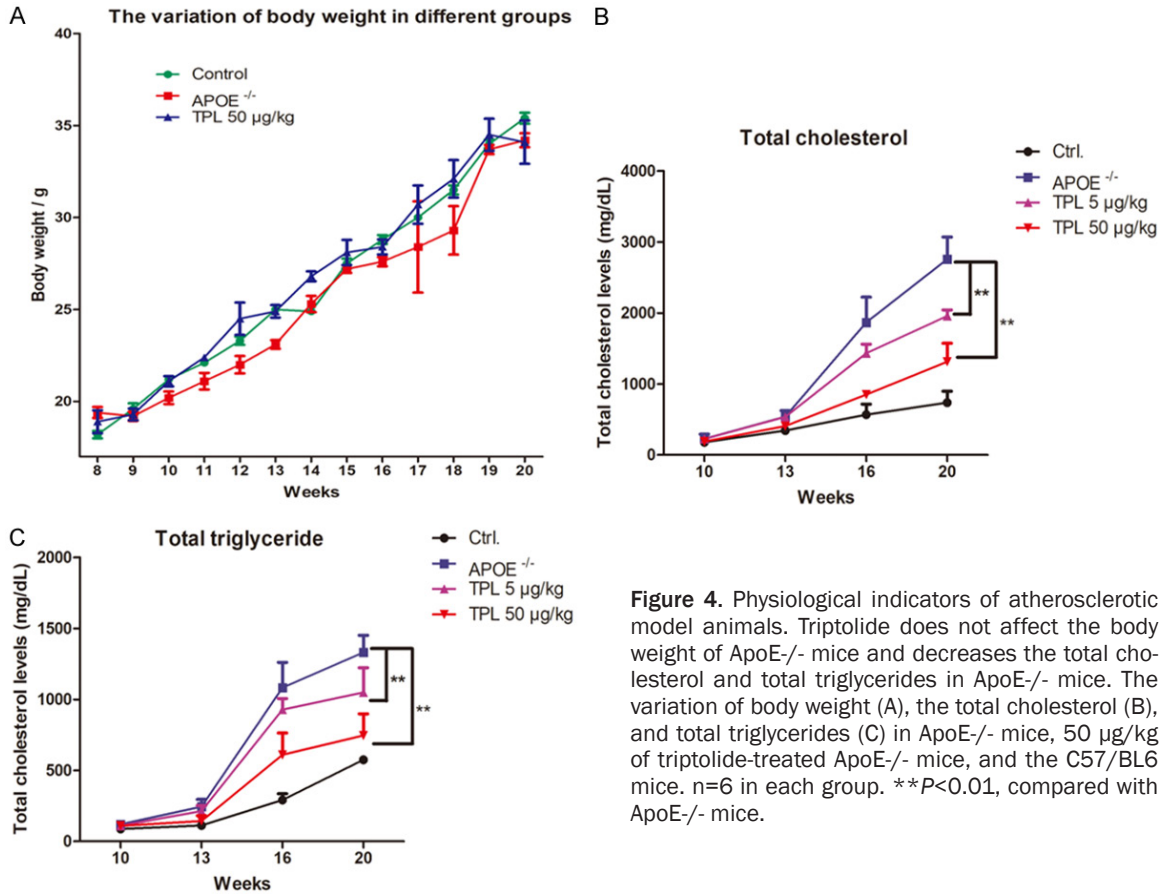
Abnormal lipid metabolism is accompanied with progression of atherosclerosis. Plasma cholesterol and triglycerides are risk factors inducing and promoting the progression of atherosclerosis. Here, total cholesterol and total triglycerides were determined, as described in Materials and methods. Results showed that

total cholesterol (**Figure 4B**) and total triglyceride (**Figure 4C**) levels were gradually increased since the 10<sup>th</sup> week, compared with control mice with corresponding backgrounds. Interestingly, ApoE<sup>-/-</sup> mice treated with 5 µg/kg of triptolide and 50 µg/kg of triptolide had lower levels of total cholesterol and total triglycerides than ApoE<sup>-/-</sup> mice (\*\* $P < 0.01$ , compared with ApoE<sup>-/-</sup> mice).

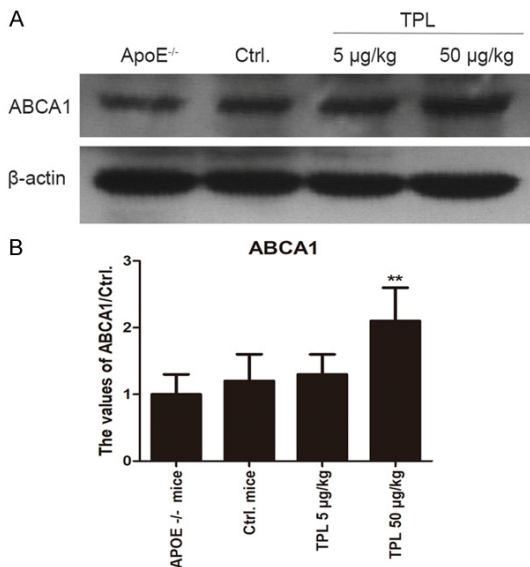
### *Triptolide contributes to expression of ABCA1 in macrophages*

Macrophage-derived foam cells have an important role in the progression of atherosclerosis. According to the *in vitro* experiment, macrophage cells were obtained from each group of triptolide treated ApoE<sup>-/-</sup> mice and control group. Macrophage-derived foam cells were

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**Figure 4.** Physiological indicators of atherosclerotic model animals. Triptolide does not affect the body weight of ApoE<sup>-/-</sup> mice and decreases the total cholesterol and total triglycerides in ApoE<sup>-/-</sup> mice. The variation of body weight (A), the total cholesterol (B), and total triglycerides (C) in ApoE<sup>-/-</sup> mice, 50 µg/kg of triptolide-treated ApoE<sup>-/-</sup> mice, and the C57/BL6 mice. n=6 in each group. \*\*P<0.01, compared with ApoE<sup>-/-</sup> mice.



**Figure 5.** Triptolide contributes to expression of ABCA1 in macrophages. The ApoE<sup>-/-</sup> mice were treated with 5 µg/kg of TPL and 50 µg/kg of TPL every twice day. At the 20<sup>th</sup> week, peritoneal macrophage cells were obtained and treated with 12.5 µg/ml oxLDL for 24 hours. A. Expression of ABCA1 was detected by Western blotting. TPL: triptolide. B. Expression

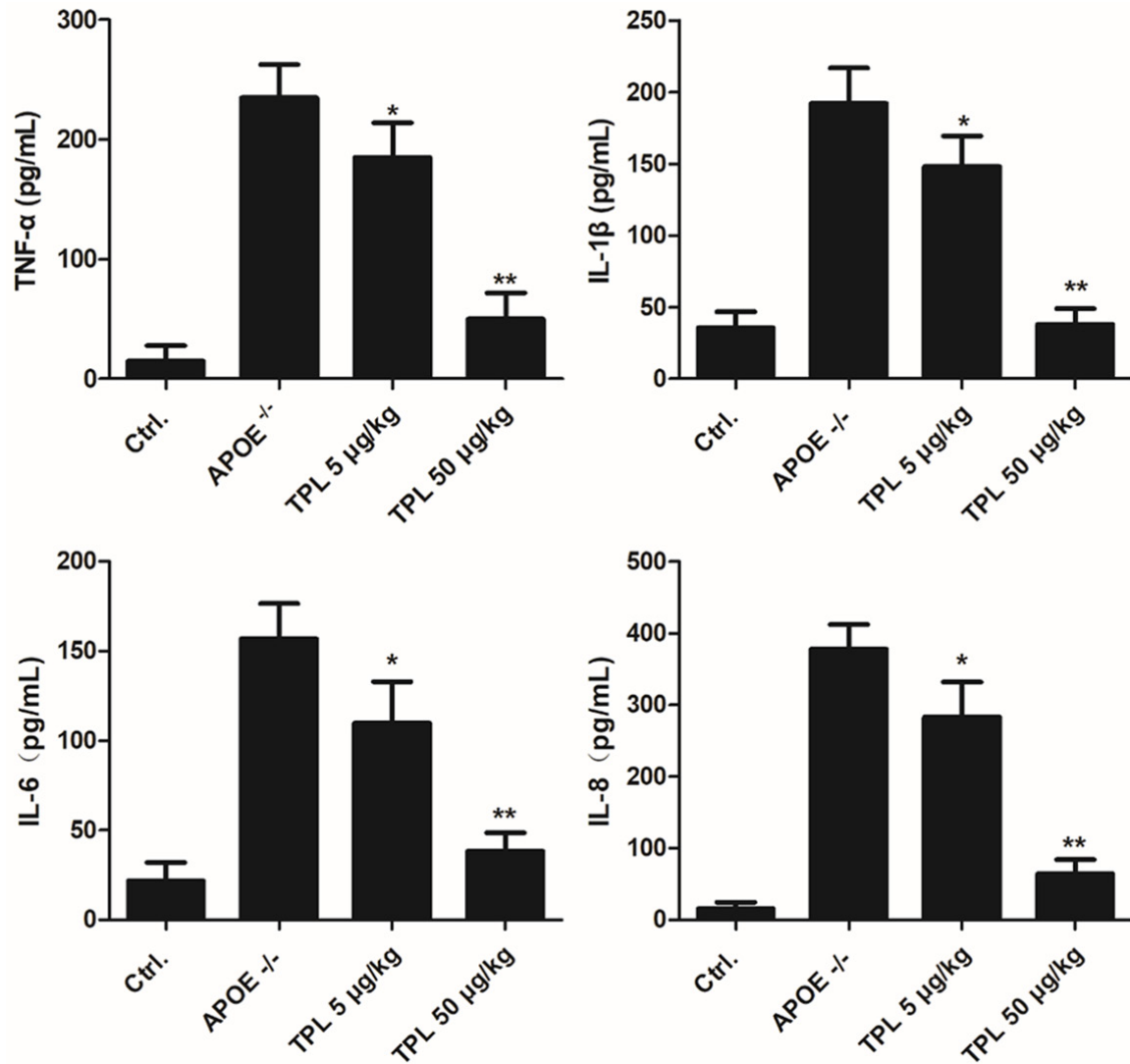
levels of ABCA1 was shown in histogram. Levels of ABCA1 in ApoE<sup>-/-</sup> mice were used as negative control. \*\*P<0.01, compared with the level of ABCA1 in ApoE<sup>-/-</sup> mice.

induced by stimulation with 12.5 µg/ml of oxLDL for 24 hours. Expression levels of ABCA1 were determined by Western blot. As shown in **Figure 5**, levels of ABCA1 were greatly increased in macrophage cells from 50 µg/kg of triptolide-cultured ApoE<sup>-/-</sup> mice. However, levels of ABCA1 in 5 µg/kg of triptolide-treated ApoE<sup>-/-</sup> mice were not obviously varied, compared to ApoE<sup>-/-</sup> mice and negative control mice.

*Secretion of inflammatory cytokines obviously inhibited in TPL-treated mice*

Chronic inflammatory reactions promote the progression of atherosclerosis. This study detected the secretion of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8, by ELISA assay. As shown in **Figure 6**, secretion of TNF-α, IL-1β, IL-6, and IL-8 was gradually decreased,

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**Figure 6.** Secretion of inflammatory cytokines is obviously inhibited in TPL-treated mice. The mice were divided into four groups and fed for 20 weeks. Peritoneal macrophage cells were treated with 12.5  $\mu\text{g}/\text{ul}$  oxLDL. Secretion of inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8, was determined by ELISA assay, as described in Materials and methods. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with ApoE<sup>-/-</sup> mice.

in a dose-dependent manner. Specifically, macrophage cells obtained from 50  $\mu\text{g}/\text{kg}$  triptolide-treated ApoE mice produced less TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 than ApoE<sup>-/-</sup> mice (**Figure 6**). Present results demonstrate that triptolide treatment decreased the secretion of inflammatory cytokines and inhibited the formation of foam cells.

### Discussion

Cardiovascular and cerebrovascular diseases are commonly seen with high incidence, characterized by chronic inflammation and abnor-

mal lipid metabolism [21, 22]. The present study used an ApoE<sup>-/-</sup> mice model, exploring the anti-inflammatory roles of triptolide in the progression of atherosclerosis of mice. It was found that secretion of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 was decreased in mice treated with 50  $\mu\text{g}/\text{kg}$  of triptolide, while expression of ABCA1 was obviously increased in macrophages.

The current study found that expression of ABCA1 in macrophages from triptolide-treated ApoE<sup>-/-</sup> mice was significantly higher than that from negative control ApoE<sup>-/-</sup> mice, suggesting that triptolide treatment increased levels of

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ABCA1 and promoted reverse cholesterol transport. This was consistent with the results of Luo et al., in which they found that expression of anti-ATP-binding cassette transporter A1 in the macrophages of ApoE(-)/(-) mice was upregulated when treated by triptolide. However, expression of LXRalpha was not obviously changed [20]. Thus, triptolide treatment inhibited the formation of foam cells and suppressed the progression of atherosclerosis.

Triptolide has been reported to attenuate inflammatory response in various disease models [12, 13, 23, 24]. As the progression of atherosclerosis is accompanied by chronic inflammatory response, this study tested the roles of triptolide in the formation of atherosclerosis. The ApoE-/- mice model is usually used to study the development and treatment of atherosclerosis [25, 26]. This study also chose ApoE-/- mice as an animal model to test the roles of triptolide in progression of atherosclerosis. This was examined by testing levels of inflammatory cytokines. Data suggests that treatment with triptolide obviously decreased the production of inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8. Ingesting modified low-density lipoprotein and cholesterol efflux affect the formation of macrophage-derived foam cells [27-29]. In the present study, 12.5  $\mu$ g/mL of ox-LDL was used to treat macrophages in triptolide-treated ApoE-/- mice and negative control mice, aiming to induce the formation of macrophage-derived foam cells. Next, cytokines production was detected by ELISA assay, finding that foam cells in ApoE-/- treated with triptolide produced less inflammatory cytokines than control mice. Results suggest that triptolide showed potent effects, inhibiting inflammatory response in the progression of atherosclerosis.

In conclusion, triptolide showed obvious effects concerning the suppression of chronic inflammatory response. It also increased expression of ABCA1, promoting reverse cholesterol transport in foam cells. Therefore, triptolide might inhibit the formation of atherosclerosis in a mice model. Results suggest the promotion of triptolide as a new agent for clinical therapy of atherosclerosis.

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### Disclosure of conflict of interest

None.

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