

Effects of Atorvastatin on Oxidized Low-Density Lipoprotein-induced Proliferation, Apoptosis, and Matrix Metalloproteinase-9 Expression in Human Macrophages

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Abstract: Atorvastatin (ATV), an HMG-CoA reductase inhibitor, is utilized in the treatment of atherosclerotic diseases. Mononuclear Macrophages (MMs) are critical immune cells involved in atherosclerosis progression, participating in processes such as foam cell formation, cytokine secretion, and the development of fibrous cap and lipid core. To further elucidate the mechanism by which ATV exerts its therapeutic effects on atherosclerosis through the modulation of healthy human MM biological processes, this work investigated the impact of ATV on the expression of matrix metalloproteinases-9 (MMP-9) in healthy human MMs induced by oxidized low-density lipoprotein (ox-LDL), along with mechanisms involved Mononuclear cells from healthy individuals were isolated using density gradient centrifugation and differentiated into macrophages, which were subsequently divided into three groups: Blank, ox-LDL, and ATV (0.01, 0.1, and 1.0 $\mu\text{mol/L}$). Cell proliferation was assessed using MTT assay, while apoptosis was evaluated by flow cytometry. The expression levels of interleukin (IL)-6, IL-1 β , tumor necrosis factor (TNF)- α , and MMP-9 mRNA were measured using quantitative real-time PCR, and MMP-9 protein EL was analyzed via Western blotting. Relative to the Blank group, ox-LDL group exhibited increased cell proliferation activity and reduced apoptosis rates, along with elevated mRNA expression levels of inflammatory genes IL-6, IL-1 β , and TNF- α . Additionally, both MMP-9 mRNA and protein expression levels were greatly higher ($P<0.05$). In contrast, the different concentrations of ATV groups showed decreased cell proliferation activity, increased apoptosis rates, and reduced mRNA expression levels expression levels of inflammatory genes IL-6, IL-1 β , and TNF- α , and decreased MMP-9 mRNA and protein expression levels versus ox-LDL group ($P<0.05$). Notably, as the concentration of ATV increased, the alterations in cell proliferation, apoptosis, inflammatory gene expression, and MMP-9 levels became more pronounced ($P<0.05$). In summary, ATV can downregulate MMP-9 expression and inhibit inflammatory gene expression, thereby influencing the proliferation and apoptosis processes of human MMs.

Keywords: Atorvastatin, Oxidized Low-density Lipoprotein, Matrix Metalloproteinases-9 and Mononuclear Macrophages

Introduction

Atherosclerosis is pathological basis for cardiovascular diseases, with endothelial cell injury, monocyte-macrophage proliferation, and vascular smooth muscle cell (VSMC) hyperplasia being significant pathological processes contributing to its development (Cheng *et al.*, 2023; Jin *et al.*, 2023). The progression of atherosclerosis is closely associated with macrophage dysfunction (Mubdt *et al.*, 2022; Wculek *et al.*, 2022). Oxidized low-density lipoprotein (ox-LDL) induces the transformation of

macrophages into foam cells and promotes the secretion of pro-inflammatory cytokines (such as interleukin (IL)-6 and tumor necrosis factor (TNF)- α) as well as matrix metalloproteinases (such as matrix metalloproteinase-9 (MMP-9)) (Attiq *et al.*, 2024). Among these, MMP-9 contributes to the degradation of collagen and elastin, thereby disrupting the fibrous cap structure; elevated MMP-9 expression is a hallmark of vulnerable plaques (Pogorielova *et al.*, 2022; Ramírez-Carracedo *et al.*, 2024). Therefore, targeting the regulation of MMP-9 expression may represent a novel strategy for plaque stabilization.

Atherosclerosis is a leading cause of coronary artery disease, with lipid metabolism disorders serving as its underlying pathological basis. Atorvastatin (ATV), a class of HMG-CoA reductase inhibitors, competitively blocks activity of HMG-CoA reductase, thereby inhibiting cholesterol biosynthesis (Kronborg *et al.*, 2023). By suppressing cholesterol production, ATV dramatically reduces LDL-cholesterol (C), apolipoprotein B, and triglycerides, thereby ameliorating the progression of atherosclerosis (Oh *et al.*, 2021). In addition to its lipid-lowering effects, ATV also inhibits VSMC proliferation and migration, while promoting their apoptosis, which can improve blood rheology and viscosity, consequently enhancing cardiac function, endothelial function, and coagulation parameters (Xue *et al.*, 2020). Therefore, ATV is widely applied for atherosclerotic diseases.

Although the MMP family comprises numerous members, MMP-9 has been selected as a focal point of investigation due to its distinct role in plaque instability. Clinical evidence indicates that MMP-9 expression is significantly elevated in vulnerable plaques compared to stable plaques and is directly associated with the risk of cardiovascular events (Konrad *et al.*, 2024). Therefore, elucidating the regulatory mechanisms of MMP-9 by ATV may provide a novel therapeutic target for enhancing plaque stability. This work analyzed the impacts of ATV on cell proliferation, apoptosis, and MMP-9 expression. The findings aimed to provide a theoretical foundation for understanding the pathogenesis of atherosclerotic diseases and for developing effective treatment strategies.

Related Research

As research on atherosclerotic diseases deepens, understanding their pathogenesis, screening for novel diagnostic and therapeutic biomarkers, and identifying treatment targets have become crucial areas of investigation.

LDL is subject to various factors that can induce oxidative modification in the bloodstream, leading to the formation of ox-LDL. ox-LDL can be internalized by macrophages via scavenger receptors, a process that promotes lipid accumulation and foam cell formation (Hao *et al.*, 2023). Moreover, ox-LDL exhibits enhanced cytotoxicity, causing damage to endothelial cells (Zhang *et al.*, 2022). It serves as an indicator for predicting the risk and prognosis of atherosclerosis. Cao *et al.* (2019) investigated RAW264.7 macrophages and found that ox-LDL induction led to the formation of foam cells, a decrease in cell viability, an enhance in intracellular lipid accumulation, and the emergence of a senescent phenotype (Hao *et al.*, 2019). Tomar *et al.* (2022) employed multisolvent simulation and other computational methods to analyze the role of the scavenger receptor ox-LDL receptor 1 (LOX-1) in atherosclerotic signaling pathways. They found that modulating the interaction between ox-LDL and LOX-1

could prevent or slow the progression of atherosclerosis. Ox-LDL acts crucially in atherosclerosis progression; therefore, reducing ox-LDL levels may help mitigate the progression of the disease and lower the risk of cardiovascular events.

MMs include bone marrow-derived monocytes, peripheral blood monocytes, and tissue macrophages. These cells are crucial immune components within the body, possessing significant functions such as infection defense, tumor suppression, and immune regulation. They are also capable of stimulating the proliferation, differentiation, and functional activity of neutrophils, monocytes, and macrophages (Zelepukin *et al.*, 2024; Zhang *et al.*, 2023). MMs act essentially in atherosclerosis progression. Zerneck *et al.* (2023) integrated twelve immune cell single-cell RNA sequencing (scRNA-seq) datasets from the aortas of healthy or atherosclerotic mice and identified subpopulations with distinct transcriptomic signatures within resident, inflammatory, and foam cell macrophage populations. Edgar *et al.* (2021) found that elevated extracellular glucose in macrophages promotes the expression of pro-inflammatory genes and pro-atherosclerotic functional characteristics through a glycolysis-dependent mechanism. Notably, different subtypes of MMs play distinct roles in the progression of atherosclerosis. For instance, M1 macrophages are primarily located in the lipid core region of atherosclerotic plaques and exhibit pro-inflammatory functions, while M2 macrophages are mainly found in the shoulders and periphery of the plaques, serving anti-inflammatory and reparative roles. The interplay and balance between these different macrophage subtypes collectively influence the progression of atherosclerosis.

ATV is a statin-class lipid-lowering medication that exerts its effects by inhibiting HMG-CoA reductase activity in the liver, thereby reducing cholesterol synthesis and lowering LDL-cholesterol and triglycerides in the bloodstream (Yin *et al.*, 2023). ATV has been shown to reduce the risk of cardiovascular diseases. Yao *et al.* (2023) demonstrated that PM2.5 exposure can lead to the occurrence of atherosclerotic events. However, administration of ATV via intraperitoneal injection in a PM2.5-induced atherosclerotic rat model can counteract the progression of atherosclerosis by inhibiting the release of pro-inflammatory cytokines and reducing serum lipid levels. Xiong *et al.* (2020) found that ATV can inhibit endoplasmic reticulum stress induced by ox-LDL and reduce the aortic plaque area in atherosclerotic mouse models, while also decreasing the infiltration of CD3⁺ T cells and macrophages within the local plaques. Li *et al.* (2023) utilized network pharmacology to predict the common targets of ATV in its anti-atherosclerotic and anti-tumor effects. They discovered that ATV dramatically downregulates the expression of co-pathogenic genes MMP-9, MMP-12, CD36, and FABP4, thereby exerting therapeutic effects against

atherosclerosis. These findings underscore the important role of ATV in the treatment of atherosclerosis.

ATV Treatment of ox-LDL-Induced Human MMs: Experimental Procedure

Sources of Experimental Subjects, Drugs, and Reagents

Fasting venous blood samples were collected from six healthy male individuals aged 25 to 41 years at the ** Hospital Health Examination Center in June 2023. The participants were informed about the study and provided written informed consent. The research protocol received approval from the Ethics Committee of ** Hospital. The sample size was determined a priori using G*Power software based on an anticipated large effect size ($d = 1.2$, derived from our pilot data), with $\alpha = 0.05$ and power $(1 - \beta) = 0.8$. This study included six healthy male volunteers. The sample size was determined based on a priori power analysis (Power = 0.8, $\alpha = 0.05$) and was consistent with the results of preliminary experiments. Future studies will expand the sample size and include participants of different sexes to enhance generalizability.

The utilized experimental drugs and reagents included fetal bovine serum (FBS) (MFCD00132239; Sigma-Aldrich, USA); RPMI-1640 culture medium (MFCD00217820; Sigma-Aldrich, USA); CD14 antibody (14-0149-82; Thermo Fisher Scientific, USA); ox-LDL (308068-14-6; Sigma-Aldrich, USA); ATV; 3-(4,5)-dimethylthiazolazo(-z-y1)-3,5-diphenyltetrazolium bromide (MTT) assay kit (298-93-1; Sigma-Aldrich, USA); Annexin V-FITC/PI apoptosis detection kit (V35123; Thermo Fisher Scientific, USA); PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) reverse transcription kit (RR047A; Beijing Baorui Medical Technology Co., Ltd., China); TB Green® Premix Ex Taq™ II (Tli RNaseH Plus), Bulk real-time fluorescence quantitative PCR kit (RR820L; Beijing Baorui Medical Technology Co., Ltd., China); Trizol reagent (15596026CN), Radio Immunoprecipitation Assay (RIPA) lysis buffer (89901), Bicinchoninic Acid (BCA) protein assay kit (23225), Polyvinylidene fluoride (PVDF) membrane (22860), MMP-9 antibody (MA5-32705), β -actin antibody (MA1-140), goat anti-human IgG antibody (62-8420), and Enhanced chemiluminescence (ECL) substrate (34577) (all from Thermo Fisher Scientific, USA).

Isolation, Cultivation, and Identification of Human MMs

According to the density gradient centrifugation methodology (Vijay *et al.*, 2023), heparinized venous blood was divided into ten aliquots of 3 mL each, which were then mixed with an equal volume of RPMI-1640 medium. The mixture was slowly applied to centrifuge tubes containing an equal proportion of lymphocyte separation medium and centrifuged at 2000 rpm for 15

minutes. The cloudy intermediate lymphocyte layer was collected and mixed with 3 mL of phosphate-buffered saline (PBS), then centrifuged at 1500 rpm for washing the cells three times. Subsequently, 5 mL of RPMI-1640 medium plus 10% FBS was applied and mixed. The cell suspension was transferred to a 12-well plate at a volume of 2 mL/well and incubated in a 37°C, 5% CO₂ humidified incubator (Model 3131; Thermo Fisher Scientific, USA). Cells at the fourth passage and in the logarithmic growth phase were identified. Initially, cells were cultured for 48 hours in serum-free RPMI-1640 medium with 40 ng/mL PAM until they differentiated into macrophages, after which the cells were rinsed thrice with RPMI-1640 medium. Identification of monocyte-derived macrophages was performed using a specific fluorescently labeled CD14 antibody.

Grouping and Treatment of Human MMs

Human monocyte-derived macrophages were randomly rolled into five groups. The preparation and concentration verification of ox-LDL (100 μ g/mL) were performed using the BCA assay to ensure consistency across experimental groups. All reagents were from the same lot number, and standardized operating procedures were followed to ensure reproducibility:

1. Blank group: conventional culture of human monocyte-derived macrophages.
2. Ox-LDL group: cultured human monocyte-derived macrophages were induced with medium containing 100 μ g/mL ox-LDL for 48 hours.
3. 0.01 μ mol/L ATV group (0.01 μ mol/L ATV): after 2 hours of induction with ox-LDL, human monocyte-derived macrophages were cultured for 48 hours in medium containing 0.01 μ mol/L ATV.
4. 0.1 μ mol/L ATV group (0.1 μ mol/L ATV): after 2 hours of induction with ox-LDL, human monocyte-derived macrophages were cultured for 48 hours in medium containing 0.1 μ mol/L ATV.
5. 1.0 μ mol/L ATV group (1.0 μ mol/L ATV): after 2 hours of induction with ox-LDL, human monocyte-derived macrophages were cultured for 48 hours in medium containing 1.0 μ mol/L ATV.

MTT Assay for Detecting Proliferation of Human MMs

Human monocyte-derived macrophages were seeded at 2×10^4 cells per well in a 96-well plate. Once the cell growth density reached over 85%, cells were subjected to treatment as per the designated groups. The cells were further cultured for 24, 48, 72, and 96 hours, during which 10 μ L of 5 mg/mL MTT solution was applied to each well. After a 4-hour incubation period, the culture was terminated, and the original medium was discarded. Then, 100 μ L of dimethyl sulfoxide was applied to each well, mixed thoroughly, and incubated for an additional 10

minutes. The optical density at 570 nm wavelength was measured using an automated microplate reader (Model YP-96-1; Shandong Youyunpu Optoelectronic Technology Co., Ltd., China).

Detection of Apoptosis in Human MMs by Flow Cytometry

After treatment, cells from each group were collected and rinsed thrice with PBS to remove residual culture medium. A single-cell suspension of 200 μ L was prepared. Subsequently, 10 μ L of Annexin V-FITC working solution and 5 μ L of PI working solution were applied, followed by 300 μ L of 1 $\times$ binding buffer. The mixture was gently mixed and incubated in the dark at 25°C for 15 minutes. Apoptosis rates were then assessed employing a flow cytometer (CytoFLEX SRT; Beckman Coulter, USA).

Detection of mRNA Expression in Human MMs by Real-Time Quantitative PCR

After treatment, cells from each group were harvested and lysed thoroughly using Trizol reagent to extract RNA. The concentration and purity of the extracted RNA were assessed. Following the instructions provided with the reverse transcription kit, RNA was reverse transcribed to synthesize complementary DNA (cDNA). Subsequently, according to the guidelines of the real-time quantitative PCR kit, cDNA was used as a template to determine the relative expression levels of the target genes IL-6, IL-1 β , TNF- α , and MMP-9 mRNA. IL-6: (upstream) 5'-ACTCACCTCTTCAGAACGAATTG-3', (downstream) 5'-CCATCTTTGGAAGGTTTCAGGTTG-3'; IL-1 β : (upstream) 5'-AGCTACGAATCTCCGACCAC-3', (downstream) 5'-CGTTATCCCATGTGTCGAAGAA-3'; TNF- α : (upstream) 5'-AGGTGTTTATACGGGAGCTGA-3', (downstream) 5'-GCATTGGTCTGCAAGTGAATCTC-3'; MMP-9: (upstream) 5'-GGGACGCAGACATCGTCATC-3', (downstream) 5'-TCGTCATCGTCGAAATGGGC-3'; GAPDH: (upstream) 5'-CTCACCGGATGCACCAATGTT-3', (downstream) 5'-CGCGTTGCTCACAATGTTTCAT-3'. Using GAPDH as the reference gene, the relative expression levels of the target genes were calculated according to the $2^{-\Delta\Delta CT}$ method.

Detection of MMP-9 Protein Expression in Human MMs by Western Blotting

Following cell collection, total protein was extracted using RIPA buffer. After protein concentration determination via BCA method, 50 μ g of protein was loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were then transferred to PVDF membrane, subsequently blocked at

25°C with blocking buffer for 2 hours. Following this, primary antibodies against MMP-9 and β -actin were applied and the membrane was incubated overnight at 4 °C. Post washing, a horseradish peroxidase-conjugated secondary antibody was applied, and membrane was incubated at 25°C for 1 hour. Subsequent to washing, ECL detection was performed, and protein bands were visualized employing a gel imaging system (GoodImage2000pro; Shanghai Licai Biotechnology Co., China). Using β -actin as a control protein, the relative optical density of MMP-9 was quantified using *ImageJ*.

Statistical Processing

Statistical analyses were performed using SPSS 24.0 (IBM Corp., USA). All experimental data are expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test for comparisons between treatment groups and the ox-LDL control group. A value of $P < 0.05$ was considered statistically significant.

ATV Treatment of ox-LDL-Induced Human MMs: Experimental Results

Identification of Human Monocyte-Derived Macrophages

During the development of atherosclerosis, monocytes differentiate into macrophages. Monocyte-derived macrophages influence atherosclerosis progression and the stability of plaques through various mechanisms, including the formation of foam cells, secretion of cytokines, development of fibrous caps and lipid cores, and interactions among different macrophage subtypes (Lazzarini *et al.*, 2023; Tomas *et al.*, 2021; Zhang *et al.*, 2023). Therefore, intervention strategies targeting monocyte-macrophage dynamics may provide novel approaches for treating atherosclerosis. In Fig. 1A, the induced human monocyte-derived macrophages exhibited irregular shapes, with some cells adhering to the culture surface, indicating their differentiation into macrophages. Fig. 1B illustrates that flow cytometric analysis revealed a CD14 positivity rate of 99.4% in the induced human monocyte-derived macrophages, confirming their identity as monocyte-derived macrophages and validating their suitability for subsequent experimental studies. In this study, ox-LDL stimulation resulted in a 2.3-fold increase in intracellular lipid droplet area in macrophages ($P < 0.01$), indicating a significant enhancement in foam cell formation (Fig. 1A). Lipid droplet staining using Oil Red O revealed abundant red-stained lipid droplet accumulation in the cytoplasm of the ox-LDL group, whereas treatment with ATV led to a dose-dependent reduction in lipid droplet area.

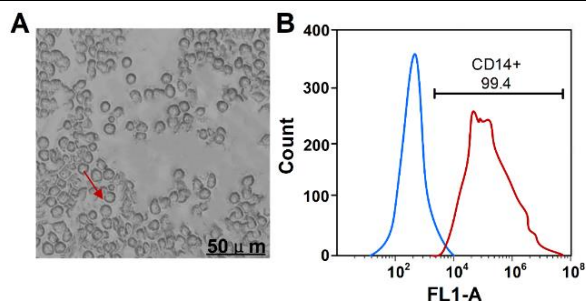


Fig. 1: Identification of human monocyte-derived macrophages (A: microscopic observation ($\times 200$, scale bar: $50\ \mu\text{m}$); red arrows indicate adherent macrophages; B: flow cytometric analysis of surface antibody CD14)

Effect of ATV on the Proliferation of ox-LDL-Induced Human MMs

ox-LDL is a potent cellular activator that induces macrophage proliferation by activating the MAPK signaling pathway and increasing intracellular calcium ion concentration, acting imperatively in the pathogenesis and progression of atherosclerosis (Wang *et al.*, 2022). Moreover, ox-LDL can cause endothelial cell damage and activate monocytes to release inflammatory mediators, thereby contributing to the atherosclerotic process (Zheng *et al.*, 2023). ATV suppresses ox-LDL-induced macrophage proliferation. Relative to the untreated control, ox-LDL markedly enhanced macrophage proliferation ($P < 0.05$), whereas ATV inhibited this effect in a dose-dependent manner (Fig. 2).

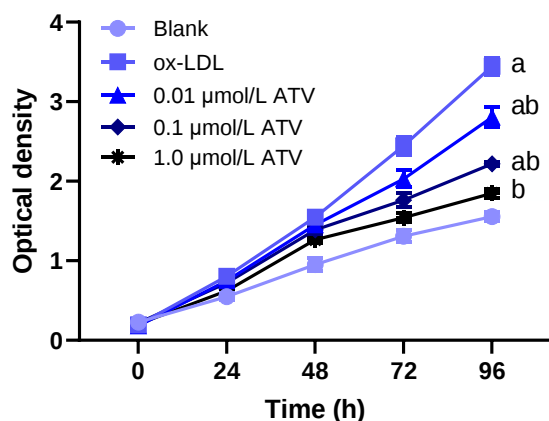


Fig. 2: Comparison of proliferation activity of human monocyte-derived macrophages among groups (^a $P < 0.05$ vs. blank group, ^b $P < 0.05$ vs. ox-LDL group (in all Figs).) The inhibitory effect of ATV on proliferation showed a dose-dependent manner.

These findings suggest that ATV may slow the progression of atherosclerosis by curbing the excessive proliferation of ox-LDL-stimulated macrophages and thereby reducing foam-cell accumulation within plaques. ATV, as a statin medication, can reduce plaque formation and growth by lowering LDL-C levels, thereby contributing to the treatment of atherosclerosis. In Fig. 2, all concentrations of ATV (0.01 , 0.1 , and $1.0\ \mu\text{mol/L}$) significantly reduced cell proliferation activity compared to the ox-LDL group ($P < 0.05$). Furthermore, the inhibitory effect of ATV demonstrated a clear dose-dependent trend, with higher concentrations resulting in stronger inhibition. The concentrations of ATV used in this study (0.01 – $1.0\ \mu\text{mol/L}$) encompass its clinically relevant plasma levels (0.1 – $1.0\ \mu\text{mol/L}$), consistent with peak plasma concentrations observed following oral administration. This suggests that the experimental findings may have potential clinical relevance.

Influence of ATV on the Apoptosis of ox-LDL-Induced Human MMs

Guo *et al.* (2022) analyzed the detrimental influences of ox-LDL on vascular endothelial cells and found that ox-LDL can activate apoptotic pathways in endothelial cells, inhibit the expression of endothelial junction proteins, and affect cell migration, deformation, and formation, ultimately leading to disruption of the endothelial barrier function (Guo *et al.*, 2022). In Fig. 3, the apoptosis rate of human monocyte-derived macrophages in ox-LDL group was markedly lower than in the other four groups ($P < 0.05$). Unlike vascular endothelial cells, a reduced apoptosis rate in monocyte-derived macrophages allows for sustained uptake of ox-LDL, leading to increased formation and accumulation of foam cells, thereby exacerbating the pathological processes of atherosclerosis (Chang *et al.*, 2021; Yang *et al.*, 2020). Compared to ox-LDL group, the apoptosis rate of human monocyte-derived macrophages increased gradually with the rising concentration of ATV, and the differences in apoptosis rates among the groups were considerable ($P < 0.05$). Plaque rupture is one of the major causes of acute myocardial infarction and other cardiovascular events; therefore, a decreased apoptosis rate in monocyte-derived macrophages may increase the risk of these events. Chen *et al.* (2022) found that the addition of ATV to metoprolol treatment dramatically reduced the area of cerebral infarction in patients with atherosclerosis and improved plaque scores. Moreover, when the apoptosis rate of monocyte-derived macrophages is reduced, the inflammatory factors and growth factors they release may stimulate the proliferation and migration of smooth muscle cells, thereby accelerating atherosclerosis progression. Therefore, it is proposed that ATV may slow the

progression of atherosclerosis by promoting increased apoptosis in monocyte-derived macrophages, which in turn suppresses foam cell formation and influences plaque stability. The apoptosis rate of macrophages in the ox-LDL group was significantly decreased (Fig. 3), which may exacerbate lipid core expansion within plaques by impairing foam cell clearance. In contrast, ATV promoted apoptosis in a dose-dependent manner, which may help limit necrotic core formation and thereby enhance plaque stability.

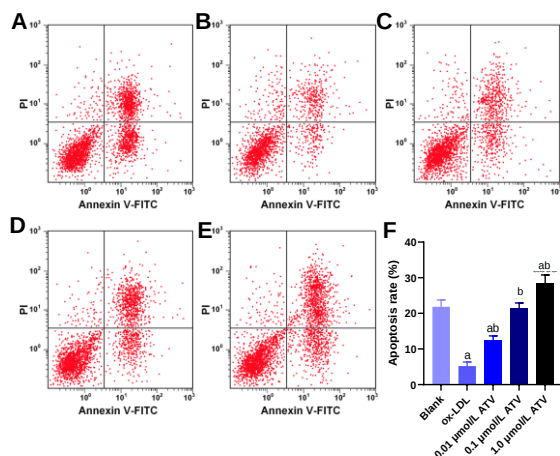


Fig. 3: Comparison of apoptosis rates in human monocyte-derived macrophages among groups (A-E: flow cytometry plots showing apoptosis rates for the Blank group, ox-LDL group, 0.01 μmol/L ATV group, 0.1 μmol/L ATV group, and 1.0 μmol/L ATV group, respectively; F: statistical comparison of apoptosis rates. (^a $P < 0.05$ vs. blank group, ^b $P < 0.05$ vs. ox-LDL group. The inhibitory effect of ATV on proliferation showed a dose-dependent manner.)

Impact of ATV on Expression levels of Inflammatory Genes in ox-LDL-Induced Human MMs

Inflammatory cell infiltration is a hallmark of unstable plaques in atherosclerosis. Monocyte-derived macrophages are key cells in the inflammatory process of atherosclerosis; their adhesion to the arterial wall leads to the secretion of a large amount of pro-inflammatory cytokines and cell adhesion molecules, exacerbating the inflammatory response (Cai *et al.*, 2023; Mehta *et al.*, 2021). This inflammatory response serves as a significant driving force for the onset and progression of atherosclerosis. IL-6 stimulates the activation and migration of other immune cells, thereby intensifying the level of inflammation; IL-1β is involved in the regulation of IL-6 and TNF-α, participating in various autoimmune inflammatory responses and cellular activities; TNF-α transmits information through specific receptors on the cell membrane, promoting biological activities such as cell proliferation and differentiation, immune regulation,

and inflammation mediation. In Fig. 4, the relative mRNA expression levels of the inflammatory genes IL-6, IL-1β, and TNF-α in the Blank group of human monocyte-derived macrophages were the lowest, while the mRNA expression levels of these inflammatory genes in ox-LDL group were significantly higher than in those in the other four groups ($P < 0.05$). In Table 1, ATV dose-dependently inhibited the expression of IL-6, IL-1β, and TNF-α, consistent with its clinical effects in reducing inflammatory infiltration within plaques. Lu *et al.* (2023) suggested the similar result, who stimulated primary macrophages with ox-LDL to mimic the atherosclerotic microenvironment. They found that, after induction, the intracellular calcium ion concentration and reactive oxygen species levels increased, along with a notable elevation in the release of IL-6, IL-18, IL-1β, and TNF-α. Moreover, relative to ox-LDL group, the relative mRNA expression levels of IL-6, IL-1β, and TNF-α in human monocyte-derived macrophages gradually decreased with increasing concentrations of ATV, with drastic differences in mRNA expression levels among groups ($P < 0.05$). This indicates that ATV exerts an anti-inflammatory effect, which can reduce the exacerbation of the inflammatory response in ox-LDL-induced human monocyte-derived macrophages, thereby further protecting the cardiovascular system. This effect is significant for slowing atherosclerosis progression and reducing the risk of cardiovascular diseases.

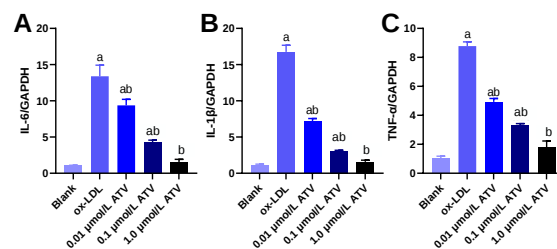


Fig. 4: Comparison of relative expression levels of inflammatory genes in human monocyte-derived macrophages among groups (A: relative expression level of IL-6; B: relative expression level of IL-1β; C: relative expression level of TNF-α. (^a $P < 0.05$ vs. blank group, ^b $P < 0.05$ vs. ox-LDL group.)

Influence of ATV on the expression of MMP-9 in ox-LDL-Induced Human MMs

MMP-9, as an important enzyme, plays critical roles in degrading components such as collagen in the vascular matrix, promoting the infiltration of inflammatory cells, and influencing the migration and proliferation of VSMCs (Luchian *et al.*, 2022). It was noted that the expression level of MMP-9 is greatly higher in unstable plaques compared to stable plaques. Moreover, elevated levels of MMP-9 may serve as potential predictive biological

markers for the occurrence of cardiovascular events (Chen *et al.*, 2023). In Fig. 5, the relative mRNA and protein expression levels of MMP-9 in the Blank group of human monocyte-derived macrophages were the lowest, while those in ox-LDL group were notably superior to the other four groups ($P<0.05$). This finding is consistent with Liao *et al.* (2021)'s observation that MMP-9 mRNA and protein expression levels were markedly increased following ox-LDL induction in THP-1 macrophages. Ox-LDL can stimulate monocyte-derived macrophages to express MMP-9 and increase the activity of MMPs, which is crucial for promoting the degradation of the extracellular matrix within atherosclerotic plaques, ultimately causing formation of vulnerable plaques.

Additionally, compared to ox-LDL group, the relative mRNA and protein expression levels of MMP-9 in human monocyte-derived macrophages gradually decreased with increasing concentrations of ATV ($P<0.05$) (Table 1). The dose-dependent downregulation of MMP-9 (Figs. 5A-B) is closely associated with plaque stability. Studies demonstrated that reduced MMP-9 levels decrease collagen degradation and strengthen the fibrous cap structure, further supporting the potential role of ATV in preventing plaque rupture. ATV inhibited MMP-9 protein expression in a dose-dependent manner, an effect that may be mediated by blockade of the NF- κ B pathway. However, the phosphorylation status of NF- κ B requires validation in subsequent experiments.

Table 1: Effects of different concentrations of ATV on mRNA expression of inflammatory cytokines and MMP-9 protein levels in ox-LDL-induced human monocyte-derived macrophages

Group	IL-6 mRNA (relative expression level)	IL-1 β mRNA (relative expression level)	TNF- α mRNA (relative expression level)	MMP-9 protein (relative expression level)
Blank group	1.00 $\pm$ 0.12	1.00 $\pm$ 0.15	1.00 $\pm$ 0.10	1.00 $\pm$ 0.08
ox-LDL group	4.35 $\pm$ 0.41 ^a	3.89 $\pm$ 0.38 ^a	3.67 $\pm$ 0.33 ^a	3.95 $\pm$ 0.45 ^a
0.01 μ mol/L ATV	3.12 $\pm$ 0.29 ^b	2.87 $\pm$ 0.24 ^b	2.65 $\pm$ 0.21 ^b	3.02 $\pm$ 0.32 ^b
0.1 μ mol/L ATV	2.03 $\pm$ 0.18 ^b	1.95 $\pm$ 0.17 ^b	1.82 $\pm$ 0.15 ^b	2.11 $\pm$ 0.23 ^b
1.0 μ mol/L ATV	1.45 $\pm$ 0.14 ^b	1.32 $\pm$ 0.12 ^b	1.28 $\pm$ 0.11 ^b	1.56 $\pm$ 0.19 ^b

Note: ^a $P<0.05$ vs. blank control group; ^b $P<0.05$ vs. ox-LDL group

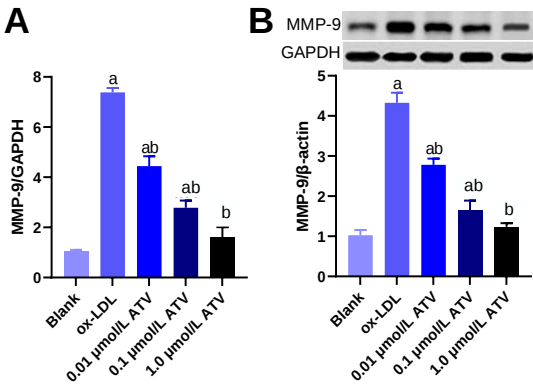


Fig. 5: Comparison of relative expression levels of MMP-9 in human monocyte-derived macrophages among groups (A: relative expression level of MMP-9 mRNA; B: relative expression level of MMP-9 protein. (^a $P<0.05$ vs. blank group, ^b $P<0.05$ vs. ox-LDL group.)

In atherosclerosis, the migration and proliferation of VSMCs are critical steps in plaque formation and development. MMP-9 facilitates the migration of VSMCs by degrading the extracellular matrix. Ma *et al.* (2023) found that ATV treatment in patients with acute coronary syndrome markedly reduced serum MMP-9 levels. This indicates that ATV can inhibit the increase in MMP-9 expression levels in human monocyte-derived macrophages induced by ox-LDL. This effect may help stabilize plaques, reduce inflammatory cell infiltration, lower the risk of cardiovascular events. The mechanism

by which ATV inhibits MMP-9 expression likely involves the synergistic interaction of multiple pathways. ox-LDL promotes MMP-9 transcription through activation of the NF- κ B pathway, whereas ATV can inhibit HMG-CoA reductase, reducing the production of isoprenoid intermediates and thereby blocking the Rho/ROCK-NF- κ B signaling cascade. ATV may also indirectly suppress MMP-9 expression by upregulating miR-29b, a hypothesis that requires further validation through gene silencing experiments. Additionally, ATV may influence MMP-9 expression indirectly via multiple target pathways; future studies incorporating gene knockout or inhibitor experiments are needed to clarify the specificity of these mechanisms. Although previous studies have confirmed the anti-inflammatory effects of ATV, this study is the first to reveal its dual mechanism of dose-dependent regulation of macrophage apoptosis and MMP-9 expression. Notably, 1.0 μ mol/L ATV significantly promoted apoptosis (Fig. 3), an effect that has not been extensively explored in prior research. Our findings also demonstrate a linear correlation between the inhibitory effect of ATV on MMP-9 and its plasma concentration (Fig. 5), providing new evidence to optimize clinical dosing strategies.

Conclusion

ATV can inhibit the exacerbation of the inflammatory response in human monocyte-derived macrophages induced by ox-LDL, suppress cell proliferation, promote apoptosis, and inhibit MMP-9 mRNA and protein

expression levels. These mechanisms collectively provide a scientific rationale and clinical foundation for the use of ATV in treating atherosclerosis. However, this work also has certain limitations, such as the reliance on *in vitro* cellular experiments to validate the effects of ATV on atherosclerosis. Future research should establish *in vivo* animal models to further analyze the underlying mechanisms of action. The sample size in this study was relatively small ($n=6$), although it was determined by a power analysis. Future studies with larger sample sizes are needed to reinforce our findings. The findings of this study are based on *in vitro* cellular experiments; future research should employ atherosclerosis animal models to further validate the pharmacological effects and underlying mechanisms of ATV *in vivo*. Subsequent investigations may integrate single-cell sequencing techniques to elucidate the specific regulatory effects of ATV on macrophage subsets. Additionally, an ApoE^{-/-} atherosclerotic mouse model could be established to assess the impact of ATV on plaque MMP-9 activity and clinical endpoints, such as plaque rupture incidence.

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Authors Contributions

Bensheng Liu: Conceptualization, methodology, funding acquisition, writing original draft, writing review & editing, supervision.

Huifan Li: Investigation, Formal analysis, Data curation.

Yan Wang: Investigation, Validation, Visualization.

Li Zhang: Methodology, Formal analysis, Data curation.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest

The authors declare that they have no conflicts of interest to report regarding the present study.

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