

Over-expression of Apolipoprotein J Inhibits Cholesterol Crystal-Induced Inflammatory Responses via Suppressing NLRP3 Inflammasome Activation in THP-1 Macrophages

(apolipoprotein J (clusterin) / interleukin 1 β / tumour necrosis factor α / THP-1 macrophages / inflammation)

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Abstract. Apolipoprotein J (clusterin) is a component of high-density lipoproteins, the high level of which is reversely correlated with the risk of coronary heart disease. In addition, it exerts anti-inflammatory and anti-apoptotic effects on endothelial cells and inhibits smooth muscle cell migration and proliferation, indicating that it may play a protective role in cardiovascular disease. However, the exact mechanisms by which this occurs remain unclear. This study aimed to clarify these underlying protective mechanisms by researching the inhibitory effects of apolipoprotein J *via* the NOD-like receptor protein 3 pathway on the inflammation induced by cholesterol crystals in THP-1 macrophages. In culture, THP-1 macrophages were infected with adenoviral vectors containing apolipoprotein J genes and subsequently treated with cholesterol crystals. The inflammatory cytokines interleukin-1 β , interleukin 18 and tumour necrosis factor α were quantitatively

measured with ELISA kits. NOD-like receptor protein 3, cysteinyl aspartate specific proteinase 1 and interleukin 1 β were evaluated by Western blot and PCR analysis. As a result, apolipoprotein J expression was found to remarkably decrease the levels of inflammatory cytokines, including tumour necrosis factor α , interleukin 18 and interleukin 1 β , secreted by THP-1 macrophages. It was also found capable of inhibiting the levels of NOD-like receptor protein 3, cysteinyl aspartate-specific proteinase 1 and interleukin 1 β both at the protein and mRNA levels. In the current study, we revealed that over-expression of apolipoprotein J attenuated the inflammation induced by cholesterol crystals through inhibition of the NOD-like receptor protein 3 inflammasome pathway.

Introduction

Atherosclerosis is a disease characterized by the accumulation of low-density cholesterol lipoprotein (LDL) and immune cell infiltration. LDL and its components, such as oxidized LDL, cause vascular inflammation, leading to the accumulation of lipid-filled atherosclerotic plaques (Hansson and Hermansson, 2011; Gisterå and Hansson, 2017). Moreover, when the amount of cholesterol is beyond the capacity of elimination of macrophages, it will precipitate as cholesterol crystals (CCs) (Katz et al., 1976). CCs exist in all stages of atherosclerosis, from lipid streaks and fibrous plaques to complex lesions, and are considered as the symbolic component of atherosclerosis, playing an important role in its onset. CCs are closely related to plaque instability, and the mechanisms that induce plaque rupture include mechanical injury and inflammation induction. Mechanical injury occurs because the volume of cholesterol increases when it crystallizes (Ordovas-Montanes and Ordovas, 2012). In the arterial wall, this local volume increase will lead to distortion and even tear of the plaque surface. Sharp needle-like crystals can also pierce the surface of the plaque, leading to local endothelial

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Abbreviations: ApoJ – apolipoprotein J, ASC – apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain, CC – cholesterol crystal, FBS – foetal bovine serum, HDL – high-density lipoprotein, ICAM-1 – intercellular adhesion molecule 1, LDL – low-density lipoprotein, MAC – membrane attack complex, MCP-1 – monocyte chemotaxis protein 1, MOI – multiplicity of infection, MMP-9 – metalloproteinase 9, NLR – NOD-like receptor, NLRP – NOD-like receptor protein, NOD – nucleotide-binding oligomerization domain, PMA – phorbol 12-myristate 13-acetate, SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis, VCAM-1 – vascular cell adhesion molecule 1.

injury. After the plaque rupture, cholesterol crystals can also be released into the circulation and spread to downstream blood vessels, causing cholesterol crystallinity embolism and vasodilation dysfunction. Moreover, recent studies have found that tiny CCs not only have physical and chemical properties, but, more importantly, can function as an endogenous inflammatory substance to induce local inflammation. CCs engulfed by macrophages can lead to lysosomal rupture, release of lysosomal content, activation of the NOD-like receptor protein (NLRP) 3 inflammasome, and activation of caspase-1, with a consequent increase in the expression of interleukin 1 β (IL-1 β) and promotion of atherosclerotic inflammation (Staff et al., 1999; Niyonzima et al., 2020). Duewell et al. (2010) found that in the early stages of atherosclerotic plaque formation, tiny CCs and inflammatory invasive cells emerge, triggering activation of the NLRP3 inflammasome and caspase-1, with release of mature IL-1 β . Rajamäki et al. (2010) further demonstrated that NLRP3 inflammasome activation depends on cholesterol-mediated K⁺ outflow, crystallization, and lysosomal degradation.

NLRP3 belongs to the family of NOD-like receptors (NLRs), a large family of intracellular sensors. NLRP3 is composed of NOD-like receptor 3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), and aspartate-specific cysteine proteinase (ASCP). NLRP3 is the core of the inflammasome, which consists of a central nucleotide oligomer domain, a C-terminal leucine repeat domain, and an N-terminal hot protein domain (Ozaki et al., 2015). NLRP3 is activated by various stimuli to form a massive protein complex known as the inflammasome, which then activates caspase-1 and promotes the maturation and secretion of inflammatory cytokines IL-1 β and IL-18 (Strowig et al., 2012).

Apolipoprotein J (ApoJ), also known as clusterin, is a multifunctional glycoprotein in human plasma, with a relative molecular weight of approximately 70–80 kDa and 449 amino acids (Park et al., 2014). ApoJ is widely distributed in bodily tissues and fluids and is involved in a variety of physiological functions in the human body (de Silva et al., 1990; MRC/BHF Heart Protection Study Collaborative Group, 1999; Han et al., 2012). In blood circulation, ApoJ forms high-density lipoproteins (HDLs) with Apo-AI and other lipids, and is widely present in HDL2 and HDL3. It can promote the release of cholesterol from foam cells, participate in the transport of reactive cholesterol, and exert a synergistic effect on HDL-C complexes against atherosclerosis (Jenne et al., 1991; Gelissen et al., 1998). Several studies have found that the concentration of serum ApoJ in patients with coronary heart disease was significantly higher than that in the control group, suggesting that ApoJ, as a component of HDLs, may play a role in protection against the progression of atherosclerotic diseases (Poulakou et al., 2008; Riwanto et al., 2013).

Previous research has identified that increased ApoJ expression relieved injured blood vessels by inhibiting

migration, adhesion and proliferation of smooth muscle cells (Kim et al., 2009). Moreover, ApoJ has been reported to exhibit intra- and extracellular interactions with inflammation-associated molecules, such as complement factors, NF κ B inhibitor α , and TGF- β , suggesting that it plays a crucial role in modulating conditions associated with inflammation (Liu et al., 2018). In this study, we aimed to determine whether ApoJ over-expression contributes to the anti-inflammatory effects against CC-induced inflammation and to clarify the underlying mechanism.

Material and Methods

Cell culture and differentiation

Since phorbol 12-myristate 13-acetate (PMA)-induced THP-1 macrophages can release various inflammatory mediators and are usually used to research human inflammatory diseases, we chose these cells as our experimental model (Daigneault et al., 2010). The THP-1 cell line was obtained from the cell bank of the Chinese Academy of Sciences Institute of Biochemistry and Cell Biology, Shanghai Institutes for Life Science. The cells were cultured at a density of 2×10^5 cells/ml in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 2 mmol/l L-glutamine (Gibco, Thermo Fisher Scientific, NY). Cells were incubated at 37 °C with 5% CO₂. The medium was changed every two days. THP-1 cells (2×10^5 cells/ml) were seeded in 6-well culture plates and treated for 72 h with 100 ng/ml PMA (Sigma-Aldrich, St. Louis, MO). When the cells reached 80 % confluence, we changed to RPMI 1640 medium supplemented with 10% FBS (Wang et al., 2020).

Preparation of cholesterol crystals

Cholesterol (Sigma-Aldrich) was dissolved in 95% ethanol (12.5 g/l), heated to 60 °C, filtered while still warm with filter paper to allow crystallization, and then left at room temperature (22–25 °C) (Rajamäki et al., 2010). Flat, rhomboid, and relatively large (1–10 mm) CCs formed. The crystals were filtered, autoclaved, ground with a sterile mortar and pestle to a size range of 1–5 mm, and stored at –20 °C until use. CCs were tested for endotoxin using limulus amoebocyte lysate (Bioendo, Xiamen, China).

Adenoviral infection of THP-1 macrophages

The pAD-ApoJ-IRES-EGFP adenoviral vector was used to generate ApoJ-over-expressing adenovirus, with pAD-IRES-EGFP serving as a control. Adenoviral vectors were obtained from Syngentech (Biotechnology Company, Beijing, China). THP-1 macrophage cells were infected with the indicated adenoviruses at a multiplicity of infection (MOI) of 200 : 1 in serum-free DMEM. The supernatant was removed after 4 h and replaced with DMEM containing 10% FBS. After 36 h, the supernatant was removed again, and THP-1 macrophages were subjected to different treatments.

Analysis of cytokine secretion

To determine the best CC-treatment concentration for THP-1 macrophages, cells were treated with CCs at four different concentrations (0.5, 1.0, 1.5, and 2.0 mg/ml). THP-1 macrophages (2×10^5 cells/well) were cultured and incubated with CCs in serum-free culture medium for 24 h at 37 °C and 5% CO₂. We observed the level of IL-1 β to determine the best concentration of CCs, as previous studies have found that CCs activate the NLRP3 inflammasome, resulting in the release of inflammatory cytokines, such as IL-1 β , and formation of atherosclerotic lesions (Düewell et al., 2010; Rajamäki et al., 2010; Niyonzima et al., 2020). Therefore, IL-1 β , IL-18 and TNF- α were chosen as the main indicators to conduct the experiments. In subsequent experiments, CC treatment was applied after THP-1 macrophage infection and all the cytokine levels in culture media samples were determined using enzyme-linked immunosorbent assays (ELISA, R&D SYSTEMS, Mineapolis, MN) at the appropriate concentration of CCs.

Western blotting

Cells were lysed using the radioimmunoprecipitation assay buffer. Protein samples of cell lysates were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins in the gel were transferred onto a polyvinylidene difluoride membrane (Millipore, Burlington, MA), blocked for 1 h at room temperature with 2.5% TBST milk (10 mM Tris HCl [pH 8.0], 150 mM NaCl, 0.5% Tween-20, 3% skim milk), and probed with anti-ApoJ, anti-NALP3 antibody, anti-caspase-1 p20 antibody, anti-caspase-1 antibody, or anti-actin antibody (Santa Cruz Biotechnology, Dallas, TX). The membranes were incubated in HRP-conjugated secondary antibody solution (1 : 10,000) for 2 h at room temperature. Colour detection images were collected using conventional image scanning, and the grey values were analysed using ImageJ (NIH, MD, USA, v1.8.0).

PCR analysis

Total RNA was isolated from cells using the TRIzol[®] reagent (Thermo Fisher Scientific), and RNA quality and concentration were measured with a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). Approximately 1 μ g of total RNA was reverse-transcribed into complementary DNA using a Fast Quant cDNA Kit

(Tiangen Biotech, Beijing, China) in accordance with the manufacturer's protocol. Quantitative PCR was performed using a Real-time PCR Detection System (Bio-Rad, Hercules, CA) (95 °C for 15 min followed by 40 cycles of 95 °C for 10 s and 63.8 °C for 30 s, with a final extension step of 65 °C for 5 s). *ACTB* was used as an internal reference to measure the normal expression levels. The difference in gene expression was calculated using the 2- $\Delta\Delta C$ method (Livak and Schmittgen, 2001). The primer sequences used are listed in Table 1.

Statistical analysis

Data are expressed as the mean \pm SD. One-way ANOVA analysis with Duncan's test or a two-tailed Student's *t*-test was used for statistical analysis, and *P* < 0.05 was considered statistically significant. All experiments were performed at least three times.

Results

The optimal concentration of CC treatment

We investigated whether the CC treatment activated inflammation in THP-1 macrophages with CCs at concentrations of 0.5–2.0 g/ml. The results showed that with 2.0 g/ml of CCs, the level of IL-1 β was the highest (Fig. 1). Based on these findings, we chose this concentration for CC treatment in subsequent experiments.

Over-expression of ApoJ in THP-1 macrophages

The expression of ApoJ was induced by recombinant adenovirus transfection. eGFP-positive cells were observed by fluorescence microscopy (Olympus, IX73, Tokyo, Japan) (Fig. 2). ApoJ expression increased significantly in Ad-ApoJ-transfected THP-1 macrophages compared to the control adenovirus-infected group, as determined by Western blot (Fig. 3).

Over-expression of ApoJ reduces expression of the NLRP3 inflammasome

We used real-time RT-PCR and immunoblotting to determine whether ApoJ inhibition of CCs stimulates the inflammatory response in THP-1 macrophages. As shown in Fig. 4, the mRNA expression of NLRP3, caspase-1, and IL-1 β was remarkably increased (*P* < 0.05) in THP-1 macrophages and THP-1 macrophages treated with CCs. However, the levels of NLRP3, caspase-1 and

Table 1. Primer sequences

Gene	Forward	Reverse
<i>NLRP3</i>	5'-TGGCTGTAACATTCGGAGATTG-3'	5'-GAAGTCACCGAGGGCGTTGT-3'
<i>CASP1</i>	5'-GGAAACAAAAGTCGGCAGAG-3'	5'-ACGCTGTACCCAGATTTTG-3'
<i>IL1B</i>	5'-GGCCCTAACAGATGAAGTGCT-3'	5'-TGCCGCCATCCAGAGG-3'
<i>ACTB</i>	5'-GAGACCTTCAACACC CCAGCC-3'	5'-GGATCTTCATGAGGTAGTCAG-3'

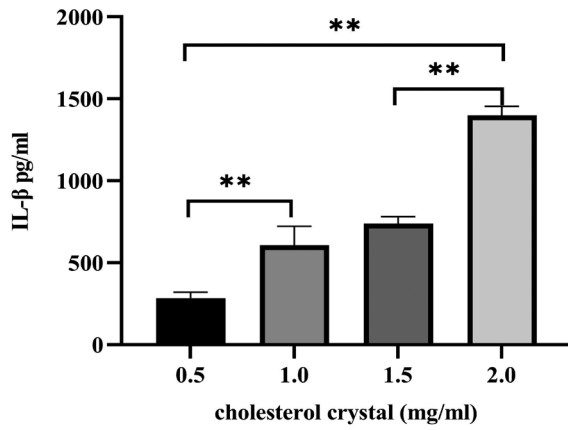


Fig. 1. Effects of ApoJ on IL-1 β secretion in THP-1 macrophages. THP-1 macrophages were treated with CCs at 0.5, 1.0, 1.5, and 2.0 mg/ml, culture medium was collected for analysing the levels of IL-1 β . The levels of IL-1 β were significantly increased by CCs at 2.0 mg/ml. These data were obtained from three independent experiments; **P < 0.001.

IL-1 β were significantly decreased (P < 0.05) in the ApoJ over-expression group compared to the pAD-IRES-EGFP group. Furthermore, to check whether the anti-apoptotic pathway employed by ApoJ involved NLRP3 inflammasome signalling pathway activation, we analysed the NLRP3, and caspase-1 p20 and p45 protein expression levels. CC treatment induced higher NLRP3, caspase-1 p20 and p45 expression than in the pAD-IRES-EGFP group, while their expression was significantly attenuated by ApoJ (P < 0.05, Fig. 5).

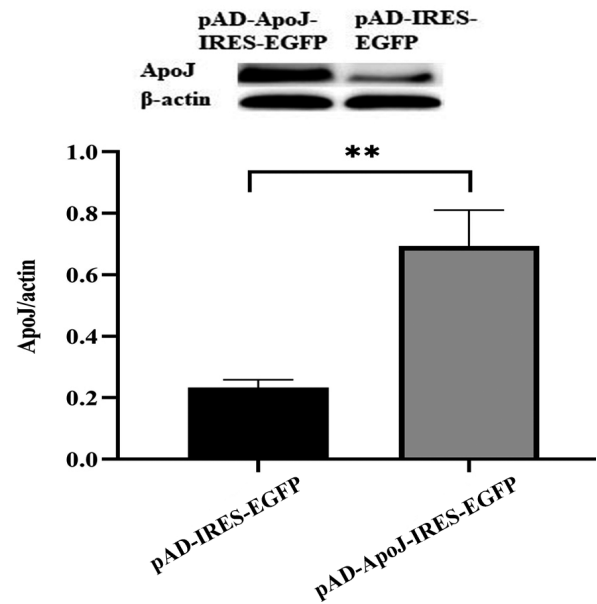


Fig. 3. Western blot analysis of ApoJ protein levels in THP-1 macrophages after adenoviral vector transfection. The pAD-ApoJ-IRES-EGFP group displayed high expression when compared with the pAD-IRES-EGFP group. These data were obtained from three independent experiments; **P < 0.001.

ApoJ inhibits the CC-stimulated expression of TNF- α , IL-18, and IL-1 β

The levels of TNF- α , IL-18 and IL-1 β cytokines were determined by ELISA in cell culture medium. The concentrations of TNF- α , IL-18 and IL-1 β were significant-

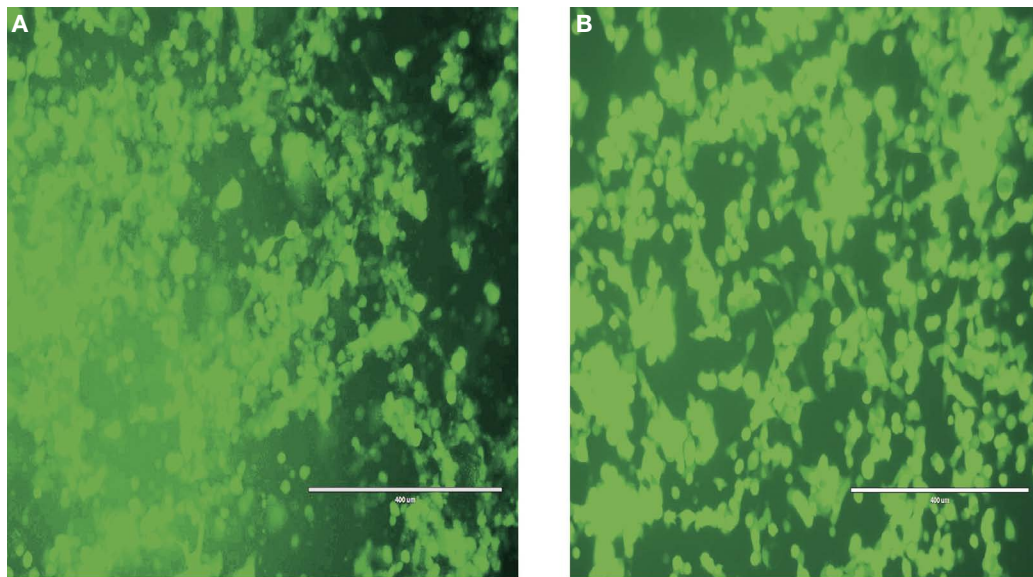


Fig. 2. ApoJ expression in THP-1 macrophages after adenoviral infection observed by fluorescence microscopy with green fluorescent protein ($\times 200$). **A.** THP-1 macrophages with pAD-IRES-EGFP adenoviral vector (control). **B.** THP-1 macrophages with pAD-ApoJ-IRES-EGFP adenoviral vector.

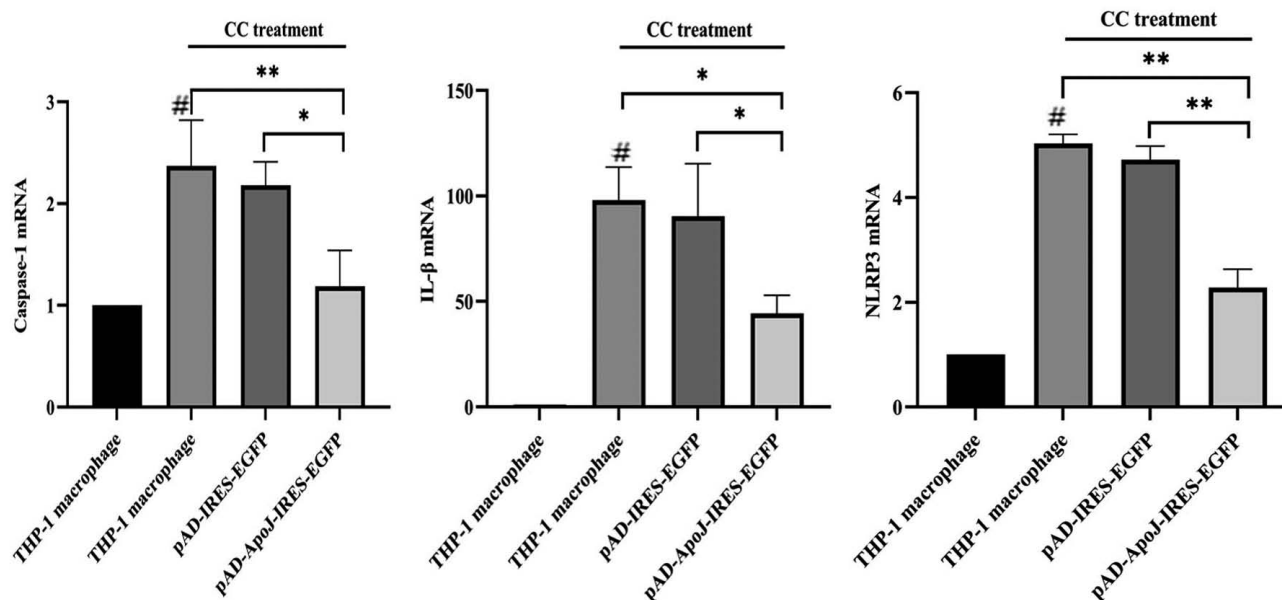


Fig. 4. The effect of ApoJ over-expression in THP-1 macrophages after transfection. After treatment with CCs (2.0 mg/ml), mRNA expression of NLRP3, caspase-1, IL-1 β was remarkably increased ($\#P < 0.001$ vs. THP-1 macrophages without CC treatments). mRNA of NLRP3, caspase-1, IL-1 β was significantly decreased in the pAD-ApoJ-IRES-EGFP adenoviral vector group. These data were obtained from three independent experiments; $**P < 0.001$, $*P < 0.05$.

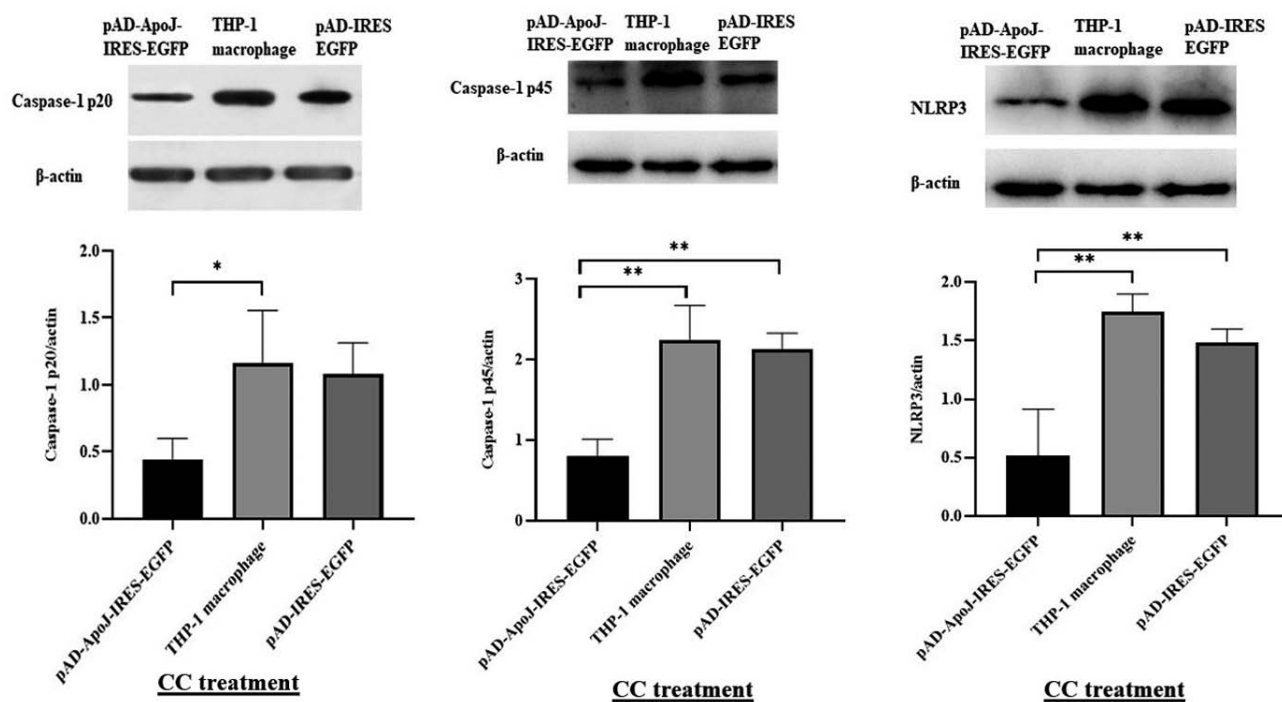


Fig. 5. Western blot analysis of NLRP3, caspase-1 (p20), caspase-1 (p45) after treatment of pAD-ApoJ-IRES-EGFP-transfected macrophages with CCs (left columns), $**P < 0.001$, $*P < 0.05$; these data were obtained from three independent experiments.

ly lower in the ApoJ-over-expressing group than in the pAD-IRES-EGFP and THP-1 macrophage groups with CC treatment. However, no significant difference was observed between the pAD-IRES-EGFP and THP-1 macrophage groups with CC treatment (Fig. 6).

Discussion

In this study, we established THP-1 macrophages with high expression of ApoJ to determine whether over-expression of ApoJ inhibits the CC-induced inflammatory

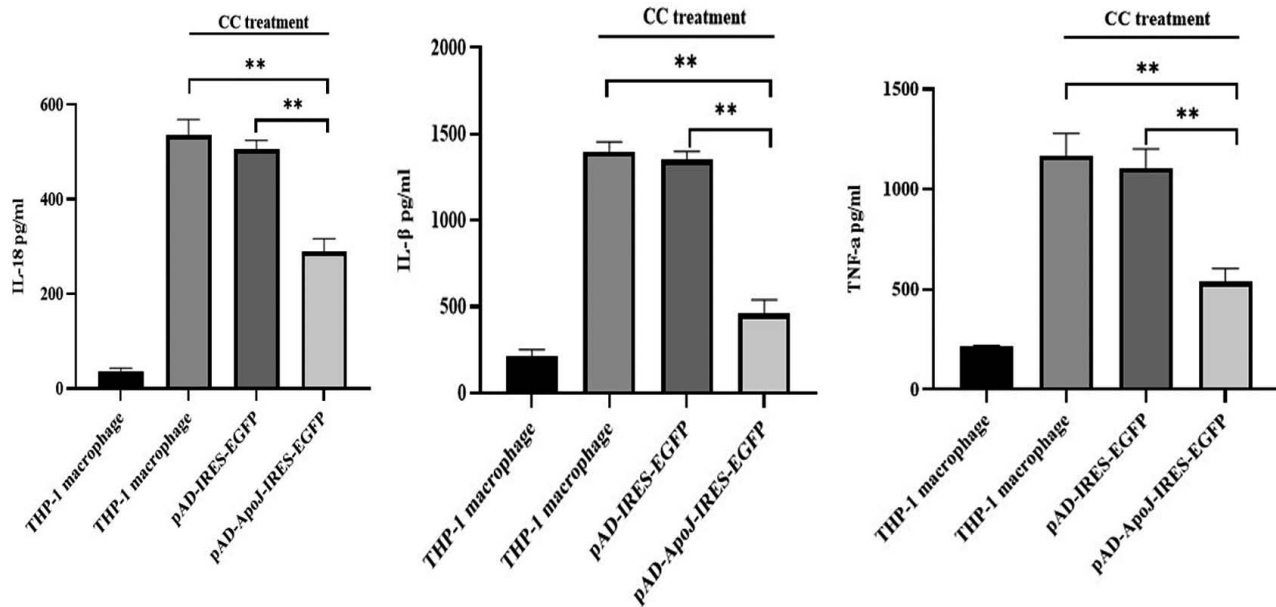


Fig. 6. THP-1 macrophages treated with CCs at a concentration of 2.0 mg/ml for 24 h after transfection with pAD-ApoJ-IRES-EGFP. The levels of IL-1 β , IL-18 and TNF- α from the culture media were analysed. ** $P < 0.001$, * $P < 0.05$; these data were obtained from three independent experiments.

response, and attempted to unravel the inflammasome pathway activator responsible for these effects. We found that over-expression of ApoJ ameliorated the inflammatory response compared to that in the control group. Moreover, we found that ApoJ inhibited NLRP3 inflammasome signalling pathway activation in the presence of CCs. This study is the first experimental evidence that ApoJ inhibits the CC-induced inflammatory response in THP-1 macrophages. Furthermore, this study also demonstrated that ApoJ suppresses activation of the NLRP3 inflammasome through the NLRP3/caspase-1 signalling pathway.

A previous study found that ApoJ was only slightly expressed in normal myocardial tissue, whereas in patients with acute myocardial infarction, ApoJ was present on the surface of the damaged myocardium together with the membrane attack complex (MAC), where they may be involved in the removal of damaged or necrotic tissue (Kim et al., 2009). This previous study suggests that ApoJ is closely related to the occurrence and development of atherosclerotic lesions. As a major stress protein, ApoJ has anti-inflammatory and cardiovascular protective effects (Corrado et al., 2010). Vandijk et al. (2010) injected ApoJ intravenously in mice with myocardial infarction and found that ApoJ was involved in regulating the inflammatory response, could reduce the infarct size and improve the survival rate.

Considering the anti-inflammatory and vascular protective effects of ApoJ, over-expression of ApoJ may have a more potent effect. *In vitro* experiments showed that adenovirus-mediated high expression of ApoJ could inhibit the mRNA expression of TNF- α -induced monocyte chemotaxis protein 1 (MCP-1), intercellular adhe-

sion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and metalloproteinase 9 (MMP-9). ApoJ inhibits DNA synthesis in vascular smooth muscle, thereby inhibiting migration and proliferation of vascular smooth muscle and protecting endothelial cells (Yanni et al., 2014). In our study, we obtained comparable results for TNF- α expression when ApoJ was present at high levels in THP-1 macrophages. Moreover, we also investigated the relevant inflammatory signalling pathway of NLRP3/caspase-1 and the cytokines downstream of the pathway, and found that high levels of ApoJ could markedly reduce the levels of NLRP3 and caspase-1 by inhibiting both gene and protein expression, while also hindering excretion of inflammatory cytokines TNF- α , IL-1 β and IL-18.

Inflammation has an extremely important role in the development of atherosclerosis and is a causative factor in its pathogenesis. There has also been a growing number of recent clinical trials targeting important inflammatory factors, such as INF- γ , TNF- α , IL-6, and IL-1 β (Chang et al., 2014; Harden et al., 2015; Cacciapaglia et al., 2018; Ridker et al., 2018). Since over-expression of ApoJ can inhibit important inflammatory pathways and multiple inflammatory factors, it could be a potential target for the anti-inflammatory treatment of atherosclerosis.

In summary, ApoJ over-expression attenuated CC-induced inflammation in THP-1 macrophages. This occurred *via* a decrease in the excretion of inflammatory cytokines TNF- α , IL-1 β , and IL-18 and inhibition of the inflammatory NLRP3/caspase-1 signalling pathway. Moreover, we believe that ApoJ could be a therapeutic target for atherosclerosis, particularly in the inhibition of cardiovascular inflammation.

Conflict of interest

The authors declare that they have no competing interests.

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