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C1q/TNF-related protein-9 attenuates atherosclerosis through AMPK-NLRP3 inflammasome singling pathway

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ABSTRACT

Backgrounds: C1q tumor necrosis factor-related protein 9 (CTRP9) has been suggested to exert an atheroprotective effect by modulating the inflammation, foam cell formation, endothelia and smooth muscle cell function via Adenosine Monophosphate Activated Protein Kinase (AMPK) pathway. On the other hand, the NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome plays an critical role in the atherosclerosis development, which is regulated by the AMPK. However, whether the CTRP9 affects the activity of NLRP3 inflammasome during the atherosclerosis development remains unclear, which would be elucidated in the current study.

Methods: The macrophage cells were stimulated with the oxidized low-density lipoprotein (ox-LDL) and also treated with the recombinant CTRP9 in the meantime. The activation of NLRP3 inflammasome was determined by measuring the releasing of IL-1β and caspase-1 p10 via ELISA and western blot, respectively. Then the AMPK was inhibited in macrophages by Dorsomorphin. Finally, the CTRP9-AMPK-NLRP3 inflammasome pathway was validated in the mouse model of atherosclerosis.

Results: The CTRP9 could down-regulate the expression of NLRP3 protein and also the activity of NLRP3 inflammasome in the ox-LDL activated macrophages. Inhibiting the AMPK significantly restored the activities of NLRP3 inflammasome. In the apolipoprotein E-deficient mice, lentiviral expression of CTRP9 could suppress the atherosclerosis development, which could be abolished by AMPK inhibition.

Conclusion: Our data here indicated that the CTRP9 showed atheroprotective function via CTRP9-AMPK- NLRP3 inflammasome pathway.

1. Introduction

Atherosclerosis is a chronic inflammation related disease, normally resulting in cardiovascular diseases, which is the leading cause of the mortality and morbidity in the world [1–3]. During the atherosclerosis development, the oxidized low-density lipoproteins (ox-LDL) could stimulate the macrophage to secrete pro-inflammatory cytokines, such as IL-1β and IL-18, to promote the inflammation process [4,5]. Among those important regulator and mediators involved into this inflammation process, the activation of NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome is one of the most important players [6,7]. It has been proposed that targeting the NLRP3 inflammasome might be a promising approach to suppress atherosclerosis [8].

The NLRP3 inflammasome is a protein complex containing NLRP3, ASC (apoptosis-associated speck-like protein containing a caspase-1 recruitment domain), and caspase-1 [9]. The activation of NLRP3 protein could induce the autocatalysis of pro-caspase-1, resulting in the caspase-1 cleavage and activation. The activated caspase-1 further cleave the immature pro-inflammatory cytokines, such as pro-IL-1β, resulting in cytokine maturation and inflammation responses [10].

CTRP9 (C1q/TNF-related protein 9) is a newly identified paralog of adiponectin with anti-inflammation activities [11]. There is only one locus in the mouse genome and two loci in human genome, CTRP9A and CTRP9B, while the CTRP9B is expressed at extremely low levels [11,12]. It has been demonstrated that the CTRP9 has protective roles in the regulation of obesity, diabetes, and hyperlipidemia, which are vital risk factors for atherosclerosis [13]. CTRP9 activates the AMPK (adenosine monophosphate-activated protein kinase) to enhance the glucose uptake [12], fat metabolism [14], autophagy [15], NO (nitric oxide) production [16] and also suppress the inflammation [17]. Recombinant lentiviral over-expression of the CTRP9 in the ApoE$^{-/-}$ (apolipoprotein E double knock-out) mice fed with the high-fat diet reduced the atherosclerotic lesion size [18]. And it has been proposed

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as one novel therapeutic target for treating cardiovascular diseases, especially the atherosclerosis [19–22]

The CTRP9 has been suggested to exert an atheroprotective effect via AMPK pathway [12,14–17]. And the activated AMPK could suppress the activities of NLRP3 inflammasome [23–26]. However, whether the CTRP9 affects the activity of NLRP3 inflammasome during the atherosclerosis development remains unclear, which would be elucidated in the current study.

2. Methods and materials

2.1. Cell culture and treatment

The monocyte/macrophage cell line THP-1 (human) and RAW 264.7 (mouse) were purchased from ATCC (American Type Culture Collection, USA). Cells were cultured in RPMI-1640 medium (Gibco, USA) plus 10% fetal bovine serum (Gibco) and 1% of penicillin-streptomycin solution (Thermo Fisher Scientific). The THP-1 derived macrophage was induced by induction medium (RPMI-1640 plus 100 ng/ml PMA (Sigma) and 0.3% (m/V) bovine serum albumin (Thermo Fisher Scientific)) for 3 days. Macrophage cells were firstly stimulated with ox-LDL at 50 μg/ml for 24 h, followed by another 48 h of 50 μg/ml ox-LDL plus 0.3 μg/ml, 1 μg/ml or 3 μg/ml CTRP9 [27,28]. The activity of AMPK was inhibited in macrophages by 10 μM Dorsomorphin 2HCl (Sigma).

2.2. Quantitative PCR

Total RNA was extracted with Trizol Reagent (Thermo Scientific). The cDNA was prepared by All-in-One™ First-Strand cDNA Synthesis Kit (Genecopoeia, USA) according to the manufacturer's instructions. Quantitative PCR was performed with SYBR Green qPCR Mix (Takara). The relative gene expression level was determined by by $2^{-\Delta\Delta Ct}$ method and the GAPDH was used as the internal control. Primers sequences were as follows: NLRP3 forward: 5′-AGCCAAGAATCCACAGTGTA ACC-3′; NLRP3 reverse: 5′-AGTGTTGCCTCGCAGGTAAAG-3′; GAPDH forward: 5′-CATCAAGAAGGTGGTGAA-3′; GAPDH reverse: 5′-TGTTGA AGTCAGAGGAGA-3′.

2.3. Western blot and enzyme-linked immuno sorbent assay (ELISA)

Cellular proteins were prepared by using RIPA lysis buffer, separated by SDS-PAGE, transferred to PVDF membrane and detected by the first and secondary antibody probing. To measure the caspase-1 p10 secretion, the cell culture medium or mice serum was pre-cleared by brief centrifugation, and the supernatant/serum was concentrated by tenfold using Amicon Ultra-4 centrifugal filter with molecular weight cutoff at 3 kDa before analysis. Primary antibodies were as follows: anti-NLRP3 (NBP2–12446, Novus Biologicals), anti-IL-1β/IL-1F2 (AF-201-NA, Novus Biologicals), anti-Caspase-1 p10 (MBS821314, MyBioSource, USA), anti-pro-Caspase1 + p10 + p12 (ab179515, Abcam, USA), and anti-GAPDH (NB300-221, Novus Biologicals). ELISA was performed with IL-1β ELISA Kit (Abcam) according to the manufacturer's instructions.

2.4. Animal model

ApoE$^{-/-}$ mice (male, 6-week-old) with C57BL/6 background were obtained from South Laboratory Animal Center, China. The mice were kept in specific pathogen free regions. All mice were given a High Fat Diet (HFD) containing 0.25% cholesterol and 15% cocoa butter for 4 weeks before indicated treatments. The HFD treated mice were randomly divided into four groups for indicated treatments (n = 8 per group): the CTRP9 group (intravenous injection of lentivirus expressing the CTRP9, 2×10^7 TU/mouse), the CTRP9 plus Dorsomorphin group, GFP group (intravenous injection of null lentivirus expressing GFP, 2×10^7 TU/mouse), and Control group (equal volume of PBS alone). Twelve weeks later, all the mice were euthanized for subsequent study. The AMPK inhibitor Dorsomorphin (10 mg/kg) was administrated via tail-vein injection once per week for 12 weeks [29]. MCC950 (inhibitior of the NLRP3 inflammasome, Sigma) was injected intraperitoneally (50 mg/kg) twice per week. The animal study was approved by Animal Care and Use Committee of Yiwu Central Hospital.

The whole aorta was rapidly removed and washed in PBS. Half of the aortas was fixed in 4% neutral formaldehyde for histological analysis, and the other half was frozen and store in liquid nitrogen for molecular studies. The lipid core was identified by oil red O (Sigma, USA) staining. For the en face staining by oil red O, the adventitia of aortas were stripped, followed by the dissection of aortic arches. And then the aortas were readily visualized by oil red O staining after fixation. The lesion areas of the aorta were measured by ImageJ.

2.5. Statistics

Data were expressed as mean (± SE) and analyzed by a SPSS software package (SPSS Standard version 13.0, SPSS Inc, USA). Unpaired Student's *t* test and one way ANOVA were used as appropriate to assess the statistical significant of difference. *P* values under 0.05 were considered statistically significant.

3. Results

To determine whether the CTRP9 could affects the activity of NLRP3 inflammasome, the human macrophages derived from THP-1 cells and mouse macrophages (RAW264.7) were stimulated with ox-LDL for 3 days. As expected, the ox-LDL could significantly up-regulated the NLRP3 (Fig. 1A), and its down-stream targets caspase-1 p10 (Fig. 1C) and IL-1β (Fig. 1D). Interestingly, the CTRP9 could suppress the up-regulated NLPR3 expression by the ox-LDL treatment at both mRNA and protein levels (Fig. 1A and B). The CTRP9 treatment also inhibited the expression of NLRP3 inflammasome, including the caspase-1 p10 (Fig. 1C), IL-1β (Fig. 1D) and the pro-IL-1β (Fig. 1E). Thus the CTRP9 could inhibit the activity of NLRP3 inflammasome in a dose dependent manner.

Because the CTRP9 could activate the AMPK pathway [12,14–17]. And the activated AMPK could suppress the activities of NLRP3 inflammasome [23–26]. Then we were wondering whether the AMPK was involved into the inhibitory effects of CTRP9 on NLRP3 inflammasome. Indeed, the AMPK inhibitor restored the activity NLRP3 inflammasome suppressed by CTRP9, including the secretion of caspase-1 p10 (Fig. 2A) and IL-1β (Fig. 2B), the protein level of pro-IL-1β (Fig. 2C). However, the AMPK inhibition did not affect the expression of NLRP3 at both mRNA and protein levels (Fig. 2D and E). Therefore, the CTRP9 suppressed the activity of NLRP3 inflammasome through activating AMPK.

To further confirm the involvement of AMPK pathway during the CTRP9 induced suppression of NLRP3 inflammasome, the atherosclerosis mouse model was established. As described before, the lentiviral over-expression of CTRP9 could inhibit the atherosclerosis development (Fig. 3A and B) [18]. However, this inhibitory effects was abolished when the mice were co-treated with the AMPK inhibitor Dorsomorphin (DM) (Fig. 3A). The NLRP3 inflammasome inhibitor MCC950 and the MCC950 plus CTRP9 showed similar inhibitory effects on atherosclerosis (Fig. 3B). Furthermore, the suppressive effects of CTRP9 on the NLRP3 inflammasome was also attenuated by the AMPK inhibitor Dorsomorphin (DM) (Fig. 3C). The NLRP3 inflammasome inhibitor MCC950 and the MCC950 plus CTRP9 showed similar inhibitory effects on NLRP3 inflammasome (Fig. 3D). Thus, the CTRP9 could suppress the NLRP3 activities through AMPK during the atherosclerosis development in the mice.

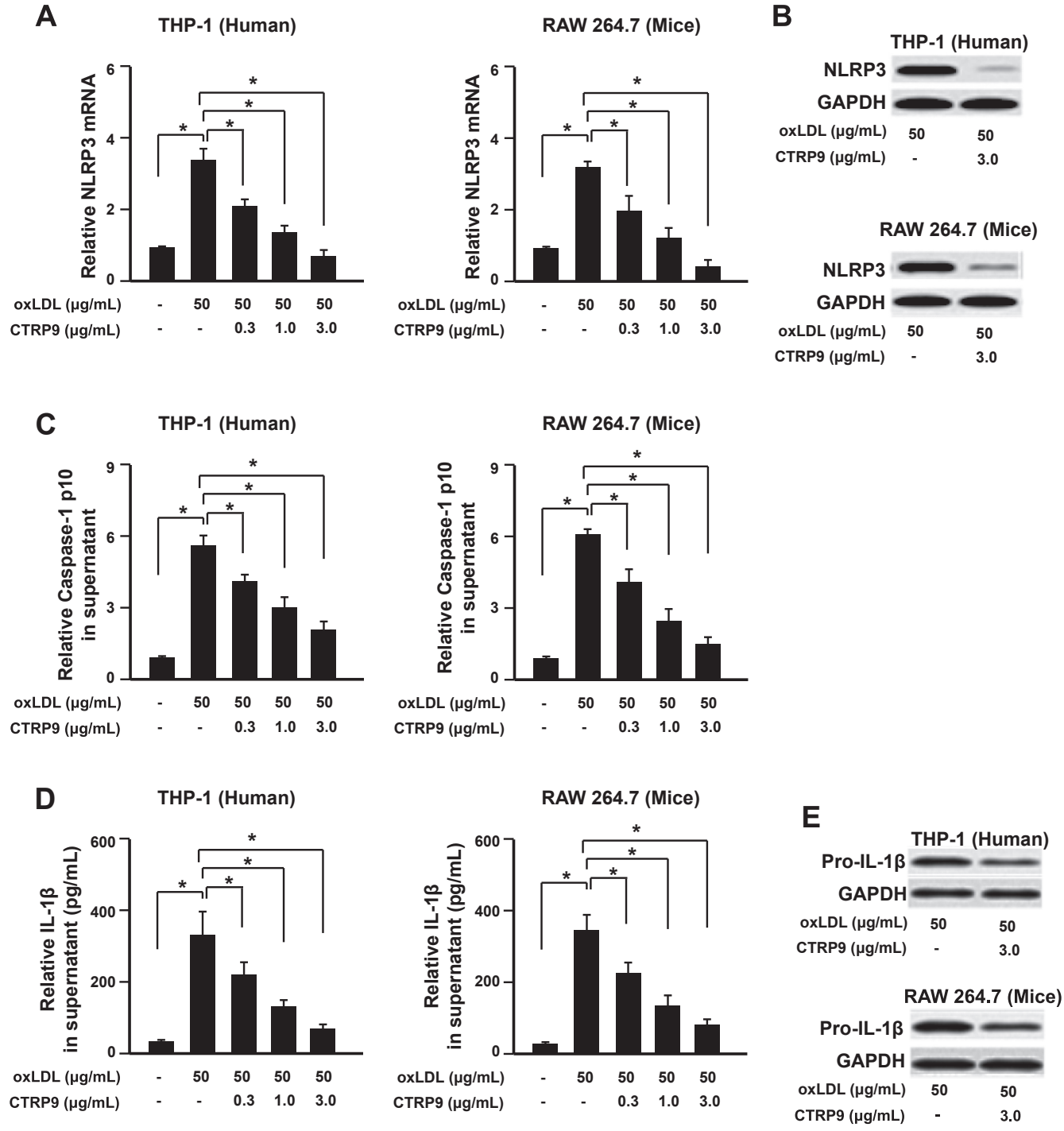

Fig. 1. CTRP9 suppressed the activation of NLRP3 inflammasome induced by ox-LDL in human and mouse macrophages. Macrophages were treated with ox-LDL for 24 h, followed by ox-LDL plus CTRP9 treatment for 48 h. (A) Relative mRNA levels of cellular NLRP3 were determined by qPCR in human and mouse macrophages (n = 3). (B) Western blot showed the protein levels of cellular NLRP3 after CTRP9 treatment. (C) The levels of caspase-1 p10 in cell culture medium after treatment were determined by western blot. The band intensity was quantified by ImageJ (n = 3). (D) The levels of IL-1β in cell culture medium after treatment were determined by ELISA (n = 3). (E) Western blot showed the protein levels of cellular pro-IL-1β after CTRP9 treatment. "−" indicates no treatment; * indicates $P < 0.05$.

4. Discussion

CTRP9 has been suggested to exert an atheroprotective effect by modulating the inflammation, foam cell formation, endothelia and smooth muscle cell function via AMPK pathway [12,14–22]. On the other hand, it has been widely acknowledged that the activation of NLRP3 inflammasome in macrophages and endothelial cells contributes to the atherosclerosis development [6,7]. And the AMPK could suppress the activity of NLRP3 inflammasome in macrophage [30,31]. However, whether the CTRP9 affects the activity of NLRP3 inflammasome during the atherosclerosis development remains unclear.

Our data here demonstrated that the CTRP9 could down-regulate the expression of NLRP3 protein and also the activity of NLRP3 inflammasome in the ox-LDL activated macrophages. Inhibiting the AMPK significantly restored the activities of NLRP3. In the mouse model of atherosclerosis, lentiviral expression of CTRP9 could suppress

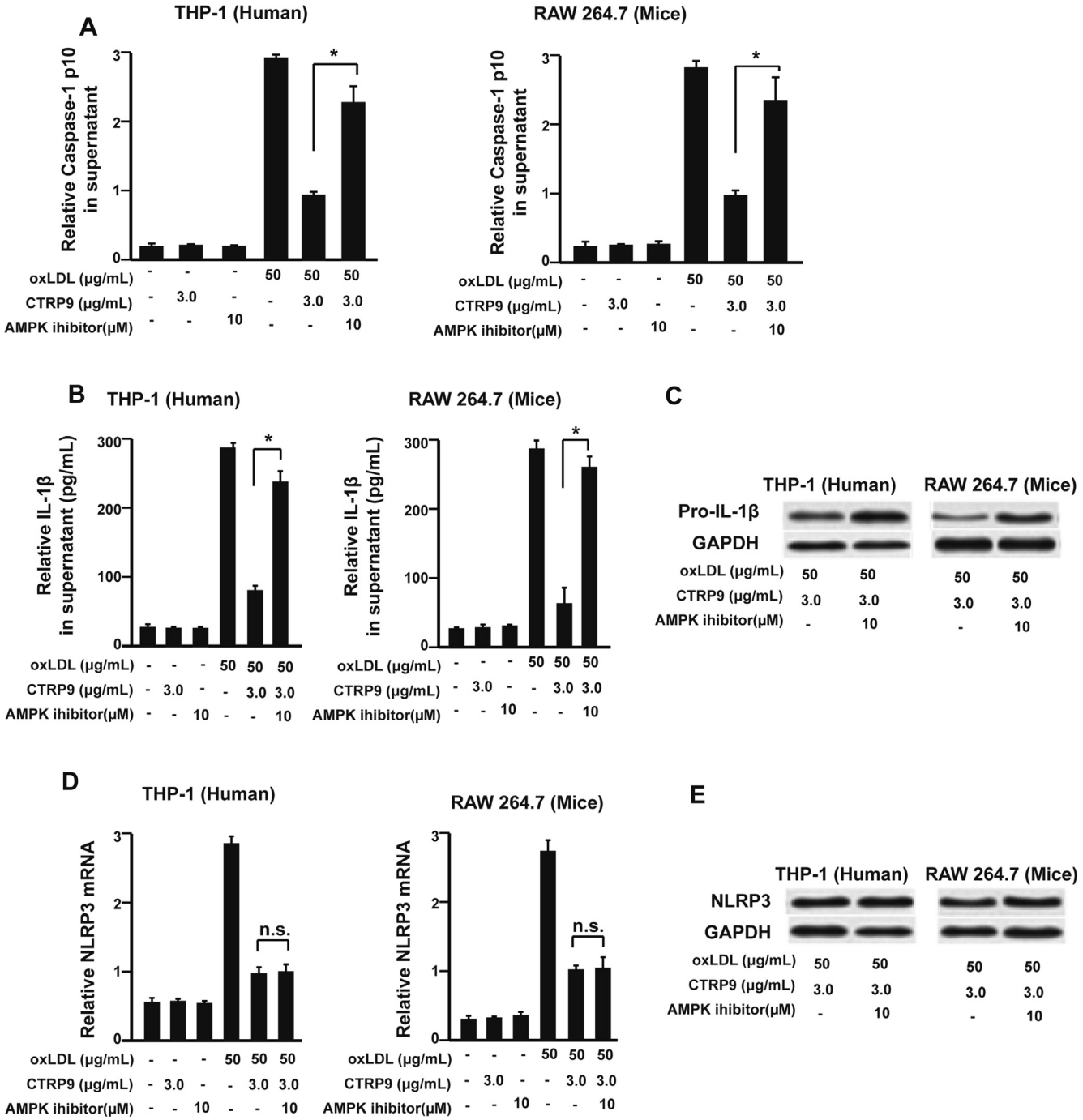

Fig. 2. CTRP9 suppressed the activation of NLRP3 inflammasome through AMPK. Macrophages were treated with ox-LDL for 24 h, followed by ox-LDL plus CTRP9 and AMPK inhibitor (Dorsomorphin 2HCl) treatment for 48 h. (A) The levels of caspase-1 p10 in cell culture medium after treatment were determined western blot. The band intensity was quantified by ImageJ. (B) The levels of IL-1β in cell culture medium after treatment were determined by ELISA (n = 3). (C) Western blot showed the protein levels of cellular pro-IL-1β after CTRP9 treatment. (D) Relative mRNA levels of cellular NLRP3 were determined by qPCR in human and mouse macrophages (n = 3). (E) Western blot showed the protein levels of cellular NLRP3 after CTRP9 plus Dorsomorphin treatment. "−" indicates no treatment; * indicates $P < 0.05$.

the atherosclerosis development, which could be abolished by AMPK inhibition.

In summary, we illustrated here that the CTRP9 showed atheroprotective function via CTRP9-AMPK- NLRP3 inflammasome pathway.

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Declaration of competing interest

The authors declared that there is no conflict of interest.

Data accessibility

All data have been presented in the figures. And other related information are available under request to the corresponding author.

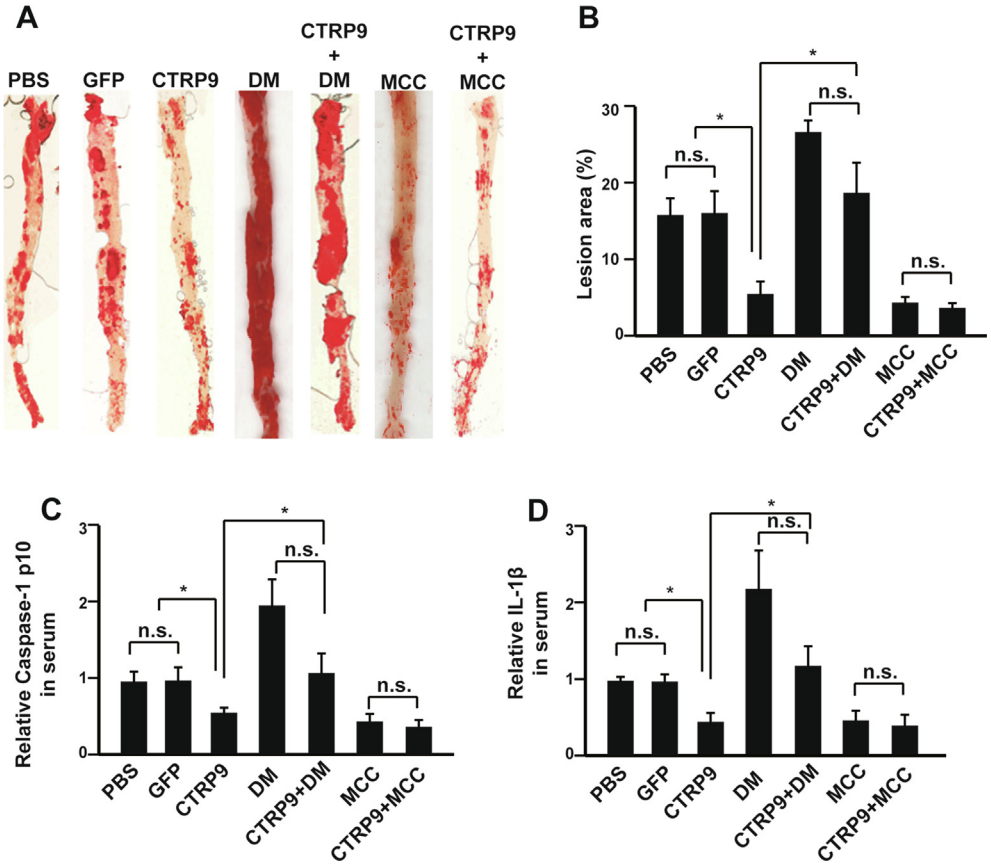

Fig. 3. CTRP9 suppressed the activation of NLRP3 inflammasome through AMPK during the atherosclerosis development in mice. The mouse model of atherosclerosis was established by feeding the ApoE$^{-/-}$ (apolipoprotein E double knock-out) mice with the high-fat diet. The mice were treated with lentiviral expressing CTRP9 with or without AMPK inhibitor Dorsomorphin (DM) (n = 8 for each group). (A) Representative images of plaques in en face analysis of aortas with oil red O staining. (B) The atherosclerosis lesion areas in the thoracoabdominal aorta were measured (n = 8). (C) The levels of caspase-1 p10 in the mice serum after treatment were determined by western blot. The band intensity was quantified by ImageJ (n = 8). (D) The levels of IL-1β in the mice serum after treatment were determined by ELISA (n = 8). N.S. indicates no significant difference; * indicates $P < 0.05$, DM: Dorsomorphin; MCC: MCC950.

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