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Pravastatin improves atherosclerosis in mice with hyperlipidemia by inhibiting TREM-1/DAP12

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Abstract. – OBJECTIVE: To study the protective effect of pravastatin on blood vessels in mice with hyperlipemia.

MATERIALS AND METHODS: Apolipoprotein E (ApoE)$^{-/-}$ and triggering receptor expressed on myeloid (TREM)-/ApoE$^{-/-}$ mice were selected and fed with high-fat food, which were then subdivided into the control group and the pravastatin intervention group. C57BL/6J mice were used as controls. Oil Red O staining was used to stain aortas and sections so as to observe the level and basic composition of plaques. Immunohistochemistry was applied to detect inflammatory cells expression in aortic plaques. Real-time polymerase chain reaction (PCR) was employed to detect the expressions of TREM-1, tumor necrosis factor-alpha (TNF-α), and interleukin-1 (IL-1) messenger ribonucleic acids (mRNAs) in vascular tissues of mice in different groups, and the expressions of TREM-1, DNAX-activating protein of molecular mass 12 (DAP12), TNF-α, and IL-1 were detected via Western blotting technique.

RESULTS: Pravastatin reduced the area of atherosclerotic plaques and improved the plaque formation by reducing lipid deposits and alleviating plaque inflammatory responses. In the pravastatin group, the expression of TREM-1 in the aorta atherosclerotic plaque of mice was decreased, the expressions of TREM-1 and DAP12 genes and proteins in vascular tissue cells declined, and the expressions of the downstream inflammatory factors, TNF-α, IL-1 were reduced.

CONCLUSIONS: Pravastatin improves atherosclerosis (AS) in mice by inhibiting TREM-1/DAP12.

Key Words
Pravastatin, TREM-1, Atherosclerosis.

Introduction

Atherosclerosis (AS), as the first killer of human deaths today, has caused great harm to human health globally. AS in patients with a very complex pathogenesis is the result of multiple factors. In recent years, more and more research results[1,2] have proved that AS is a chronic inflammatory disease. Scholars[3,4] have revealed that immune cells such as monocytes, macrophages, and lymphocytes can secrete inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), and adhesion molecules during AS inflammation, thereby allowing inflammatory cells to enter atherosclerotic plaques and accelerating the pathological process of AS.

Triggering receptors expressed on myeloid cells-1 (TREM-1) is a member of the TREM family, which is mainly expressed in neutrophils, mature monocytes, and macrophage membranes, and can strengthen the inflammatory response. Studies[5-7] have manifested that overexpression of TREM-1 can reduce the release of the anti-inflammatory cytokine IL-10 and increase the secretion and release of inflammatory cytokines and chemokines, thus leading to a positive feedback response. After TREM-1 binds to a ligand, the cytoplasmic tail region of TREM-1 can bind to DNAX-activating protein of molecular mass 12 (DAP12) to form a receptor complex (TREM-1/DAP12), and the immunoreceptor tyrosine-based activation motif (ITAM) tyrosine residue in the DAP12 intracytoplasmic region is phosphorylated. This reaction initiates the phosphorylation of a series of downstream receptor enzymes, triggers the mobilization of intracellular calcium ions, activates transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), transcribes genes encoding pro-inflammatory cytokines, and ultimately allows cells to secrete large amounts of pro-inflammatory cytokines and cell surface molecules[8-10]. TREM-1 has the effect of mediating inflammation amplification and has been a new focus of anti-inflammatory treatment of AS in recent years[11,12].

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Pravastatin is a class of hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitor drugs and has been widely used clinically as a lipid-lowering drug. However, researches on AS have confirmed that pravastatin not only reduces the prevalence rate of cardiovascular events from the lipid-lowering aspect, but also exerts a significant anti-inflammatory effect[13], so it can be assumed that pravastatin can improve AS in many ways by reducing lipids and alleviating inflammation as well.

Materials and Methods

Animals and Reagents

A total of 40 8-week-old Apolipoprotein E (ApoE)$^{-/-}$ mice weighing (24.5+0.5) g, 40 8-week-old TREM^{-}/ApoE$^{-/-}$ mice weighing (23.3+0.2) g and 20 8-week-old C57BL/6J mice weighing (17.20±0.48) g were selected. The nutritional and mental statuses of all animals were normal. These animals were provided by the Experimental Animal Center of Nanjing University. TREM-1, DAP12, TNF-α, and IL-1 antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA), TREM-1, DAP12, TNF-α, and IL-1 primers from Shanghai Bioengineering Co., Ltd. (Shanghai, China), kits for Oil Red O staining from Beyotime Biotechnology Co., Ltd. (Shanghai, China), ly-6C and CD45 antibody from Cell Signaling Technology (CST) (Danvers, MA, USA), and ribonucleic acid (RNA) reverse transcription and quantitative polymerase chain reaction (qPCR) kits from TaKaRa (Otsu, Shiga, Japan). This study was approved by the Animal Ethics Committee of Jining Municipal Government Hospital of Shandong Province Animal Center.

Modeling and Grouping

A total of 40 ApoE$^{-/-}$ mice and 40 TREM^{-}/APOE$^{-/-}$ mice were fed with high-fat food (added with 0.15% cholesterols and 21% lard oil) for 6 weeks to prepare a hyperlipidemia mouse model. After successful modeling, 20 ApoE$^{-/-}$ mice and 20 TREM^{-}/ApoE$^{-/-}$ mice were randomly selected as the pravastatin group treated with intragastric administration of 10 mg/(kg·d) pravastatin for 6 consecutive weeks. Then, the left 40 were selected as the control group receiving intragastric administration of distilled water, and C57BL/6J mice were taken as the blank control group given with high-fat food throughout the process. The environment was maintained at (21±2)°C, with (50±15)% relative humidity and a 12-h light/dark cycle. Eight weeks later, mice were sacrificed for further data analysis.

Oil Red Staining

The mice underwent solid instead of liquid fasting overnight, and the chest cavity of them was opened after anesthesia to expose the heart. The heart and aorta were removed under the aseptic condition, fixed in 10% formalin, dehydrated, routinely embedded with paraffin, cut into sections with the thickness of about 5 μm and allowed to stand overnight at 60°C. After that, toluene dewaxing and gradient alcohol dehydration were conducted. Four identical sections were taken from the aortic root of each mouse. Two sections were conservatively taken at the interval of 100 μm, which were subjected to Oil Red O staining, followed by observation under an optical microscope. Subsequently, the medical image analysis software (Image-Pro Plus IPP, Media player, Silver Springs, MD, USA) was adopted to measure plaque area (PA), cross-sectional area (CSA) of blood vessels in sections, and an average was taken from 4 sections of each specimen.

Immunohistochemical Staining

Paraffin-embedded arterial tissues were cut into sections, followed by dewaxing with xylene. Then, the sections were dehydrated with gradient alcohol, incubated with warm deionized water containing 0.3% H_2O_2 for 30 min, and blocked with serum after the removal of endogenous peroxides. After that, the primary antibody was added for incubation at 4°C overnight. The next day, the IgG antibody horseradish peroxidase (HRP) was added for incubation, and the mixed solution prepared using the avidin-biotin complex (ABC) kit was added dropwise for culturing. After 10 min of 3,3'-diaminobenzidine (DAB) color development, the sections were counterstained with hematoxylin and observed under an optical microscope after washing, dehydration and transparentization.

Western Blotting

The extracted tissues were ground with liquid nitrogen and diluted with normal saline. The supernatant was taken after the tissues were let stand on ice. Then, the tissues were centrifuged at 4°C for 5 min, and the supernatant was discarded. Precipitates were resuspended with radioimmunoprecipitation assay (RIPA) lysate containing

phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China), after which the tissues were centrifuged at 4°C and 16,000 g for 15 min, and the supernatant was taken for protein quantitation. The proteins were added with the load sample buffer solution and heated for denaturation. Subsequently, membrane transfer was carried out after electrophoresis using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and the membrane was blocked with 5% skim milk for 2 h. The primary antibody was added for incubation at 4°C overnight, followed by washing with Tris-buffered saline with Tween 20 (TBS-T) 3 times with 10 min each time. The corresponding secondary antibody was added for incubation at room temperature for 1 h, followed by washing with TBS-T 3 times with 10 min each time. Finally, the protein expression level of different samples was detected by enhanced chemiluminescence (ECL) assay (Thermo Fisher Scientific, Waltham, MA, USA).

Real-Time qPCR

A suitable amount of tissue samples were taken, added with TRIzol (Invitrogen, Carlsbad, CA, USA) and lysed at room temperature for 5 min. 1/5 volume of chloroform was added with the tissue lysate, fully shaken until fully emulsified without delamination and let stand at room temperature for 5 min. After centrifugation at 4°C and 12,000 g for 15 min, the supernatant was collected and added to the Eppendorf (EP) tube, the same volume of isopropanol was added to be mixed evenly, and the mixed solution was let stand at room temperature for 10 min. After centrifugation at 4°C and 12,000 g for 10 min, the supernatant was discarded, precipitates were washed with 75% ethanol, and then, continuously centrifuged for 5 min, and the ethanol was discarded, followed by desiccation for 2-3 min. Then, the precipitates were dissolved with 20 μL sterile diethylpyrocarbonate (DEPC)-treated water. After the RNA concentration was measured, reverse transcription was performed according to the reverse transcription kit instructions. PCR reaction system: 10 μL TB Green Premix Ex TaqII (2×), 0.4 μL ROX Reference Dye or Dye II (50×), 0.8 μL upstream and 0.8 μL downstream primers (10 μmol/L), 2 μL complementary deoxyribonucleic acid (cDNA) solution, and 6 μL sterilized ddH_2O (20 μL reaction system in total). PCR cycle parameters: a total of 40 cycles of predenaturation at 95°C for 30 s, 95°C for 5 s, 60°C for 34 s, and fluorescence collection at 60°C. Data were collected using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as a housekeeping gene, and the relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Data were expressed as mean ± standard deviation and analyzed using paired or unpaired *t*-test. One-way ANOVA was employed for comparisons among multiple groups, and the Student-Newman-Keuls (SNK) post-hoc test was conducted for pairwise comparisons. $p<0.05$ represented that the difference was statistically significant. Data were analyzed on Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA), and then, plotting was performed using GraphPad software (Version X; La Jolla, CA, USA).

Results

Pravastatin Improved AS in Mice

Oil Red O staining was conducted for the gross aorta specimens of each group of mice, and the ratio of PA to the blood vessel cross-sectional area (CSA) was compared. Figure 1A shows Oil Red O staining of aortas of these five groups of mice, Figure 1B shows Oil Red O staining of aortic sections and Figure 1C displays the ratio of Oil Red O plaque staining area to the blood vessel CSA. Under a microscope, almost no plaques were observed in wild-type mice, and atheromatous plaques were evident in the aorta of untreated $ApoE^{-/-}$ mice, accounting for approximately 25% while the plaques accounted for about 10% in $ApoE^{-/-}$ mice treated with pravastatin. Oil Red O staining revealed that the content of lipid vacuoles indirectly represented the relative content of lipids. In the pravastatin group, the lipid vacuoles in mouse aorta plaques were significantly less than those in the control group (Figure 1B). Labeled CD45 and macrophage ly-6C was applied as an index to detect atherosclerotic inflammatory responses (Figure 2A-2B). In addition, inflammatory cells and macrophages were significantly reduced in the plaques of pravastatin-treated $ApoE^{-/-}$ mice compared with those in the control group (Figure 2B). These results indicate that pravastatin can effectively reduce the area of atherosclerotic plaques, reduce the lipid content in plaques, and alleviate the plaque inflammatory response.

TREM-1 Knockout Reduced AS Degree in Mice and Significantly Lowered the Plaque Inflammation Index

TREM-1 played a key role in the inflammatory response during the formation of AS. In TREM^{-}/ApoE$^{-/-}$ mice, Oil Red O staining of aortic sections displayed that atherosclerotic plaques occupied about 18% of the arterial area. The difference was statistically significant in comparison with untreated ApoE$^{-/-}$ mice (p<0.05), but the differences were not statistically significant in comparisons with pravastatin-treated ApoE$^{-/-}$ mice and TREM^{-}/ApoE$^{-/-}$ mice (p>0.05) (Figure 1A-1B). CD45 and Ly-6c labeling illustrated that macrophages in TREM^{-}/ApoE$^{-/-}$ mouse plaques were reduced compared with those in untreated ApoE$^{-/-}$ mouse plaques (Figures 2A-2D). qPCR and Western blotting results manifested that TREM-1 knockout could lower the gene and protein expression levels of DAP12 and inflam-

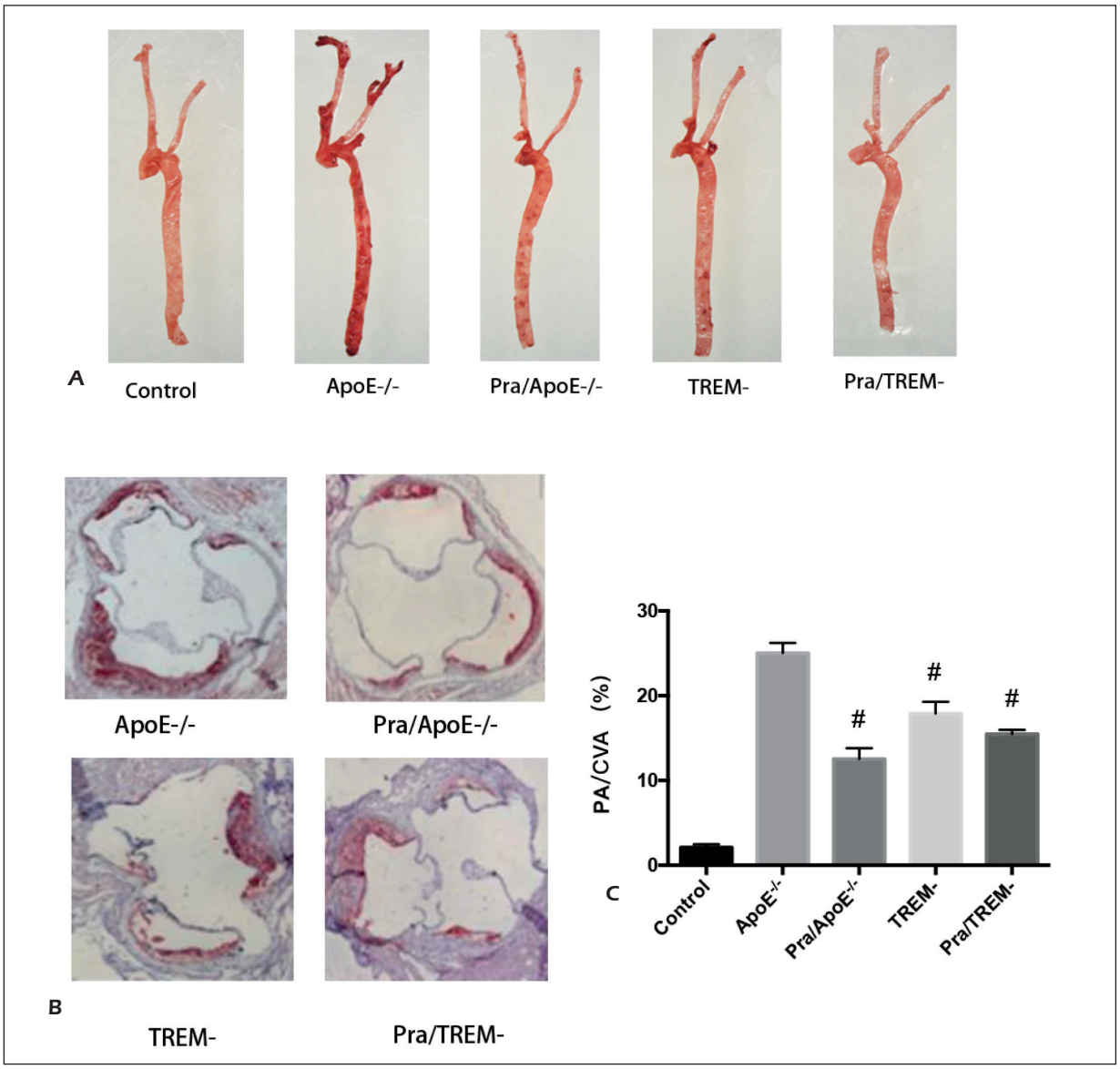

Figure 1. A, Oil Red O staining images of the aortas of control group (WT), ApoE$^{-/-}$ group (ApoE$^{-/-}$), pravastatin+ApoE$^{-/-}$ group (Pra/ApoE$^{-/-}$), TREM-/ApoE$^{-/-}$ group (TREM-), and pravastatin+TREM-/Apoe$^{-/-}$ group (Pra/TREM-). **B**, Oil Red O staining images of control group (WT), ApoE$^{-/-}$ group (ApoE$^{-/-}$), pravastatin+ApoE$^{-/-}$ group (Pra/ApoE$^{-/-}$), TREM-/ApoE$^{-/-}$group (TREM-), and pravastatin+TREM-/Apoe$^{-/-}$ group (Pra/TREM-), with scale plate of 100 μM. **C**, Aortic plaque area is expressed as the ratio of PA to CVA. $^{\#}p$<0.05 vs. ApoE$^{-/-}$ (n=5).

matory cytokines, TNF-α and IL-1, in the mouse aorta (Figure 3A-3F). Therefore, knocking out TREM-1 gene could decrease atherosclerotic plaques in mice, reduce the lipid content in plaques, inhibit the levels of inflammatory cytokines and significantly reduce inflammatory responses.

Pravastatin Suppressed TREM-1/DAP12 and Decreased Downstream Inflammatory Cytokines

TREM-1 could bind to DAP12 and trigger phosphorylation of downstream receptor enzymes, thus enabling cells to produce a large number of inflammatory cytokines. To further investigate the effects of pravastatin on the TREM-1/DAP12 pathway and downstream inflammatory cytokines, the gene and protein expression levels of TREM-1, DAP12, TNF-α, and IL-1 in mouse aorta tissues were examined (Figures 3-4). The messenger ribonucleic acid (mRNA) and protein expression levels of TREM-1 and DAP12 were significantly increased in untreated ApoE$^{-/-}$ mice, but significantly decreased in the pravastatin treatment group (Figure 3A-3F). The expression levels of TNF-α, IL-1 genes and proteins went up in untreated ApoE$^{-/-}$ mice, and pravastatin treat-

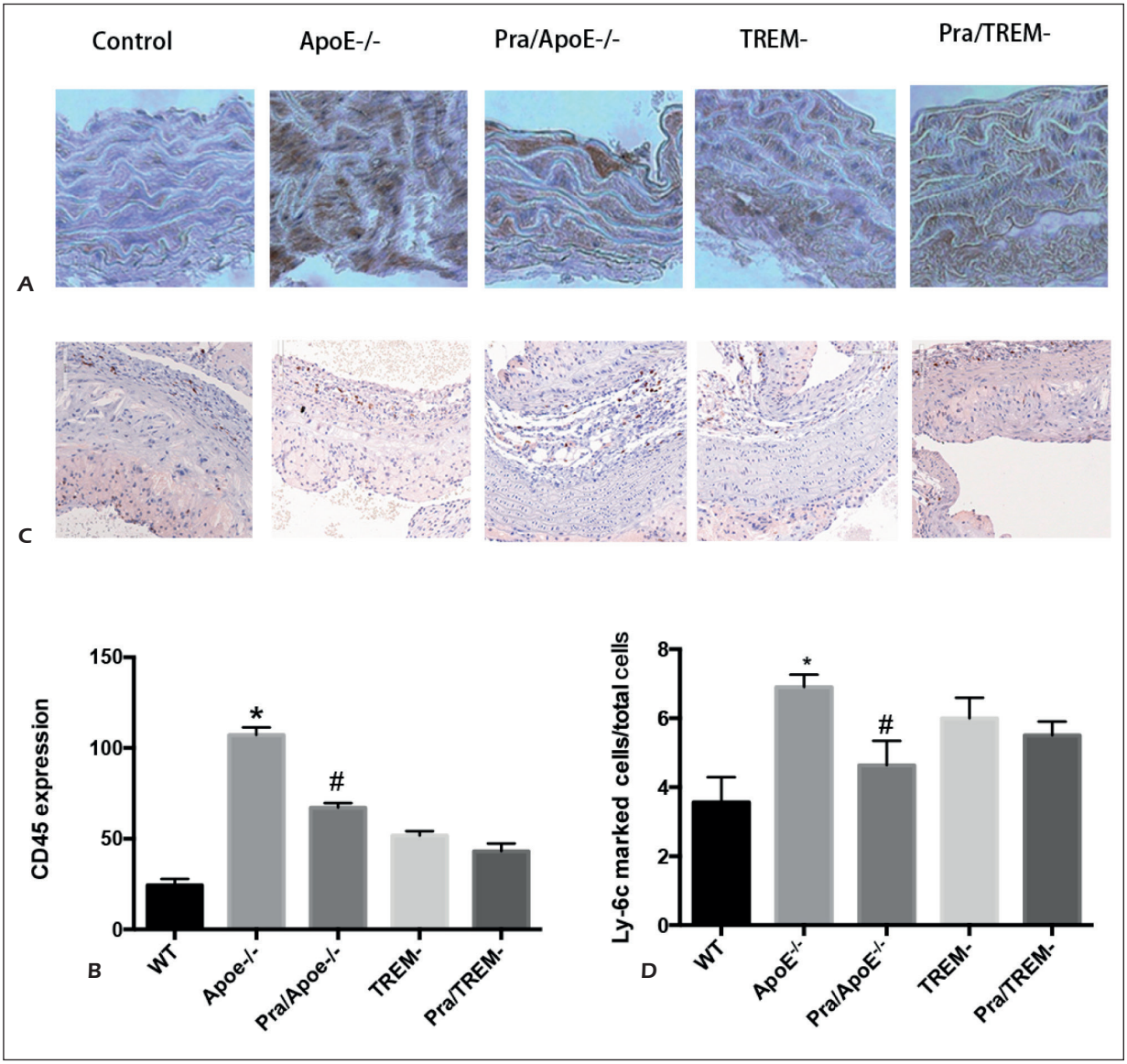

Figure 2. A, CD45 staining images of WT, ApoE$^{-/-}$, Pra/ApoE$^{-/-}$, TREM-, and Pra/TREM-. **B**, The expression level of cd45-labeled cells. *p<0.05 vs. WT, and #p<0.05 vs. ApoE$^{-/-}$ (n=5). **C**, Ly-6C staining images of WT, ApoE$^{-/-}$, Pra/ApoE$^{-/-}$, TREM-, and Pra/TREM-. **D**, The percentage of ly-6C-labeled macrophages in total cells. *p<0.05 vs. WT, and $^{\#}p$<0.05 vs. ApoE$^{-/-}$ (n=5).

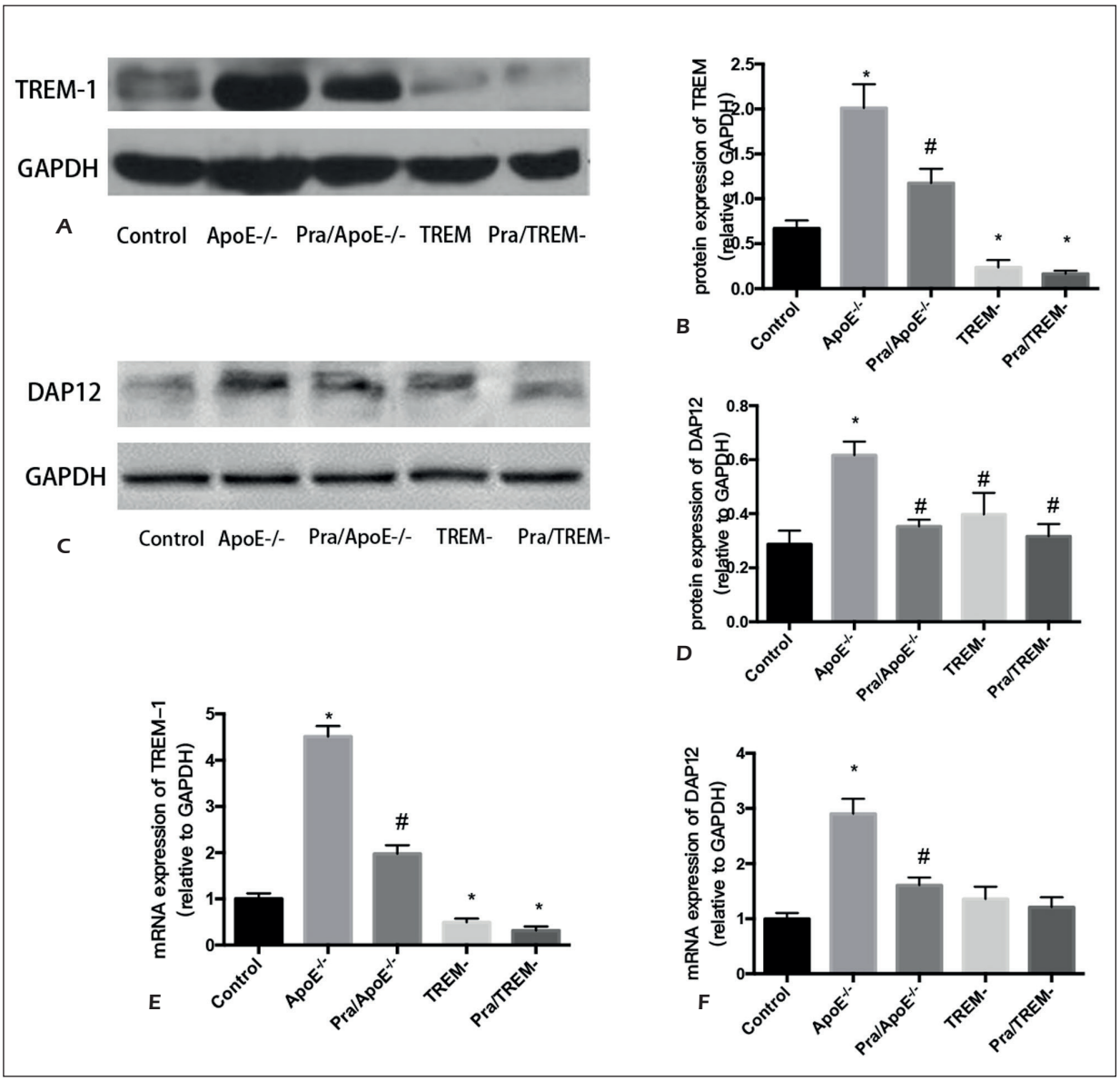

Figure 3. ***A-B***, Images of TREM-1 protein content in WT, ApoE$^{-/-}$, Pra/ApoE$^{-/-}$, TREM-, and Pra/TREM- via Western blotting detection. ***C-D***, Images of DAP12 protein content in WT, ApoE$^{-/-}$, Pra/ApoE$^{-/-}$, TREM-, and Pra/TREM- via Western blotting detection. ***E-F***, Images of TREM-1/DAP12 mRNA content in WT, ApoE$^{-/-}$, Pra/ApoE$^{-/-}$, TREM-, and Pra/TREM- via Real-time PCR detection. *p<0.05 vs. WT, and $^{\#}p$<0.05 vs. ApoE$^{-/-}$ (n=5).

ment could reduce these inflammatory cytokines. In untreated TREM^{-}/ApoE$^{-/-}$ mice, the expression levels of TREM-1 and DAP12 declined, and the expression levels of downstream TNF-α, IL-1, and other inflammatory cytokines were remarkably lower than those in the untreated ApoE$^{-/-}$ mice. The expression levels of downstream TNF-α, IL-1, and other inflammatory cytokines were not statistically different from those in untreated TREM^{-}/ApoE$^{-/-}$ mice and pravastatin-treated ApoE$^{-/-}$ mice (Figure 4). The above results suggest that pravastatin can act on TREM-1/DAP12 and suppress the production and release of downstream inflammatory cytokines, TNF-α, and IL-1.

Discussion

Pravastatin is a lipid-lowering drug widely used in the treatment of atherosclerotic heart disease, which can reduce serum cholesterol and low-den-

sity lipoprotein contents, thereby reducing the occurrence rate of cardiovascular events[14]. However, in addition to lipid-lowering effects, pravastatin also has anti-inflammatory and protective effects on the vascular endothelium. Inflammation has been proven to be an important step in the formation of atherosclerotic plaques. Therefore, pravastatin inhibits the formation and release of inflammatory cytokines and slow down the inflammatory response, thus reducing atherosclerotic plaque formation, which is an important role in lipid lowering[15].

This work manifested that pravastatin could effectively reduce the area of atherosclerotic plaque. The plaque content was analyzed by H&E staining, which revealed that in plaques of the pravastatin group, both lipid and macrophage contents were markedly reduced, and the reduction of macrophages could decrease the formation of foam cells and the deposition of cholesterols[16,17]. Therefore, pravastatin could improve the formation of atherosclerotic plaques through both lipid-lowering and anti-inflammatory effects.

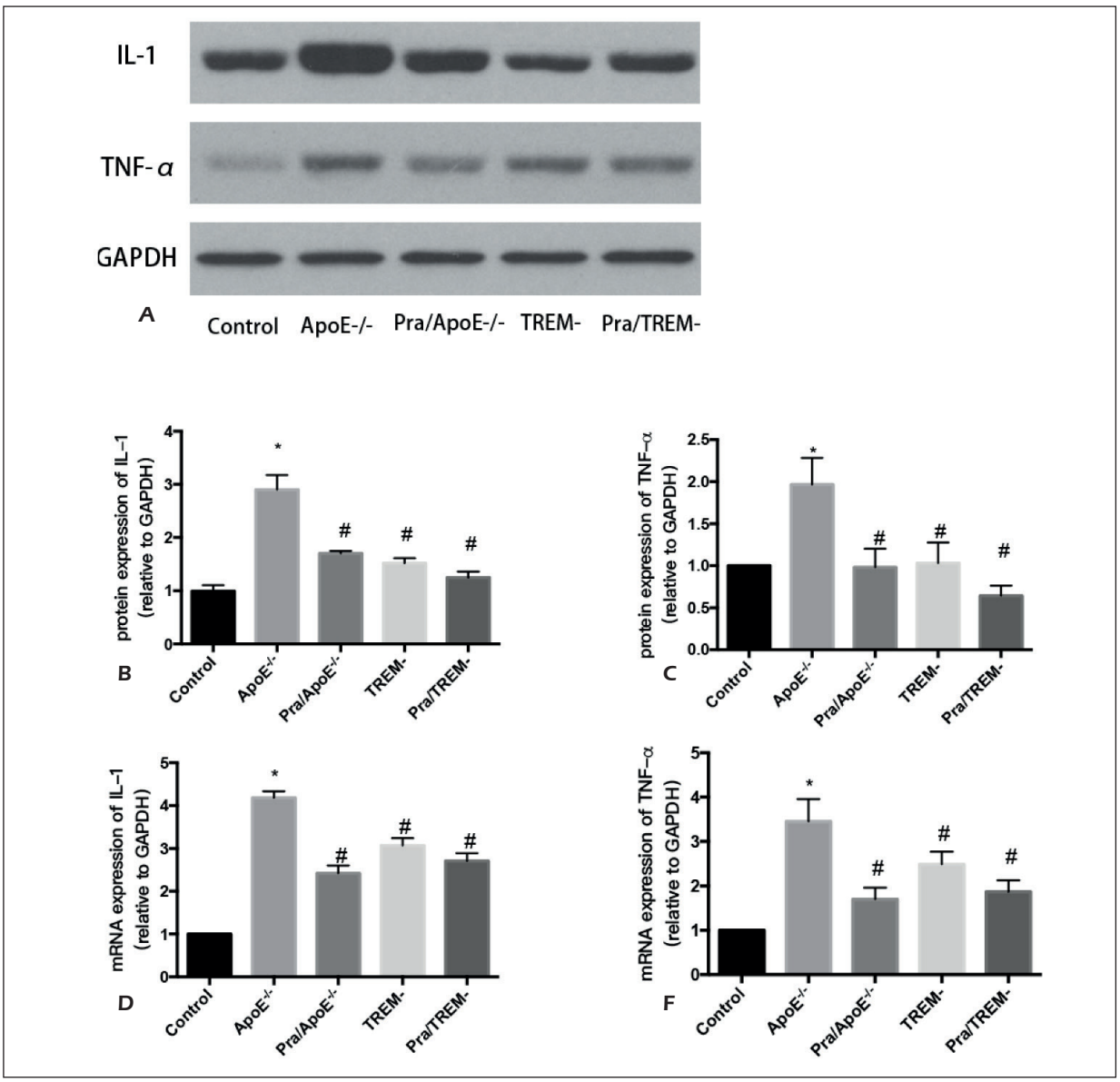

Figure 4. A-C, Images of IL-1 and TNF-α protein content in WT, ApoE$^{-/-}$, Pra/ApoE$^{-/-}$, TREM-, and Pra/TREM- via Western blotting detection. **D-E**, Images of IL-1 and TNF-α mRNA content in WT, ApoE$^{-/-}$, Pra/ApoE$^{-/-}$, TREM-, and Pra/TREM- via Real-time PCR detection. *p<0.05 vs. WT, and $^{\#}p$<0.05 vs. ApoE$^{-/-}$ (n=5)

TREM-1 mediates inflammation amplification and the complex formed by TREM-1 binding to DAP12 phosphorylates downstream receptor enzymes, triggers the mobilization of intracellular calcium, and promotes the production and release of inflammatory cytokines[8]. In this study, it was found that after TREM-1 knockout, the area of atherosclerotic plaques, the lipid content, and the number of macrophages were decreased significantly, indicating that TREM-1 plays important roles in the formation of atherosclerotic plaques and the inflammation process.

The anti-inflammatory effect independent of lipid-lowering effect of pravastatin is of great importance in reducing the occurrence rate of AS, relieving AS course, and ultimately, reducing the occurrence rate of cardiovascular events. TREM-1 is a crucial inflammatory cytokine, but the relationship between pravastatin and TREMM-1 and their roles in the formation of atherosclerotic plaques remain unknown. In this investigation, we showed that administration of pravastatin reduced the expression of TREM-1 and DAP12 in mouse aortic tissues and plaques, and further inhibited the expression of TREM-1/DAP12 downstream inflammatory cytokines, such as TNF-α, IL-1, and MCP-1. The effect of pravastatin on reducing inflammatory cytokines in TREM-1 knockout mice was not statistically different from that in pravastatin-treated ApoE$^{-/-}$ mice. These results suggested that the anti-inflammatory effect of pravastatin is achieved by TREM-1/DAP12. However, whether there are other pathways that play roles in pravastatin's inhibition of inflammatory responses remains to be confirmed.

Conclusions

We found that pravastatin inhibited the formation of TREM-1/DAP12 complex by suppressing the inflammation triggering factor TREM-1. This further impeded the synthesis and release of downstream inflammatory cytokines such as TNF-α and IL-1, improved the formation of atherosclerotic plaques in mice with hyperlipemia and slowed down inflammatory responses during plaque formation, thereby reducing the occurrence rate of cardiovascular disease.

Conflict of Interests

The authors declare they have no conflict of interest.

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