

# Tacrolimus reduces atherosclerotic plaque formation in ApoE<sup>-/-</sup> mice by inhibiting NLRP3 inflammatory corpuscles

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**Abstract.** Effect of tacrolimus on atherosclerotic plaques and its influence on Nod-like receptor protein 3 (NLRP3) inflammatory pathway were studied by establishing the mouse model of atherosclerosis. The mice were divided into 3 groups: C57BL/6 mouse group (WT group), ApoE<sup>-/-</sup> mouse group (ApoE<sup>-/-</sup> group) and ApoE<sup>-/-</sup> mouse + tacrolimus intervention group (ApoE<sup>-/-</sup> + Tac group). The area of atherosclerotic plaques and the pathological morphologic changes were observed. The NLRP3, interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-18, NLRP3 inflammatory corpuscles, pro-inflammatory factors IL-1 $\beta$  and IL-18 in the aorta were analyzed. The area of atherosclerotic plaques in ApoE<sup>-/-</sup> mice was increased significantly, and it was significantly reduced after tacrolimus intervention. After tacrolimus intervention, the arterial intima became obviously thinner and no obvious cholesterol crystals were observed. The macrophage infiltration in atherosclerotic plaques was significantly increased, and the content of smooth muscle cells was also increased. The levels of serum IL-1 $\beta$ , IL-18 and NLRP3 in ApoE<sup>-/-</sup> mice were significantly increased, and they remarkably declined after tacrolimus intervention. ROS content in atherosclerotic plaques was increased in ApoE<sup>-/-</sup> mice, and it remarkably declined after tacrolimus intervention. The protein content of NLRP3, ASC, Casp-1, IL-1 $\beta$  and IL-18 in the aorta in ApoE<sup>-/-</sup> mice was remarkably increased, and they were inhibited to some extent after tacrolimus intervention. In conclusion, it is speculated that tacrolimus may reduce the formation of AS through inhibiting ROS in macrophages and activation of NLRP3 inflammatory corpuscles and reducing the release of IL-1 $\beta$  and IL-18.

## Introduction

With the improvement of living standards, coronary atherosclerotic disease remains one of the leading causes of

morbidity and mortality worldwide (1). Coronary atherosclerosis is the pathological basis of coronary atherosclerotic heart disease (2,3). Therefore, it is of great significance to explore the pathogenesis of atherosclerosis (AS) and search effective therapeutic methods. At present, the specific mechanism of AS remains unclear, and most scholars regard it as a chronic inflammatory response (4-6). Mononuclear macrophages have the most significant role in formation of atherosclerotic plaques, and the germinal cells of the innate immune system, which exist in each stage of atherosclerotic lesions (7,8). The pro-inflammatory factors released by macrophages play key roles, in which interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 are the most important ones accelerating the development of AS (9,10). Studies have demonstrated that metabolites formed by the body can be sensed by Nod-like receptor (NLR) in the cytoplasm of macrophages, and then NLR forms a complex with apoptosis-associated speck like protein containing CARD (ASC) and caspase-1 (Casp-1). The complex, which is called inflammatory corpuscle, can promote the maturation of inflammatory cytokines (11,12). Nod-like receptor protein 3 (NLRP3), an inflammatory corpuscle most closely related to chronic inflammatory response, is a kind of pattern recognition receptor in innate immune cells that has been studied widely, which plays a decisive role in innate immunity (13). After the ligand binds to NLRP3, the formation of inflammatory corpuscles is promoted, and Casp-1 is activated, ultimately promoting the maturation and secretion of pro-IL-1 $\beta$  and pro-IL-18, so that the pro-inflammatory factors IL-1 $\beta$  and IL-18 are produced (14).

Tacrolimus, also known as FK506, is a potent immunosuppressor, which, as a first-line drug in liver, kidney and heart transplantation, has come into the market in Japan and the United States in recent years (15). At the same time, it also plays a positive role in the treatment of such autoimmune diseases as atopic dermatitis, systemic lupus erythematosus and autoimmune eye diseases (16-18). A large number of clinical studies have proved that tacrolimus can significantly reduce the incidence of early initial poor function (IPF), primary nonfunction (PNF) and delayed nonfunction (DNF) caused by ischemia-reperfusion injury after transplantation (19-21), and ischemia-reperfusion injury is essentially a non-specific inflammatory response, indicating that tacrolimus has an anti-inflammatory property. Moreover, many studies have shown that tacrolimus is topical calcineurin inhibitors (22-24). However, whether tacrolimus can affect the occurrence and

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development of AS through the anti-inflammatory effect has not been reported yet. In this investigation, the animal model of AS was established to observe the effect of tacrolimus on atherosclerotic plaques and its influence on the NLRP3 inflammatory pathway.

## Materials and methods

**Laboratory animals and models.** A total of 20 male apolipoprotein E (ApoE, a polymorphic protein involved in the transformation and metabolism of lipoproteins)<sup>-/-</sup> mice aged 6 weeks, weighing 16-18 g and 10 male C57BL/6 mice (as a wild-type control group) aged 6 weeks old weighing 16-18 g were purchased from Qingdao University Animal Center. After adaptation for 1 week, ApoE<sup>-/-</sup> mice were fed with high-fat diet (The formula: 79.85% general fodder + 15% fat + 5% yolk powder + 0.15% cholesterol), while C57BL/6J mice were fed with general fodder. This study was approved by the Animal Ethics Committee of the Third People's Hospital of Qingdao Animal Center (Qingdao, China).

**Experimental grouping and treatment.** The mice were divided into 3 groups: C57BL/6 mice group (WT group), ApoE<sup>-/-</sup> mouse group (ApoE<sup>-/-</sup> group), and ApoE<sup>-/-</sup> mouse + tacrolimus intervention group (ApoE<sup>-/-</sup> + Tac group). In ApoE<sup>-/-</sup> + Tac group, after high-fat diet for 6 weeks, tacrolimus at 3 mg/kg/day, according to pre-experimental results was intraperitoneally injected for 12 weeks.

**Extraction of aorta.** After tacrolimus intervention for 12 weeks, the blood was taken from the orbit, the mice were anesthetized and fixed on an anatomy plate, and the heart was exposed. A fine needle was inserted into the left ventricle to the ascending aorta, and 50 ml phosphate buffered saline (PBS) was slowly perfused at room temperature. The aorta was isolated under a surgical microscope, the excess adipose tissues around the aorta were removed, and the aorta was extracted from the mouse. The aortic root was embedded into OCT embedding agent, sliced into frozen sections (approximately 5- $\mu$ m thick) and stored at -20°C. In addition, a small part of the upper aortic segment was preserved in 4% paraformaldehyde, embedded into paraffin, sliced into paraffin sections (approximately 5- $\mu$ m thick) and stored at room temperature. The adipose tissues around the upper segments of thoracic aorta and abdominal aorta were removed, followed by oil red O staining.

**Oil red O staining.** Oil red O staining for aorta: The upper segments of thoracic aorta and abdominal aorta were differentiated in 60% isopropanol for a few minutes, laid on a glass slide, and added dropwise with oil red O working solution. After soaking for 2 h, the oil red O dye was discarded, followed by differentiation with 60% isopropanol. Then the differentiation solution was replaced until the whitening of aorta, and reddening of plaque. After the aorta was washed with tap water, it was observed and photographed under an optical microscope.

Oil red O staining for frozen sections: The frozen sections of aortic root were taken from the -20°C refrigerator, re-warmed at room temperature for 30 min, washed with 60% isopropanol

for 10 min, and stained with heated oil red O working solution for 10 min, followed by differentiation with 60% isopropanol for 3-10 sec until the mesenchyme became clear, washing, hematoxylin counterstaining for 1 min, and washing with tap water. The water was dried carefully with filter paper, and the sections were sealed with gelatin glycerin and observed and photographed under an optical microscope.

**Hematoxylin and eosin (H&E) staining.** The paraffin sections prepared were transparentized with xylene, deparaffinized with ethanol, soaked in tap water for 5 min, stained with hematoxylin for 5 min, differentiated with 1% hydrochloric acid alcohol for 30 sec and observed under the microscope, followed by eosin staining completely soaking tissues. Finally, the sections were sealed with neutral balsam, and observed and photographed under the optical microscope.

**Immunofluorescence staining.** The frozen sections were taken from the -20°C refrigerator, re-warmed at room temperature for 30 min and fixed in ice acetone for 10 min. After antigen retrieval for 30 min, the sections were soaked in 0.3% triton X-100 at room temperature for 30 min to rupture the cell membrane, and sealed with 5% bovine serum albumin (BSA) at room temperature for 60 min. The serum around the tissues was wiped off, and the primary antibodies ( $\alpha$ -SMA diluted at 1:400, and MOMA-2 diluted at 1:50) were added dropwise onto the sections for incubation in a wet box at 4°C overnight. The next day, the sections were taken from the refrigerator, re-warmed at room temperature for 30 min and added with secondary antibodies (diluted at 1:400 and 1:200) for incubation in the dark at room temperature for 3 h, followed by 4',6-diamidino-2-phenylindole (DAPI) counterstaining in the dark for 5 min. Finally, the sections were sealed with anti-fluorescence quenching sealing solution, covered with the cover glass, and observed and photographed under a fluorescence confocal microscope.

**Enzyme-linked immunosorbent assay (ELISA).** The blood was collected and centrifuged at 2,750 x g and 4°C for 15 min, and the supernatant was taken. The concentrations of serum IL-1 $\beta$ , IL-18 and NLRP3 in mice were measured according to the instructions of the ELISA kit.

**Aortic reactive oxygen species (ROS).** The frozen sections were taken, re-warmed for 10 min and added with dihydroethidium (DHE) prepared in the dark, followed by incubation in the dark at 37°C for 30 min. Finally, the sections were observed and photographed under the fluorescence confocal microscope.

**Western blotting.** The arterial tissues isolated were cut off, added with 1 ml protein lysis buffer, homogenized for approximately 2 min and lysed for 30 min, followed by centrifugation at 10,500 x g and 4°C for 15 min. The supernatant was taken, and the protein concentration in the aorta was detected according to the instructions of the bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). After denaturation, 50  $\mu$ g protein was slowly loaded for electrophoresis. After that, the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane

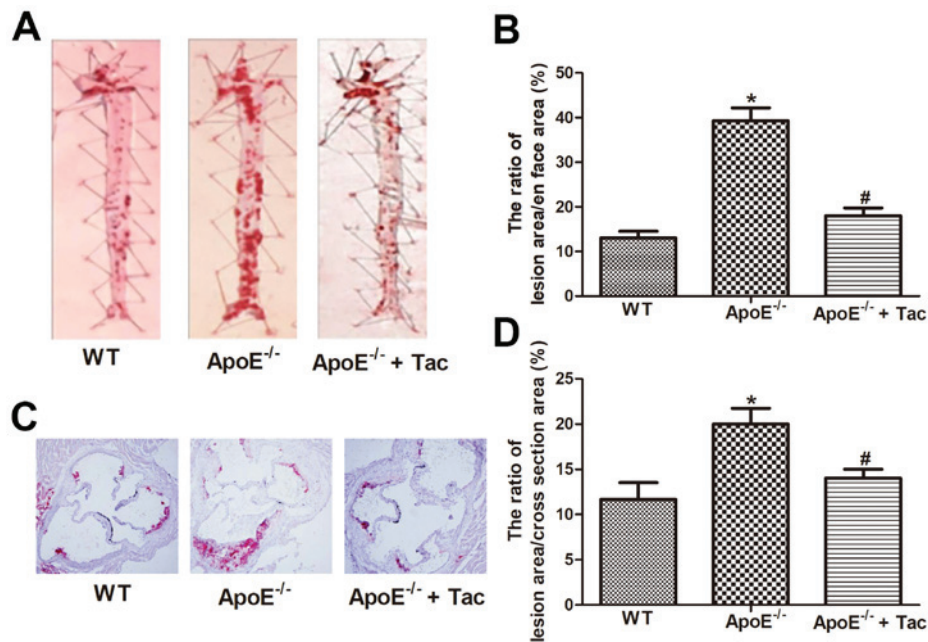


Figure 1. Tacrolimus reduces the area of atherosclerotic plaques in mice. (A) Representative images of oil red O staining of aorta (magnification of x5). (B) Analysis of the ratio of lesion area/en face area in different groups. (C) Representative images of oil red O staining of aortic root (magnification of x40). (D) Analysis of the ratio of lesion area/cross section area in different groups. \* $P < 0.05$  vs. WT group, # $P < 0.05$  vs. ApoE<sup>-/-</sup> group. WT, C57BL/6 mouse group; ApoE<sup>-/-</sup>, ApoE<sup>-/-</sup> mouse group; ApoE<sup>-/-</sup> + Tac, ApoE<sup>-/-</sup> mouse + tacrolimus intervention group.

(EMD Millipore), washed with tris buffered saline-tween (TBST) and sealed with 5% skim milk powder for 2 h, followed by incubation with primary antibodies (diluted pro rata) on a shaking table at 4°C overnight. The next day, the membrane was washed, and the protein was incubated with secondary antibodies for 2 h. After the membrane was washed again, the image was developed in an imager using the electrochemiluminescence (ECL) solution, followed by scanning and quantitative calculation.

**Statistical analysis.** Image-Pro Plus 6.0 (Silver Springs) was used for image analysis and Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp.) was used for data analysis. All data were expressed as (mean  $\pm$  SD). Comparison between groups was done using One-way ANOVA test followed by post hoc test (least significant difference).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Tacrolimus reduces the area of atherosclerotic plaques in mice.** The oil red O staining of aorta in each group revealed that the area of atherosclerotic plaques in ApoE<sup>-/-</sup> mice was increased significantly compared with that in WT mice, and the difference was statistically significant, indicating that the AS model was successfully established. After tacrolimus intervention, the area of atherosclerotic plaques was significantly reduced in ApoE<sup>-/-</sup> mice fed with high-fat diet, and the difference was statistically significant (Fig. 1A and B). The oil red O staining of aortic root further showed that tacrolimus could reduce the area of atherosclerotic plaques in ApoE<sup>-/-</sup> mice fed with high-fat diet, and there was a statistically significant difference (Fig. 1C and D).

**Tacrolimus alleviates the pathological lesions of atherosclerotic plaques in mice.** The H&E staining of aortic root in each group revealed that the thickness of the aortic wall was uniform and there was very little AS in WT mice. In the other two groups, there was intima thickening of the arterial wall in different degrees in ApoE<sup>-/-</sup> mice, the fibrous caps were formed, the thickness of the aortic wall was non-uniform and the atherosclerotic plaques were obvious. There were obvious arterial intima thickening and formation of cholesterol crystals in ApoE<sup>-/-</sup> mice fed with high-fat diet, and after tacrolimus intervention, the arterial intima became obviously thinner and no obvious cholesterol crystals were observed (Fig. 2).

**Tacrolimus reduced the macrophage infiltration in atherosclerotic plaques in mice.** To evaluate the effect of tacrolimus on inflammation in atherosclerotic plaques, the macrophage infiltration and changes in smooth muscle cells in plaques were detected using MOMA-2 and  $\alpha$ -SMA as specific markers. In atherosclerotic lesions, the macrophage infiltration was obviously increased in ApoE<sup>-/-</sup> mice compared with that in WT mice, showing a statistically significant difference, and the content of smooth muscle cells was also increased, but there was no statistically significant difference. After tacrolimus intervention, the macrophage infiltration was obviously reduced, and the content of smooth muscle cells was obviously increased in atherosclerotic lesions in ApoE<sup>-/-</sup> mice fed with high-fat diet, displaying statistically significant differences (Fig. 3).

**Tacrolimus reduces the concentrations of serum inflammatory factors in AS mice.** According to the ELISA results, the levels of serum IL-1 $\beta$ , IL-18 and NLRP3 in ApoE<sup>-/-</sup> mice fed with high-fat diet were significantly increased compared with those in WT mice, and the differences were statistically significant.



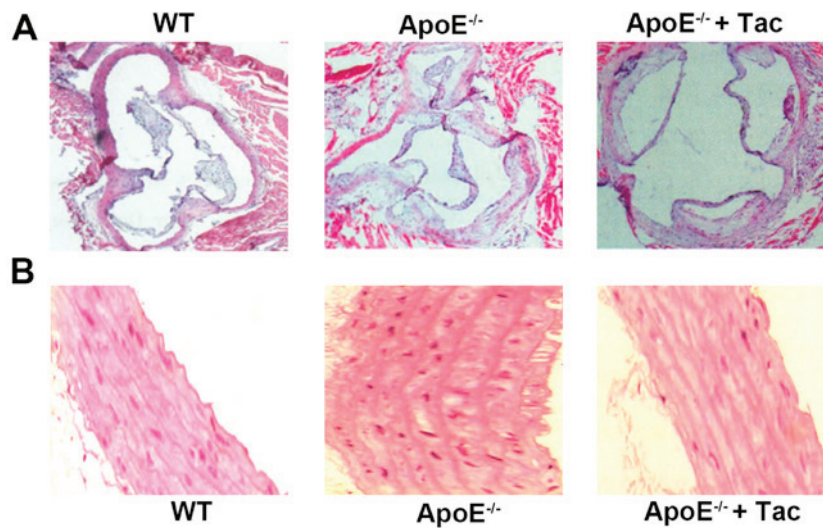


Figure 2. Tacrolimus alleviates the pathological lesions of atherosclerotic plaques in mice. (A) Representative images of H&E staining of aortic root (magnification of x40). (B) Representative images of H&E staining of intima of aorta (magnification of x400). H&E, hematoxylin and eosin; WT, C57BL/6 mouse group; ApoE<sup>-/-</sup>, ApoE<sup>-/-</sup> mouse group; ApoE<sup>-/-</sup> + Tac, ApoE<sup>-/-</sup> mouse + tacrolimus intervention group.

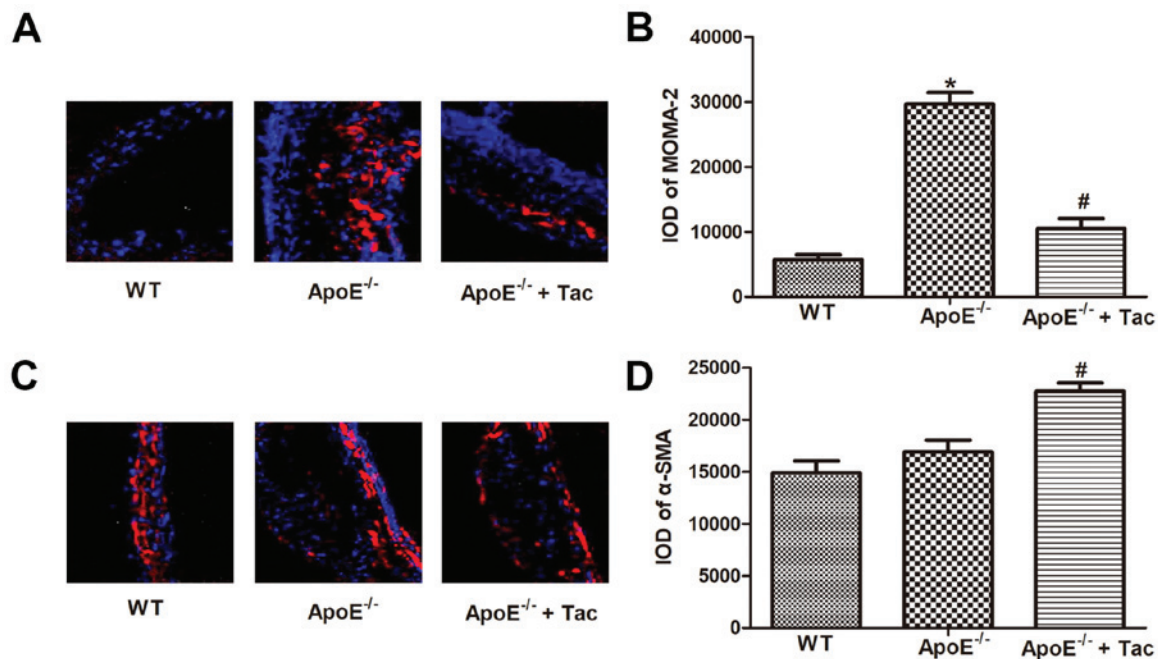


Figure 3. Tacrolimus reduces macrophage infiltration in atherosclerotic plaques in mice. (A) Representative images of immunofluorescence staining showed the expression of macrophage specific markers MOMA-2 (red) (magnification x400). (B) Analysis of the IOD of MOMA-2 in different groups. (C) Representative images of immunofluorescence staining showed the expression of smooth muscle cells specific markers α-SMA (red) (magnification of x400). (D) Analysis of the IOD of α-SMA in different groups. \*P<0.05 vs. WT group, #P<0.05 vs. ApoE<sup>-/-</sup> group. WT, C57BL/6 mouse group; ApoE<sup>-/-</sup>, ApoE<sup>-/-</sup> mouse group; ApoE<sup>-/-</sup> + Tac, ApoE<sup>-/-</sup> mouse + tacrolimus intervention group.

After tacrolimus intervention, the levels remarkably declined in ApoE<sup>-/-</sup> mice fed with high-fat diet, showing statistically significant differences (Fig. 4).

*Tacrolimus inhibits ROS production and activation of NLRP3 inflammatory corpuscles in atherosclerotic plaques in mice.* The ROS content in atherosclerotic plaques was detected using DHE. The results manifested that the ROS production in atherosclerotic plaques was remarkably increased in ApoE<sup>-/-</sup>

mice compared with that in WT mice, showing a statistically significant difference, and it was remarkably decreased after tacrolimus intervention, displaying a statistically significant difference (Fig. 5A and B). The results of western blotting showed that the protein content of NLRP3, ASC, Casp-1, IL-1β and IL-18 in the aorta in ApoE<sup>-/-</sup> mice fed with high-fat diet was obviously increased compared with that in WT mice, and the differences were statistically significant. After tacrolimus intervention, the expression of these five kinds of proteins

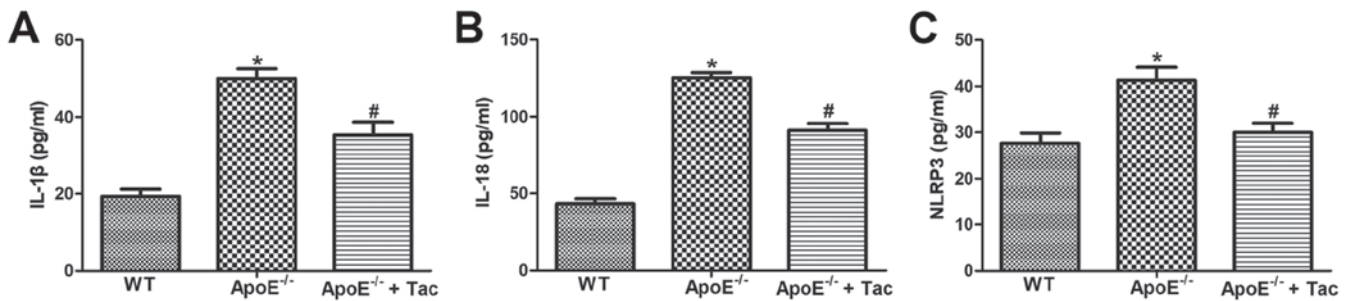


Figure 4. Tacrolimus reduces the concentrations of serum inflammatory factors in AS mice. (A) Analysis of the IL-1 $\beta$  level in different groups. (B) Analysis of the IL-18 level in different groups. (C) Analysis of the NLRP3 level in different groups. \*P<0.05 vs. WT group, #P<0.05 vs. ApoE<sup>-/-</sup> group. WT, C57BL/6 mouse group; ApoE<sup>-/-</sup>, ApoE<sup>-/-</sup> mouse group; ApoE<sup>-/-</sup> + Tac, ApoE<sup>-/-</sup> mouse + tacrolimus intervention group; IL, interleukin; NLRP3, Nod-like receptor protein 3.

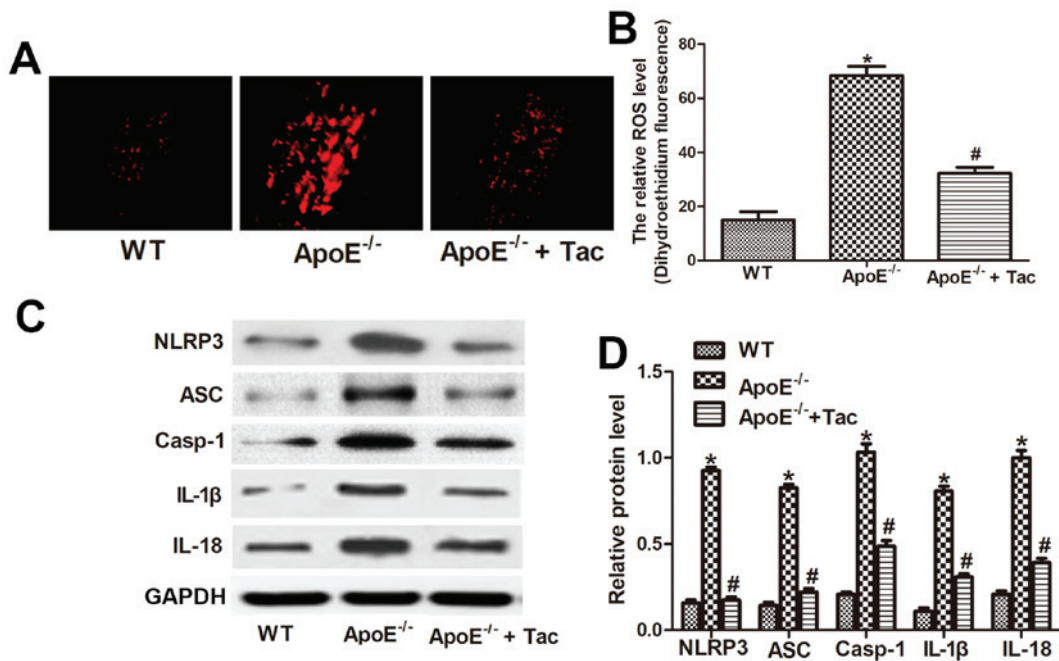


Figure 5. Tacrolimus inhibits ROS production and activation of NLRP3 inflammatory corpuscles in atherosclerotic plaques in mice. (A) The ROS content in atherosclerotic plaques detected by DHE (magnification of x400). (B) Analysis of the ROS level in different groups. (C) Western blotting showed protein level of NLRP3, ASC, Casp-1, IL-1 $\beta$  and IL-18 in different groups. (D) Analysis of protein level of NLRP3, ASC, Casp-1, IL-1 $\beta$  and IL-18. \*P<0.05 vs. WT group, #P<0.05 vs. ApoE<sup>-/-</sup> group. WT, C57BL/6 mouse group; ApoE<sup>-/-</sup>, ApoE<sup>-/-</sup> mouse group; ApoE<sup>-/-</sup> + Tac, ApoE<sup>-/-</sup> mouse + tacrolimus intervention group; IL, interleukin; NLRP3, Nod-like receptor protein 3; ROS, reactive oxygen species; DHE, dihydroethidium; ASC, apoptosis-associated speck-like protein containing CARD; Casp-1, caspase-1.

was inhibited to some extent, indicating that tacrolimus can inhibit ROS production and activation of NLRP3 inflammatory corpuscles in the aortic root of AS mice (Fig. 5C and D).

## Discussion

AS is a kind of chronic inflammatory disease caused by the imbalance of lipid metabolism and deposition of lipid-rich foam cells under the arterial wall, and inflammation is one of the important pathophysiological mechanisms of its occurrence and development (25). AS-induced cardiovascular and cerebrovascular diseases have become the main cause of human disability and death in the world, and the coronary heart disease caused by coronary AS is the main cause of death of cardiovascular diseases (1). Tacrolimus, an immunosuppressive drug widely applied in the transplantation currently, belongs to the macrolide in molecular structure,

which has anti-inflammatory property (26), but its role in AS has not been reported yet. In this investigation, the effect of tacrolimus on atherosclerotic plaques was studied via oil red O staining and H&E staining, and the results revealed that the area of atherosclerotic plaques in ApoE<sup>-/-</sup> mice fed with high-fat diet was obviously increased compared with that in WT group, suggesting that tacrolimus can inhibit the formation and development of atherosclerotic plaques in ApoE<sup>-/-</sup> mice fed with high-fat diet.

Macrophages are considered as major inflammatory cells during the formation of AS (27,28). Macrophages can develop into foam cells, and the lipid nucleus is formed after necrosis of foam cells, which is a major component of atherosclerotic plaques (29). In addition, macrophages are also the major inflammatory cellular components in atherosclerotic plaques, and a variety of pro-inflammatory factors secreted by them alter the local environment of plaques and affect the plaque

stability and disease development (27,29). The collagen fibers produced by smooth muscle cells migrating to plaques are the main source of fibrous caps in plaques, and smooth muscle cells in the fibrous cap are decreased (30,31). If inflammatory cells lead to death of smooth muscle cells, macrophages are increased, and thin fibrous caps are prone to rupture, so the plaque stability declines and they rupture easily (32,33). Therefore, inhibiting the macrophage infiltration and preventing the decrease of smooth muscle cells in plaques are the therapeutic goals for stabilizing plaques. In this study, the infiltration of macrophages and changes in smooth muscle cells in plaques were detected using MOMA-2 and  $\alpha$ -SMA as markers for macrophages and smooth muscle cells, and it was found that tacrolimus inhibited the macrophage infiltration and the decrease of smooth muscle cells in plaques in mice, thereby stabilizing plaques and inhibiting the occurrence and development of AS.

Macrophages are the main source of IL-1 $\beta$  and IL-18 produced in the body, and their accumulation in vascular lesions is the main cause of local inflammatory response and plaque formation (9,10). According to previous studies, ligands bind to NLRP3 to promote the formation of inflammatory corpuscles and activate Casp-1, ultimately resulting in maturation and secretion of pro-IL-1 $\beta$  and pro-IL-18 (14). In this investigation, the levels of serum NLRP3, IL-1 $\beta$  and IL-18 in mice were detected. The results showed that the levels of serum IL-1 $\beta$ , IL-18 and NLRP3 in ApoE<sup>-/-</sup> mice were significantly increased compared with those in WT group, and they declined in ApoE<sup>-/-</sup> mice fed with high-fat diet after tacrolimus intervention, indicating that tacrolimus possesses potential anti-inflammatory effect.

At present, there are three hypotheses on the mechanism of metabolites in the body in activating NLRP3 inflammatory corpuscles: ion channel mode, lysosome mode and ROS mode (34-36). The ROS model has been well studied at present, mainly because ROS production promotes the dissociation between thioredoxin in cells and its ligand in Txnip, the latter of which may bind to NLRP3 and lead to its activation (37). Oxidative stress is caused by the imbalance between antioxidants and ROS, and its effect of promoting AS has been widely recognized (38). Studies have demonstrated that ROS significantly promotes the occurrence and development of AS (39,40). Therefore, the expression levels of ROS and NLRP3 inflammatory corpuscles in atherosclerotic plaques were further detected, and it was found that the rapid formation of AS might be related to ROS production and activation of NLRP3 inflammatory corpuscles that promote the production and secretion of IL-1 $\beta$  and IL-18. After tacrolimus intervention, ROS production was inhibited and IL-1 $\beta$  and IL-18 were decreased, inhibiting the formation of atherosclerotic plaques.

In conclusion, it is speculated that tacrolimus may reduce the formation of AS through inhibiting ROS in macrophages and activation of NLRP3 inflammatory corpuscles and reducing the release of IL-1 $\beta$  and IL-18. However, its specific mechanism remains to be further studied.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

XL designed the study and performed the experiments, XL and XS collected the data, XL and LS analyzed the data, XL prepared the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of the Third People's Hospital of Qingdao Animal Center (Qingdao, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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