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Original Article

Tripartite motif 59 improves atherosclerotic vulnerable plaque stability of ApoE (-/-) mice by regulating the NF-κB signaling pathway

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Abstract: Object: The goal of this study was to investigate the effect of tripartite motif 59 (TRIM59) on atherosclerosis (AS) of ApoE (-/-) mice and to determine whether its mechanism was related to the NF-κB signaling pathway. Methods: AS mouse models were established by continuously feeding ApoE (-/-) mice with a high-fat diet, and then the mice were grouped and injected intraperitoneally with 6 μg/kg, 12 μg/kg and 18 μg/kg TRIM59 recombinant protein respectively. The aortic arch lesions and plaque vulnerability were observed and the inflammatory factor levels and related proteins expression were detected. Results: After treatment with different doses of TRIM59 recombinant protein, the number of atherosclerotic plaques in ApoE (-/-) AS mice decreased at different degrees, the proportion of collagen fibers in the plaque increased significantly, and both extracellular lipid accumulation and the proportion of foam cells in plaques were significantly decreased. The levels of inflammatory mediators MCP-1, ICAM-1, TNF-α and IL-1β in the serum were significantly decreased, and expression of NF-κB pathway-related proteins was also down-regulated. Conclusion: TRIM59 protein can ameliorate atherosclerotic lesions and improve the stability of sclerosing plaques, inhibiting AS inflammatory response. The mechanism may be related to regulation of the NF-κB signaling pathway by TRIM59 protein.

Keywords: Atherosclerosis, tripartite motif 59, ApoE (-/-) mice, NF-κB signaling pathway

Introduction

Cardiovascular and cerebrovascular diseases are the leading cause of death in humans, and atherosclerosis (AS) is the pre-pathological basis for most cardiovascular and cerebrovascular diseases. The pathological essence of AS is chronic inflammatory response, which is the main cause of plaque instability and rupture [1]. The nuclear factor-κB (NF-κB) signaling pathway is an important mechanism regulating inflammatory response and promoting plaque rupture. Targeting inhibition of NF-κB signaling pathway has become one of the strategies for the treatment of cardiovascular and cerebrovascular diseases [2]. Post-transcriptional modifications such as phosphorylation and ubiquitination can regulate NF-κB signaling pathway, and E3 ubiquitin ligase plays an important role in ubiquitination [3]. The tripartite motif (TRIM) family is one of the E3 ubiquitin ligase families and is involved in a variety of pathophysiological processes such as tumors, inflammation, and autoimmune disorders by regulating the NF-κB signaling pathway [4, 5]. TRIM59, a member of the TRIM family, could inhibit the activation of NF-κB signaling pathway by binding to and inhibiting activation of Toll-like receptors (TLRs) [6]. Therefore, it can be speculated that TRIM59 may participate in the atherosclerotic process through an inflammatory response. In this study, ApoE (-/-) mice were fed a high-fat diet to construct an animal model of AS and treated with TRIM59 recombinant protein. The therapeutic effect of TRIM59 on AS mice was explored, in order to provide some experimental basis for treatment.

Materials and methods

Animals and main reagents

In total, 68 clean-grade healthy male ApoE (-/-) mice were provide by Antaikang Biotechnology

(China). Each mice weighed 20-25 grams and was 6 weeks old. TRIM59 recombinant protein was purchased from Shanghai Yaoyun Biotechnology (Abnova, China). MCP-1, ICAM-1, TNF-α, IL-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Beijing NeoBioscience; TLR4, NF-B p65 and PARP-1 antibodies were purchased from Sigma-Aldrich, USA. All experiments in this study were approved by the Ethics Committee of Zhengzhou People's Hospital.

Animal grouping and processing

In total, 54 mice were randomly selected to receive high-fat diet (containing 15% fat, 0.15% cholesterol) for 8 weeks as the AS model group, and the remaining 14 as normal control group (ND group) were given normal feed. After 8 weeks, four mice were randomly selected from the two groups respectively, and the aortic root tissues were taken out under sterile conditions to prepare for paraffin sections. The samples were identified by Sudan IV and H&E staining. The remaining 50 model mice were randomly divided into 5 groups, and 10 in each group: high fat diet group (HFD group), simvastatin group (SVT group), low dose TRIM59 recombinant protein treatment group (L-rTRIM59 group), medium dose TRIM59 recombinant protein treatment group (M-rTRIM59 group), and high dose TRIM59 recombinant protein treatment group (H-rTRIM59 group). SVT group was intraperitoneally injected with 3 mg/kg simvastatin. The L-rTRIM59, M-rTRIM59 and H-rTRIM59 groups were intraperitoneally injected with 6 μg/kg, 12 μg/kg and 18 μg/kg TRIM59 recombinant protein, respectively. The ND group and the HFD group were intraperitoneally injected with the same volume of normal saline. All mice were given once a day for 8 weeks. During the administration period, the ND group continued to be fed with normal feed, and the other groups were fed a high fat diet.

Sample collection

After 8 weeks of continuous administration, the mice were fasted for 12 hours, and 1 mL of blood was taken from retroorbital venous sinus, let stand for 2 hours, and then centrifuged at 3000 rpm for 15 minutes, and the separated serum was frozen at -80°C. Then the mice were sacrificed by cervical dislocation, the chest was opened to expose the heart, the pericardial tissue were removed and the aortic arch tissue were obtained. The sample were fixed 4% paraformaldehyde solution overnight and embedded in paraffin.

Serum blood lipid index and inflammatory index detection

Serum total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride (TG) levels were detected by automated biochemical analyzer. Serum levels of MCP-1, ICAM-1, TNF-α and IL-1β were measured by ELISA, and all operations were performed strictly accordance with the kit instructions.

Sudan IV staining to observe the lesion area of aortic arch

The fat and adventitial tissues of aortic arch tissues fixed with 4% paraformaldehyde were removed under a microscope. The tissues were stained with filtered Sudan IV working solution for 6 min, decolored with 80% ethanol solution for 5 minutes, and images were collected using a Sony 3-CCD camera. The percentage of total aortic surface area covered by lesions (red) indicated the extent of atherosclerotic lesion formation.

HE staining to observe the shape of aortic arch disease

Paraffin-embedded aortic arch tissues were serially sliced with a microtome (4 μm), and were dehydrated in ethanol, stained by hematoxylin solution for 15 minutes, differentiated in 1% acid alcohol, stained with eosin for 5 minutes, dehydrated in ethanol and cleared in xylene. The specimens were dripped with gum Arabic then and covered with coverslips. Using Image-Pro image analysis software to observe extracellular lipid components and pathological morphology of tissues.

Movat staining to observe aortic arch tissue foam cells and collagen

Paraffin sections of aortic arch tissue were routinely dewaxed and incubated in Bouin solution at 50°C for 10 minutes, stood in 5% sodium thiosulfate for 5 minutes, 1% Allixin blue aqueous solution for 20 minutes, and incubated in

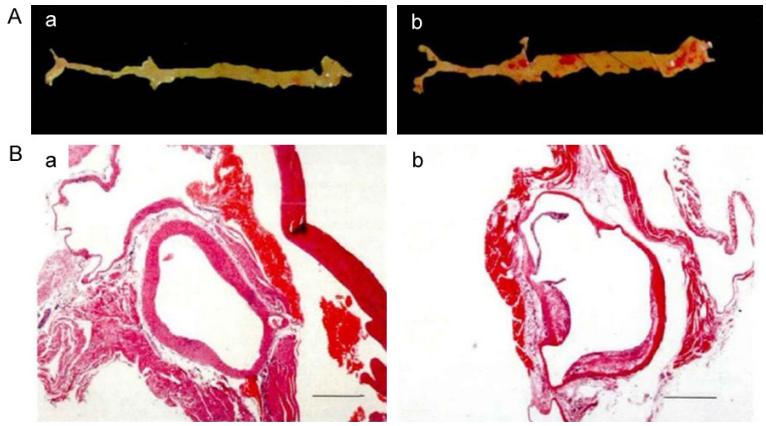

Figure 1. Atherosclerosis in ApoE (-/-) mice after high fat diet. A: Sudan IV staining; B: HE staining. a: ApoE (-/-) mice fed by normal feed, b: ApoE (-/-) mice fed by high fat diet. Bar = 500 μm.

Table 1. Effect of TRIM59 on blood lipid levels in ApoE (-/-) mice ($\bar{x} \pm s$)

Group	TC (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	TG (mmol/L)
ND	2.53 ± 0.08	0.55 ± 0.02	12.67 ± 2.11	0.28 ± 0.10
HFD	23.86 ± 1.53*	19.01 ± 1.34*	3.08 ± 0.71*	0.63 ± 0.11
SVT	22.58 ± 1.34	17.83 ± 2.06	2.88 ± 0.67	0.61 ± 0.12
L-rTRIM59	19.34 ± 1.58#	17.36 ± 1.29#	2.74 ± 0.39	0.52 ± 0.08#
M-rTRIM59	17.53 ± 1.24#	15.28 ± 1.31#	2.78 ± 0.50	0.44 ± 0.07#
H-rTRIM59	15.62 ± 1.15#	12.71 ± 1.22#	2.82 ± 0.41	0.36 ± 0.08#

Note: Compared with ND group, *P<0.05; compared with HFD group, #P<0.05.

alkaline ethanol at 70°C for 10 minutes. After rinsing, the tissues were stood in Weigert hematoxylin for 1 hour, in the saffron essence for 1 minute, in 5% phosphotungstic acid solution for 5 minutes, in glacial acetic acid for 5 minutes, respectively and washed by 95% ethanol, made transparent in xylene I and II for 15 min and sealed. Analysis was performed by using Image-Pro image analysis software.

Immunohistochemical analysis of NF-κB p65 expression

The paraffin sections of the aortic arch were dewaxed and hydrated by gradient alcohol, incubated in citrate buffer at 95°C for 15 minutes, and incubated with peroxidase blocker for 30 minutes. Rabbit anti-NF-κB p65 polyclonal antibody was incubated overnight at 4°C. Sections incubated with PBS served as negative controls and then were incubated with the FITC-labeled secondary antibody for 1 hour, washed with PBS, and the color developed by DAB solution. After being washed with tap water, it was counterstained with hematoxylin, dehydrated with gradient alcohol, made transparent with xylene, and then sealed with a neutral gum. Observation was carried out under a microscope (200 ×). Positive coloration results in brown or brownish yellow particles in the cells.

Western blot detection of related protein expression

Expression of TLR4, NF-κB p65 and PARP-1 proteins in aortic arch tissues were detected by Western blot, and β-actin was used as an internal reference.

Statistical analysis

Statistical analysis was performed using SPSS 20.0. Measurement data is expressed as mean ± standard deviation ($\bar{x} \pm s$), and differences among multiple groups were analyzed via one-way analysis of variance (ANOVA). Comparisons among multiple groups were analyzed by least significant difference (LSD) t-test. P<0.05 was considered as different statistically significant.

Results

ApoE (-/-) mouse atherosclerosis modeling

The formation of aortic atherosclerosis in ApoE (-/-) mice was observed by Sudan IV staining. It can be seen that the aorta of the AS model group fed with high fat diet for 8 weeks (**Figure 1Aa**) had different degrees of bulging and was stained with red plaque, while the aortic surface of mice in the blank group (**Figure 1Ab**) was smooth and had no plaques. The H&E staining showed that compared with the blank group (**Figure 1Ba**), there were obvious lipid cores and foam cells infiltrating into the aortic in the plaque of the AS model group (**Figure 1Bb**), and small vacuoles were visible inside in the cytoplasm, and lymphocytes and neutrophil infiltration were observed in some sections, confirming the successful construction of the AS mouse model.

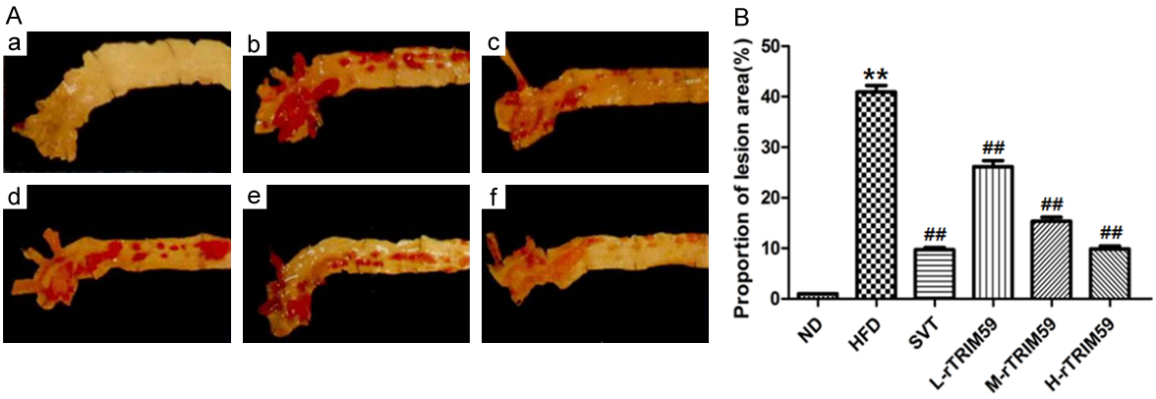

Figure 2. Effect of TRIM59 recombinant protein on the degree of aortic lesions in ApoE (-/-) mice. A: Aortic Sudan IV staining in each group; B: Percentage of lesion area (red). a: ND group, b: HFD group, c: SVT group, d: L-rTRIM59 group, e: M-rTRIM59, f: H-rTRIM59. Compared with ND group, $^{*}P<0.05$, $^{**}P<0.01$; compared with HFD group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

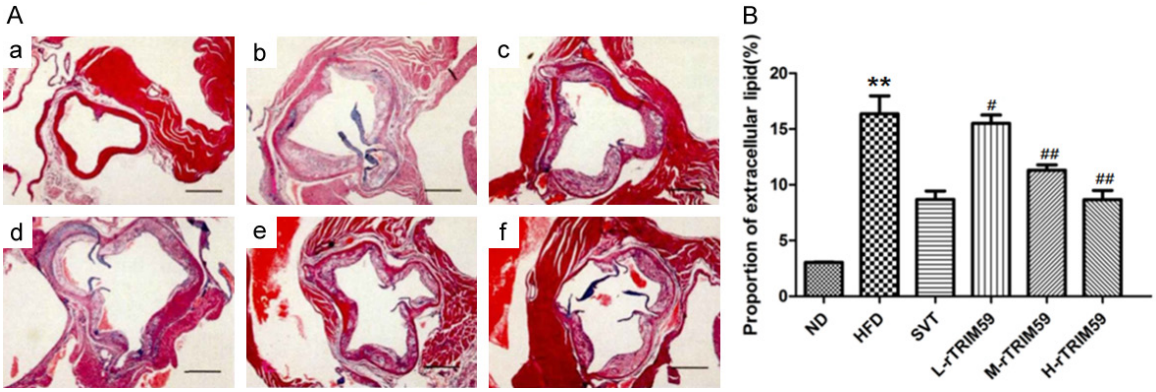

Figure 3. Effect of TRIM59 recombinant protein on the basic pathological morphology and lipid composition of atherosclerotic plaque in ApoE (-/-) mice. A: HE staining; B: Ratio of extracellular lipids in plaques. a: ND group, b: HFD group, c: SVT group, d: L-rTRIM59 group, e: M-rTRIM59, f: H-rTRIM59. Ruler = 500 μm. Compared with ND group, $^{*}P<0.05$, $^{**}P<0.01$; compared with HFD group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

Effect of TRIM59 recombinant protein on blood lipid levels in ApoE (-/-) mice

The levels of serum TC, LDL and TG in the HFD group were significantly higher than those in the ND group ($P<0.05$, **Table 1**), while the HDL level was significantly lower ($P<0.05$). Compared with the HFD group, the serum levels of TC, LDL and TG in the L-rTRIM59, M-rTRIM59, and H-rTRIM59 groups were significantly lower, while the HDL levels were not significantly changed ($P<0.05$).There were no significant difference in serum TC, LDL, HDL and TG levels between SVT group and HFD group ($P>0.05$).

Effect of TRIM59 recombinant protein on AS degree and plaque stability in ApoE (-/-) mice

Sudan IV staining: The comparison of Sudan IV staining and lesion area in each group is shown in **Figure 2**. The number of red plaques in the aortic root tissue of the HFD group was the highest, while that in the SVT, L-rTRIM59 and H-rTRIM59 groups were lower (**Figure 2A**). The area ratio which the lesion (red) covered the total aorta surface area indicated the extent of atherosclerotic lesion formation. Compared with the HFD group, the lesion area of the SVT, L-rTRIM59, M-rTRIM59 and H-rTRIM59 groups was significantly decreased ($P<0.05$, **Figure 2B**).

H&E staining: H&E staining of aortic arch tissues in each group were shown in **Figure 3**. In the HFD group, it could be seen that the lipid nucleus in the atherosclerotic plaque of the blood vessel wall was enlarged, the formation of cholesterol crystals, the proliferation of adventitial fibrous connective tissue, and lym-

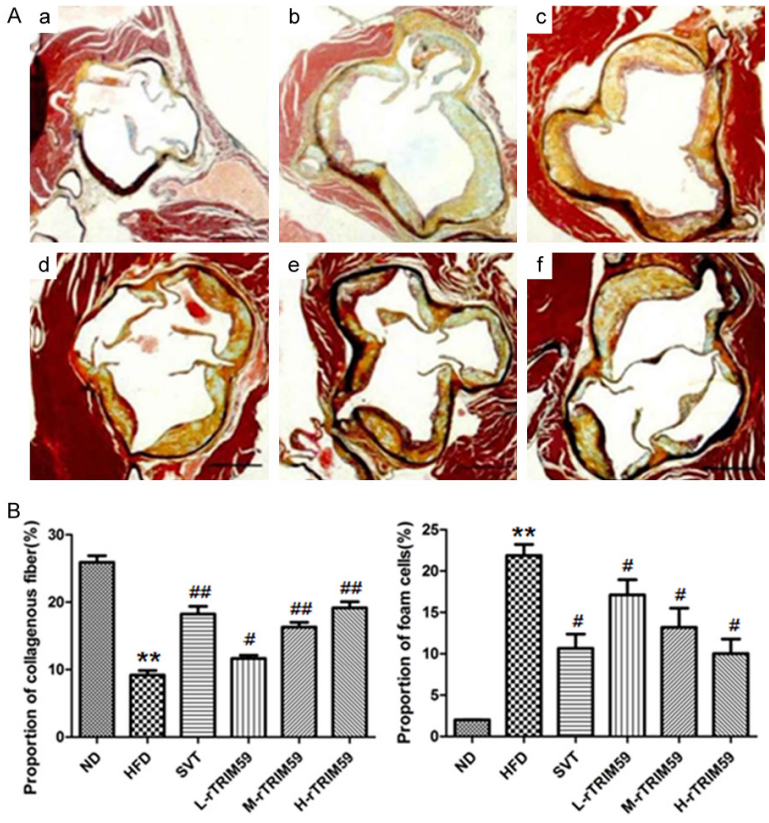

Figure 4. Effect of TRIM59 recombinant protein on atherosclerotic plaque foam cells and collagen in ApoE (-/-) mice. A: Movat staining; B: Ratio of collagen and foam cells in the plaque. a: ND group, b: HFD group, c: SVT group, d: L-rTRIM59 group, e: M-rTRIM59, f: H-rTRIM59. Ruler = 500 μm. Compared with ND group, $^{*}P<0.05$, $^{**}P<0.01$; compared with HFD group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

phocyte infiltration (**Figure 3A**), and the proportion of extracellular lipid in the plaque was significantly higher than that in the ND group ($P<0.05$). Compared with the HFD group, the proportion of extracellular lipids in the SVT, L-rTRIM59, M-rTRIM59 and H-rTRIM59 groups was significantly lower ($P<0.05$) (**Figure 3B**).

Movat staining: The effect of TRIM59 recombinant protein on foam cells and collagen in atherosclerotic plaques of ApoE (-/-) mice was evaluated by Movat staining. In the plaque of the HFD group, foam cells (lavender) were observed on the surface of the plaque, and collagen fibers (yellow) and proteoglycan (green) were deposited to form a fibrous cap, and smooth muscle cells (red) were reduced or disappeared (**Figure 4A**). As shown in **Figure 4B**, compared with the ND group, the proportion of collagen fibers in the aortic root lesions of the HFD group was significantly decreased, and the proportion of foam cells in the plaque was significantly increased ($P<0.05$). Compared with the HFD group, the proportion of collagen fibers in the plaques of SVT, L-rTRIM59, M-rTRIM59, and H-rTRIM59 mice increased significantly, and the percentage of foam cells in the plaques decreased significantly ($P<0.05$). These results suggested that TRIM59 recombinant protein can reduce extracellular lipid accumulation, inhibiting foam cell formation, increasing collagen fiber deposition, and thereby improve the stability of atherosclerotic plaque.

Effect of TRIM59 recombinant protein on the level of inflammatory mediators in ApoE (-/-) mice

The results of ELISA for the detection of inflammatory markers in each group were shown in **Figure 5**. The serum levels of MCP-1, ICAM-1, TNF-α and IL-1β in HFD group were significantly higher than those in ND group ($P<0.05$). Compared with the HFD group, the concentrations of serum inflammatory factors in SVT, L-rTRIM59, M-rTRIM59 and H-rTRIM59 groups were significantly lower ($P<0.05$), suggesting that TRIM59 can inhibit AS inflammatory response.

Effect of TRIM59 recombinant protein on NF-κB signaling pathway in ApoE (-/-) mice

The expression of NF-κB p65 protein in the aortic root of ApoE (-/-) mice detected by Immunohistochemical analysis was shown in **Figure 6A**. Compared with the ND group, the positive expression of NF-B p65 protein was increased in the aortic root tissues of the HFD group, while it was attenuated in the SVT, L-rTRIM59, M-rTRIM59 and H-rTRIM59 groups compared with the HFD group. Western blot shows that (**Figure 6B**, **6C**) expression of TLR4, NF-κB p65, and PARP-1 in the HFD group were significantly higher than those in the ND group ($P<0.05$). Compared with the HFD group, the expression of TLR4, NF-κB p65 and PARP-1 protein in SVT, L-rTRIM59, M-rTRIM59

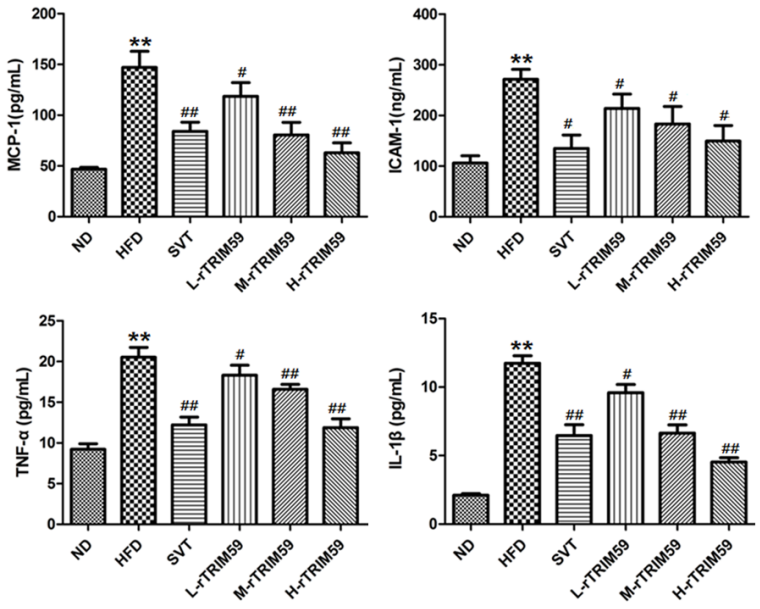

Figure 5. Effect of TRIM59 recombinant protein on the level of inflammatory mediators in ApoE (-/-) mice. Compared with ND group, $^{*}P<0.05$, $^{**}P<0.01$; compared with HFD group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

and H-rTRIM59 groups were significantly lower ($P<0.05$).

Discussion

High-fat feeding is the main means to establish AS mouse models, but the vascular plaques formed by the ApoE gene in normal AS mice are relatively stable, therefore, ApoE (-/-) mice became one of the most commonly used animals to establish vulnerable plaque AS models [7]. In this study, 6-week-old male ApoE (-/-) mice were fed by high-fat diet for 8 weeks, and it was found that the intima of the aortic root of the mice showed atherosclerotic plaque protruding to the lumen, and the collagen in the plaque was significantly reduced, confirming the initial formation of AS. Continue feeding for 8 weeks, atheromatous plaques were more typical and partially ruptured, confirming that persistent high-fat feeding could establish a vulnerable plaque AS model.

There are several theories about the pathogenesis of atherosclerosis: the theory of lipid infiltration, the theory of thrombosis, the theory of smooth muscle hyperplasia and the theory of immune inflammatory response [8]. Research has suggested that the progression of AS is actually a chronic inflammatory response involving many inflammatory cells and inflammatory mediators. The transcription factor NF-κB signaling pathway involves various pathological processes such as lipid metabolism, inflammatory immune response, apoptosis, and vascular remodeling, and is a common pathway for many factors to promote the development of AS [9]. For example, LDL, angiotensin II (Ang II), macrophages, and Chlamydia pneumoniae infection can activate NF-κB-related signaling pathways to initiate inflammation and promote AS. Animal studies have shown that NF-κB activation was found in macrophages and endothelial cells in the aortic roots of LDLR -/- mice fed with high-fat diet, and this site further develops into atherosclerotic plaques [10]; Kanters et al. [11] showed that specifically knocked out NF-κB in macrophage of LDLR -/- mice can reduce the degree of plaque damage. Therefore, inhibition of the NF-κB signaling pathway activity to inhibit the inflammatory response can achieve the purpose of preventing and treating AS diseases. However, there are certain limitations and side effects of non-selective or complete inhibition of NF-κB, exploring new targets to regulate the activity of NF-κB signaling pathway may provide new ideas for the treatment of many diseases associated with abnormal NF-κB signaling pathways. Studies have shown that the ubiquitin proteasome participates in the regulation of apoptosis, gene transcription and lysosomal digestion by acting on NF-κB signaling pathways [13], and thus participates in the development of cardiovascular diseases [14]. Versari et al. [15] found that inhibition of ubiquitin protease activity in atherosclerotic plaques can exacerbate inflammation and oxidative stress and destroy plaque stability, suggesting that the ubiquitin protease system is a protective factor for atherosclerosis. Other related studies have also shown that ubiquitin proteases can degrade damaged proteins and inhibit the process of atherosclerosis [16], and promote the formation of foam cells by degrading low-density lipoprotein, promoting

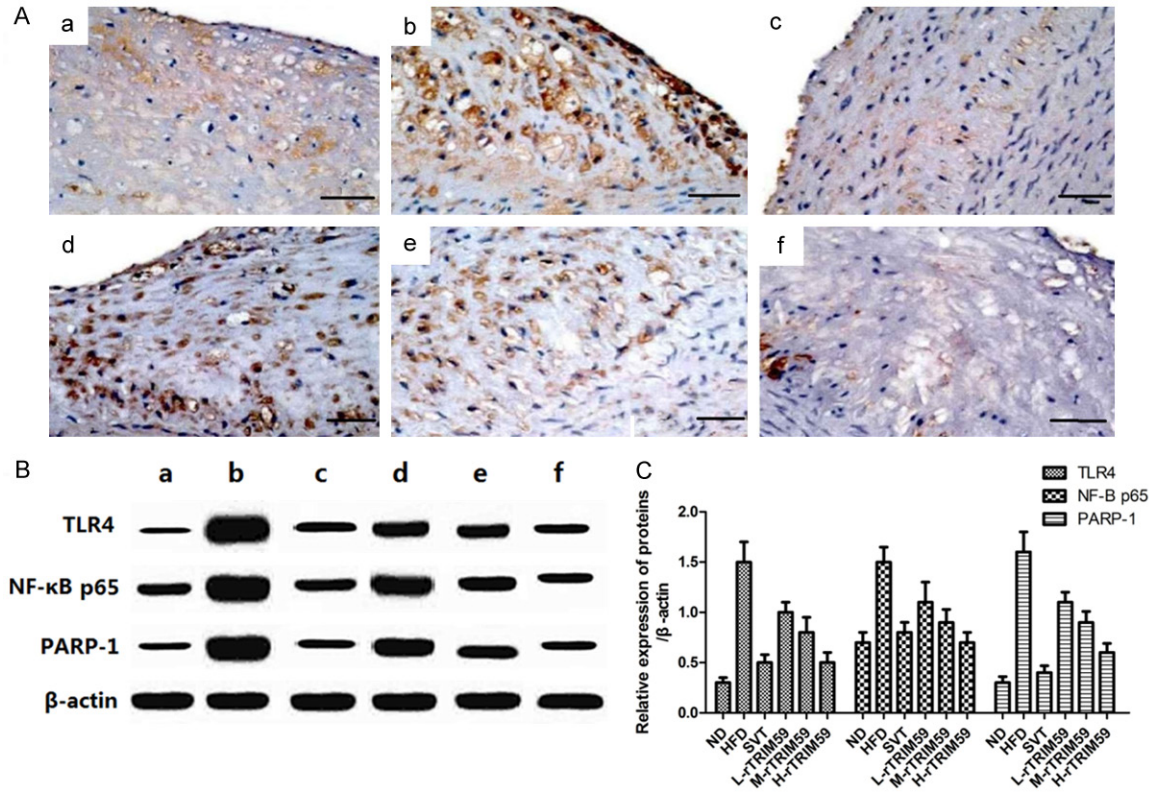

Figure 6. Effect of TRIM59 recombinant protein on NF-κB signaling pathway in ApoE (-/-) mice. A: Immunohistochemical analysis of NF-κB expression in aortic tissue; B: Western blot analysis of TLR4, NF-κB p65, and PARP-1 protein expression; C: Western blot quantitative analysis. Compared with ND group, $^{*}P<0.05$, $^{**}P<0.01$; compared with HFD group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

macrophage apoptosis [17], and affected the lipid metabolism by regulation of apolipoprotein expression [18]. E3 ubiquitin ligase is one of the components of the ubiquitin protease system. TRIM59, as a member of the RING family of E3 ubiquitin ligase, plays an important role in ubiquitination and participates in various physiological processes, such as tumors, inflammation, and autoimmune disorders [19], so it is speculated that it may play a role in the process of atherosclerosis. In this study, TRIM59 recombinant protein was used to treat ApoE -/- AS mice, and TRIM59 recombinant protein could improve the atherosclerotic lesions in AS mice, reducing the atherosclerotic plaque at the root of the aorta by increasing the collagen content in the plaque and reducing the extracellular lipid and macrophage infiltration. It has been suggested that the increased activity of TRIM59 can effectively inhibit the process of atherosclerosis, inhibiting the formation of foam cells by reducing the accumulation of extracellular lipids, promoting the deposition of collagen fibers, increasing the stability of plaque.

In the process of AS, mononuclear cells infiltrate the endothelium and induce local inflammatory reaction, which is an important pathogenesis of AS and the main cause of vulnerable plaque formation. Chemokines, adhesion molecules and inflammatory factors play an important role in this process an important pro-inflammatory mediators [20]. In the results of this study, the chemokine MCP-1, the adhesion molecule ICAM-1, and the inflammatory factors TNF-α, IL-1β in the HFD group were significantly higher than those in the blank group. At the same time, inflammatory mediators of TRIM59 recombinant protein intervention group decreased, suggesting that the improvement effect of TRIM59 on the stability of atherosclerotic plaques in AS mice may be related to the inhibition of immune inflammatory response. Inflammatory mediators can activate the NF-κB signaling pathway, and activation of this signal-

ing pathway can regulate the secretion of pro-inflammatory mediators in turn, which forming a vicious circle and promoting AS progression and plaque rupture [21]. TRIM family proteins are involved in the regulation of NF-κB signaling pathway activity, and NF-κB also regulates TRIM protein expression in both directions [22-24]. The NF-κB signaling pathway covers multiple factors such as Toll-like receptor (TLR), NF-κB, and adenosine diphosphate ribose polymerase (PARP), it mainly recognizes inflammatory stimuli through TLR4, and then activates NF-κB to transfer from cytoplasm to nucleus, thereby activating PARP-1 and downstream inflammatory factors, thereby promoting the occurrence and development of AS and plaque rupture [25]. In this study it also was confirmed that expression of TLR4, NF-κB p65 and PARP-1 protein in the aorta of the model group was significantly increased, while the TRIM59 recombinant protein could down-regulate the expression of these proteins, suggesting that TRIM59 may play an anti-AS role in regulating the NF-κB signaling pathway. Studies have shown that TRIM family proteins regulate this signaling pathway by binding to multiple targets in the NF-κB pathway. For example, TRIM12c and TRIM8 could bind to TRAF6, respectively, and activate TAK1 to positively regulate NF-κB signaling pathway [26, 27]. TRIM68 inhibits TRAF6 activation by binding to TPK-fused gene (TFG), which inhibits NF-κB signaling pathway by promoting lysosomal degradation of TAB2/3 [28, 29]. TRIM59 can mainly play a role in the inhibition of NF-κB signaling pathway by binding and inhibiting the activation of Toll-like receptors [6]. Therefore, it has been speculated that TRIM59 may protect the plaque stability of atherosclerotic mice by binding to TLR4 and inhibiting its activity, which makes it unable to activate the downstream NF-κB pathway and reduce the secretion of inflammatory mediators.

In conclusion, TRIM59 can enhance the stability of atherosclerotic plaque in ApoE (-/-) AS model mice, thereby exerting protection effect on atherosclerosis, which may be achieved by inhibiting the activation of the NF-κB signaling pathway and thereby improving the immune inflammatory response. However, this study couldn't confirm that the NF-κB signaling pathway is the only pathway for TRIM59 to inhibit AS immune inflammation, and further studies are needed to clarify the mechanism of action of TRIM59 in AS.

Disclosure of conflict of interest

None.

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