Inhibition of JAK2/STAT3/SOCS3 Signaling Attenuates Atherosclerosis in Rabbit

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Research article

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Abstract

Background : Previous studies have indicated that the JAK/STAT signaling pathway is involved in modulating arterial adventitia inflammation response. In this study, we designed experiment to further investigate the effect of JAK2/STAT3/SOCS3 signaling in rabbit atherosclerosis process.

Methods : Atherosclerosis was induced in the abdominal arteries of rabbits by atherogenic diet and endothelial damage. At the same time, animals were received either no treatment or ruxolitinib and killed after 12 weeks.

Results : H&E staining and CT scan analysis showed that rabbit atherosclerosis model was constructed successfully. Ruxolitinib, an inhibitor of the Janus kinase 2 (JAK2), substantially decreased the area of atherosclerotic plaques in rabbits treated with high fat diet and balloon injury of the aorta. Moreover, ruxolitinib substantially decreased IL-6 and TNF-α, but increased IL-10 and IL-17 levels in plasma of atherosclerotic rabbits. Additionally, ruxolitinib reduced TC and TG contents and AIP, while enhanced HDL-C level in rabbit plasma. Furthermore, we found that JAK2 and STAT3 phosphorylation were upregulated in rabbits with atherosclerosis when compared with that of the control group, and furthermore the expression of SOCS3 was also increased due to the activation of JAK2 and STAT3, while ruxolitinib inactivated JAK2 and STAT3 pathway and decreased SOCS3 expression.

Conclusion : Taken together, inhibiting the JAK2/STAT3/SOCS3 signaling pathway may be a novel method for the clinical treatment of artery atherosclerosis.

1 Background

Atherosclerosis, a complex cardiovascular disease, has been reported as a chronic inflammatory disease by the increasing studies [1, 2]. At different stages of atherosclerosis, the infiltration of various inflammatory cells, such as T cells, mast cells and macrophages, into the atherosclerotic plaques is one of the main characteristics of atherosclerosis [3]. Subsequently, the migration and proliferation of vascular smooth muscle cells (VSMCs), which attribute to the formation of neointima and atherosclerotic plaque, could be promoted by these infiltrated inflammatory cells in company with the resident vascular wall cells *via* the secretion of cytokines and growth factors [4–6].

As reported in previous studies, in the process of atherosclerotic lesion development Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway play a key role [7–9]. Suppressor of Cytokine Signaling 3 (SOCS3) can negatively regulate cytokine signaling by inhibiting JAK/STAT signaling pathway and then exert profound actions in regulating immunity and inflammation [10]. The specific JAK1/2 inhibitor—Ruxolitinib is used to treat myelofibrosis and has been approved by FDA [11]. However, whether ruxolitinib plays a key role in atherosclerosis process and JAK2/STAT3/SOCS3 signaling pathway are still not well understood.

In our study, we are committed to explore the underlying role of ruxolitinib on atherosclerosis progression. Interestingly, we found that the area of atherosclerotic plaques was substantially decreased by ruxolitinib in rabbits treated with high fat diet and balloon injury of the aorta. Moreover, ruxolitinib substantially decreased plasma levels of IL–6 and TNF-α in rabbits with atherosclerosis. Differently, the levels of IL–10 and IL–17 significantly increased in rabbit plasmas. Furthermore, we found that ruxolitinib inactivated JAK2 and STAT3 pathway and decreased SOCS3 expression. From those results, we finally concluded that inhibition of JAK2/STAT3/SOCS3 signaling attenuated atherosclerosis in rabbit.

2 Methods

2.1 Animals

Ten New Zealand male rabbits weighing 3.5~4 kg (mean 3.4 ± 0.6 kg) were purchased from Qing Long Shan Dong Wu Fan Zhi Chang (Nanjing, China). Rabbits were randomly assigned to three analyzed groups: Control (normal diet, no ruxolitinib, n = 3); Model (high fat diet and balloon injury of the aorta, n = 3); and ruxolitinib (high fat diet and balloon injury of the aorta with ruxolitinib added to the diet, n = 4). Aortic atherosclerotic plaques were induced in rabbits by high fat diet and repeated balloon injury of the aorta. Aortic injury was performed from the aortic arch to the iliac bifurcation with a 4-French Fogarty embolectomy catheter (Edwards Lifesciences) introduced through the femoral artery. All procedures were performed under general anesthesia induced by Pentobarbital (30 mg/kg) and euthanized by exsanguination. All experiments were approved by the Second Affiliated Hospital of Nanjing Medical University of Medicine Institute Animal Care and Use Committee.

2.2 CT Protocol

A dual-source CT scanner (Somatom Definition; Siemens Medical Solutions, Forchheim, Germany) was employed to perform the CT examination as described previously [12]. To ensure the entire thorax and abdomen of animals were covered by the field of view of the larger and smaller tube detector arrays, all rabbits were centrally placed in the scanner with the field of view of the second tube detector array was 260 mm. For the initial unenhanced chest CT scanning, the testing parameters were set as Zhang et al reported [12]. Hereinto, the weighted CT dose index (CTDIw) was 4–5 mGy, which was calculated according to the equation as follows: CTDIw = CTDIvol × P, where CTDIvol was volume CT dose index and P was pitch. After the injection of iopromide (300 mg/mL) with the injection dose of 2 mL/kg at a rate of 1.8 mL/sec. Subsequently, a 10-mL saline solution was injected into an internal jugular vein *via* an 18-gauge catheter prior to contrast-enhanced CT with the dual-energy mode. A bolus-tracking technique was used to trigger the CT scan and the image acquisition of the region of interest placed in the abdominal aorta started 3s later once the attenuation up to the predefined threshold of 100 HU. In addition, the other CT scanning parameters were listed following: tube voltages: 80 and 140 kVp; tube currents: 183 mA for the larger x-ray tubes and 51 mA for the smaller x-ray tubes; gantry rotation time: 0.33-second; detector collimation: 14 × 1.2-mm, pitch: 0.5; field of view: 260-mm.

2.3 Sample collection

Animals were anesthetized with an overdose of pentobarbital for abdominal aorta obtain. After carefully peeling off the adventitial layer, a part of the abdominal aorta and aorta were respectively collected and immediately snap-frozen in liquid nitrogen. In addition, the rest of the abdominal aorta was fixed with 4% formaldehyde for the routine Hematoxylin-Eosin staining. At the beginning of the study, the moment of randomization and the end of the experiment, mice 24-h post-meal plasma samples were collected.

2.4 H&E analysis

For histology, the abdominal aorta samples were fixed with 4% formaldehyde and embedded in paraffin. Then, the paraffin-embedded tissues were cut into 4 μm slices, and sections were stained using hematoxylin and eosin (H&E; Beyotime Institute of Biotechnology). Finally, all sections were scanned at an absolute magnification of 100× under a light microscope (Olympus, BX51 microscope, Tokyo, Japan). Changes in blood vessel layers were observed using a microscope (BX51; Olympus, Tokyo, Japan).

2.5 Measurement of plasma IL–6, IL–10, IL–17 and tumor necrosis factor (TNF)-α levels

Ear-edge vein blood was collected and spun at 2000g for 15 min; then plasma was separated and stored at −80℃ until use. IL–6, IL–10, IL–17 and TNF-α were measured with enzyme-linked immunosorbent assay kits (MILENIA BIOTEC, Gmbh, Germany).

2.6 Measurement of plasma TC, TG and HDL-C levels

Blood was collected from the ear-vein of rabbit with heparin anticoagulation. The plasma was separated by frozen centrifugation for 10 min at 6000×g. The contents of total cholesterol (TC), triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) were measured using biochemical test kits with the semi auto analyzer by colorimetric method at the end of treatments.

2.7 Atherogenic index plasma (AIP)

AIP is the measurement of the atherosclerotic lesion extent based on plasma lipids, and calculated using the formula AIP = TC/HDL [13].

2.8 Immunohistochemistry assay

The paraffin-embedded tissues were cut into 4 μm slices. Then, the sections were deparaffinated, hydrated and incubated with the primary antibodies at 4℃ overnight. The sections were washed with TBST (Tris-HCl buffer + 0.1% Tween 20) and then continually incubated with a secondary antibody labeled with HRP. For immunohistochemistry studies, the stain was visualized by 3, 3-diaminobenzidine (DAB) and Harris hematoxylin and analyzed under an Olympus microscope.

2.9 Western Blot Analysis

The frozen artery samples were homogenized in the RIPA lysis buffer, and protein concentration was measured by BCA Protein Assay Kit (Beyotime, Jiangsu, China). The samples were then separated by SDS-PAGE and transferred onto PVDF membrane. The membranes were blocked with 5% BSA (5% w/v in PBS + 0.1% Tween 20) and incubated with primary antibodies at room temperature. The antibodies which are against p-JAK2, JAK2, p-STAT3, STAT3, SOCS3, and β-actin were used according to the manufacturer's instructions, and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were washed in PBS containing 0.03% Tween 20 and further incubated with a peroxidase-conjugated secondary antibody for 1h. After washing, the membranes were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, USA) according to manufacturer's instructions.

2.10 Real-time PCR

Cells total RNA was extracted by Trizol reagents. RT-PCR analysis SOCS3 mRNA expression levels were quantified using HiFiScript cDNA Synthesis Kit (Cwbio, China) and Greenstar qPCR Master Mix (Bioneer, South Korea). The primer sequences used for real-time PCR were as follows: β-actin 5'-CAT CAC CAT TGG CAA TGA GC–3' and 5'-TCG TCA TAC TCC TGC TTG C–3'; SOCS3 5'-AGT TCC TGG ACC AGT ACG A–3' and 5'-TTC CTC CAC ACT GGA TTC TTG–3'. The $2^{-\Delta\Delta Ct}$ method was used for data analysis, and β-actin expression level was used for comparison with the relative mRNA expression levels for each sample.

2.11 Statistical analysis

Data are presented as means ± SD of three independent experiments. Differences between groups were analyzed by GraphPad Prism 5 software (GraphPad Software, CA, USA) with Student's t-test. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Establishment of rabbit atherosclerosis model

To verify whether rabbit atherosclerosis model was constructed successfully, the rabbit atherosclerotic plaques were detected by H&E staining and CT scan analysis. In this study, representative images of H&E staining showed that the area of atherosclerotic plaques were obviously increased in rabbits with high fat diet and aortic intimal injury when compared with control group (Fig. 1a), and CT scan analysis also showed obvious atherosclerotic imaging area (Fig. 1b), which indicated that the rabbit atherosclerosis model was constructed successfully.

3.2 Ruxolitinib alleviated atherosclerosis progress in rabbits

It has reported that the JAK/STAT signaling pathway is important in atherosclerotic lesion development [14–16], so we next explored the function of ruxolitinib, an inhibitor of JAK2, in atherosclerosis progress of rabbits. In this study, we found that the area of atherosclerotic plaques was obviously decreased after feeding with ruxolitinib in rabbits with high fat diet when compared with that of model group. Similarly, the aortic intimal injury was also alleviated by ruxolitinib as the results from H&E staining and CT scan analysis (Fig. 2a and b). Thus, the results indicated that ruxolitinib may act as an inhibitor in the rabbit atherosclerosis progress.

3.3 Ruxolitinib reduced plasma TG and TC, but enhanced HDL-C level in atherosclerotic rabbits

It is generally known that the lipid levels in blood are an important index to assess atherosclerosis progress [17]. As expected, compared with the control group, the levels of TG and TC significantly increased, while HDL-C levels significantly decreased in model group. Importantly, ruxolitinib could regulate the abnormal lipid levels, which are reflected by the decreased levels of TG and TC but the increased level of HDL-C (Table. 1). Additionally, the significantly increased AIP in model group could also be decreased by ruxolitinib ($P < 0.05$, Fig. 3).

3.4 Ruxolitinib decreased IL–6 and TNF-α levels while increased IL–17 and IL–10 levels in rabbit plasma

The enzyme-linked immunosorbent assay kits were used to detect plasma levels of IL–6, IL–10, IL–17 and TNF-α. The results showed that rabbits with atherosclerosis presented higher IL–6 and TNF-α levels than those in the control group; however, ruxolitinib substantially decreased plasma levels of IL–6 and TNF-α in rabbits with atherosclerosis (Fig. 4a~c). On the contrary, the levels of IL–10 and IL–17

significantly decreased in model group, but ruxolitinib could reverse this decrease (Fig. 4d). Together, our findings suggested that the JAK inhibitor, ruxolitinib, could inhibit inflammatory reaction in the atherosclerotic aortic wall.

3.5 Ruxolitinib alleviated atherosclerosis through inhibition of JAK2/STAT3/SOCS3 signaling

To further explore whether ruxolitinib alleviated atherosclerosis through modulating JAK2/STAT3/SOCS3 pathway, we detected the expression of SOCS3 and the phosphorylation of JAK2 and STAT3 by western blotting and found that the phosphorylation levels of JAK2 and STAT3 were significantly up-regulated in rabbits with atherosclerosis when compared with those of the control group, and furthermore the expression level of SOCS3 was also increased due to the activation of JAK2 and STAT3; however, ruxolitinib inactivated JAK2 and STAT3 phosphorylation and decreased SOCS3 expression (Fig. 5a). Additionally, RT-PCR results also indicated that the expression of SOCS3 significantly decreased by ruxolitinib at mRNA levels against rabbits in model group (*Fig. 5b)*.. Further, immunohistochemistry assay showed that high rates of p-SOCS3-positive cells were observed in the model group. Instead, after treatment with ruxolitinib, there was a significant decrease in the rates of p-SOCS3-positive cells in rabbits (Fig. 5c).From the results we have showed, we concluded that inhibition of JAK2/STAT3/SOCS3 signaling attenuated atherosclerosis in rabbit.

4 Discussion

Atherosclerosis, one of the leading causes of cardiovascular morbidity and mortality, is closely associated with the poor quality of life attribute to the limb loss and enhanced health care burden [18]. Currently, the prevalence of peripheral atherosclerotic disease is more than 200 million persons worldwide [1]. It means that we need more information about the prevalence, health costs and resource utilization of atherosclerosis in order to optimize the treatment program and decrease the occurrence and development of atherosclerosis. Meanwhile, more and more evidences have indicated that not only the formation and progression but also the rupture of atherosclerotic plaques could be drove by inflammation [19]. Thus, it will provide novel treatment strategies against atherosclerosis by focusing on the potential targets which play key roles in the inflammatory process of atherosclerosis.

Atherosclerosis, a chronic inflammatory vascular disease, accounts for the first cause of mortality worldwide [18]. In recent studies, the researchers have clearly emphasized the key roles played by immune-inflammatory balance in modulating the disease development and progression [20–22]. The growing evidences have confirmed that, some inflammatory cytokines, such as IL–6, IL–10 and TNF-α, may be responsible for the pathogenesis of atherosclerosis because these cytokines can result in the adhesion of leukocytes to vascular endothelium and foam cell formation [23, 24]. In this study, the levels of IL–6 and TNF-α were significantly increased in atherosclerotic rabbits. Importantly, ruxolitinib substantially decreased plasma levels of IL–6 and TNF-α in atherosclerotic rabbits. Additionally, IL–17, a

major effector cytokine secreted by T helper 17 (Th17) cells, is a major regulatory pathway in atherosclerosis through inhibition of IL–17 production [25. 26]. As expected, IL–10 and IL–17 levels were significantly decreased in the model group; however, ruxolitinib could delay the decrease of IL–10 and IL–17 contents. Therefore, data above suggest that inflammatory processes play important roles in atherosclerosis, and ruxolitinib could alleviate atherosclerosis through regulating the effects of inflammatory cytokines.

Additionally, hyperlipidemia is considered as one of the most important risk factors account for the further development of atherosclerosis, because severe hyperlipidemia can promote the progression of atherosclerosis and its end-points [27]. Meanwhile, the AIP acts as a critical mark of atherosclerosis as the deposition of foam cells or plaque or fatty in filtration or lipids in organs could be indicated by it [28]. In this study, high fat diet and balloon injury of the aorta could cause the increase in TG and TC contents and AIP, while resulted in the decrease in HDL-C level in model rabbits. As expected, compared with the model group, ruxolitinib could significantly reduce TG, TC and AIP, but enhanced HDL-C level. It suggested that the abnormal blood lipids were improved better in the ruxolitinib treatment group than those of the model group. Therefore, ruxolitinib can be an effective drug to alleviate atherosclerosis by regulating the lipid contents in blood.

The activation of JAK2/STAT3 pathway has been observed in atherosclerotic lesions and the activated JAK2/STAT3 signaling has been proved to play important roles in regulating the cellular activation, proliferation and differentiation of VSMCs and other vascular cells in the occurrence and development of atherosclerosis [29, 30, 31]. SOCS3 can block JAK/STAT signaling pathways to function as a negative regulator of cytokine signaling and play key roles in the regulation of cardiovascular diseases like intravascular coagulation, heart failure and even the cardiovascular atherosclerosis [32, 33]. One study has reported that SOCS3 displayed an indirect role in protecting endothelial dysfunction in atherosclerosis reflecting that IL–10 could regulate oxLDL-induced cell apoptosis by upregulating SOCS3, and the increased SOCS3 expression subsequently interrupted the activation of p38 MAPK signaling pathway in endothelial cells [33]. In this study, compared with the model group, the expression of SOCS3 was significantly down-regulated at mRNA and protein levels after ruxolitinib treatment. Meanwhile, the results from immunohistochemical analysis also demonstrated that p-SOCS3-positive cells rates were significantly decreased by ruxolitinib. Further, the expression of p-JAK2, p-STAT3 and STAT3 were also significantly down-regulated by ruxolitinib treatment compared with those of the model group. Additionally, some researches have shown that IL–17 production was enhanced after SOCS3 deletion in T cells [34, 35]. Actually, ruxolitinib inhibited the expression of SOCS3 accompanied by the increase in IL–17 production. Therefore, we have proved that ruxolitinib could attenuate atherosclerosis by the inhibition of JAK2/STAT3/SOCS3 signaling pathway, while the detailed underlying mechanisms still need further studies.

5 Conclusions

In the present work, we not only firstly demonstrated that the JAK2 inhibitor ruxolitinib could reduce the formation of aortic atherosclerotic plaque and decrease the contents of plasma TG and TC, while enhance HDL-C level in rabbit with atherosclerosis,but also confirmed that ruxolitinib regulated the secretion of inflammatory components, such as IL–6, IL–10, IL–17 and TNF-α through the inactivation of JAK2/STAT3 pathway and the downregulation of SOCS3 expression. Considering the important role of JAK2/STAT3/SOCS3 signaling pathway played in inflammatory process of atherosclerosis, the potentially therapeutic strategy may be expected for the treatment of patients with atherosclerosis.

Abbreviations

JAK: Janus kinase; STAT: Signal transducer and activator of transcription; SOCS3: Suppressor of Cytokine Signaling 3; CTDIw: Weighted CT dose index; TNF: Tumor necrosis factor; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; AIP: Atherogenic index plasma; DAB: 3, 3-diaminobenzidine; Th17: T helper 17.

Declarations

7.1 Ethics approval and consent to participate

Manipulations of animals were performed in accordance with the Guideline for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Experimental Animal Ethic Committee of the Second Affiliated Hospital of Nanjing Medical University.

7.2 Consent for publication

Yes.

7.3 Availability of data and material

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

7.4 Competing Interest

The authors declare that they have no financial, personal or professional conflict of interest.

7.5 Funding

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7.6 Authors' contributions

CQ and XLY contributed in the conception of the work, conducting the study, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. XLY and JJ contributed in establishment of rabbit atherosclerosis models. XLY, ZY and ZD contributed in H&E staining and CT scan. XLY, HWH and SC contributed in measurement of plasma various factors levels, immunohistochemistry assay, western blot and real-time PCR.

7.7 Acknowledgement

Not Applicable

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Table 1

Table 1. Ruxolitinib regulated TC, TG and HDL-C levels in rabbit plasmas (mean ± SD, n = 3 or 4).

Groups	TC（mmol/L）	TG（mmol/L）	HDL-C（mmol/L）
Control	2.68 ± 0.41	0.83 ± 0.34	1.48 ± 0.30
Model	25.24 ± 2.22	2.44 ± 1.11	0.64 ± 0.13
Ruxolitinib	21.15 ± 2.56	1.64 ± 0.75	0.91 ± 0.23

Figures

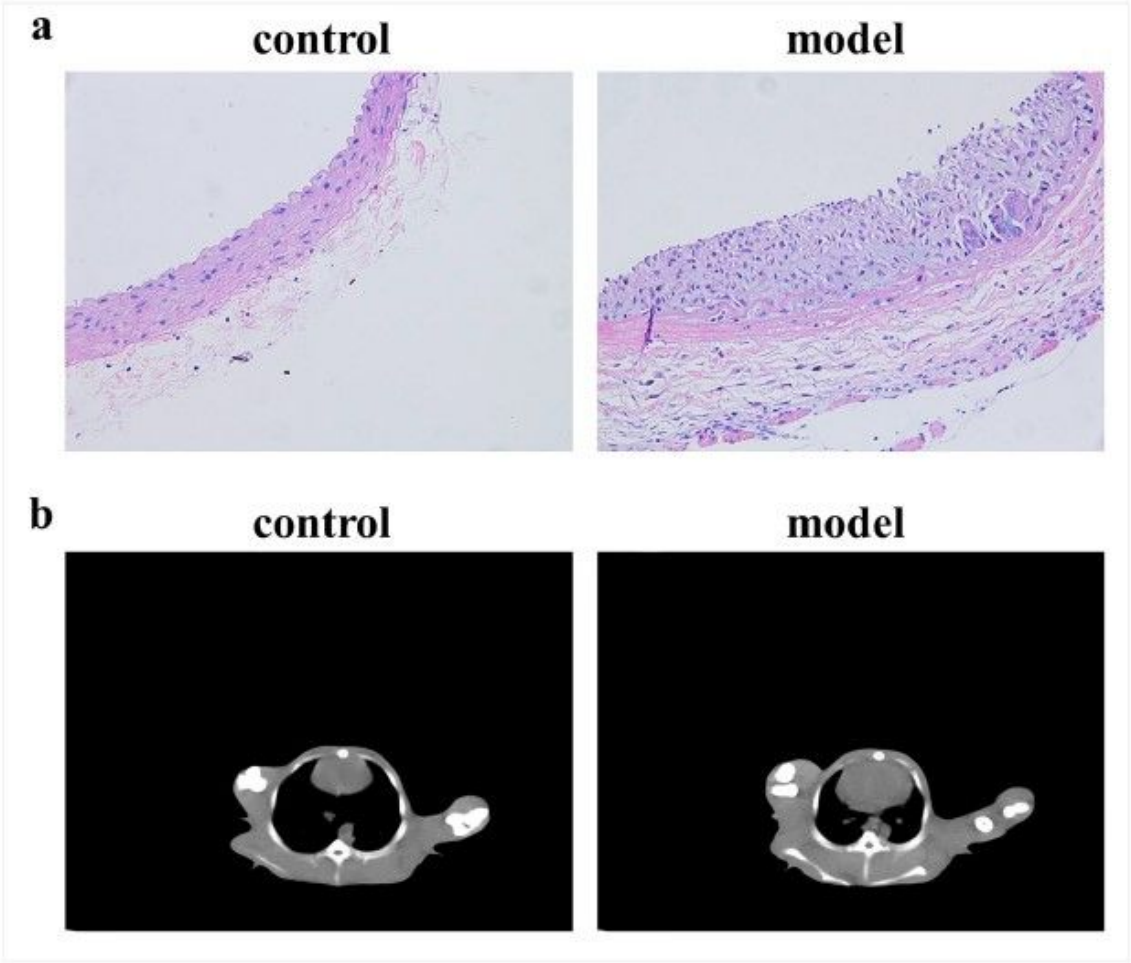

Figure 1

Establishment of rabbit atherosclerosis models. (a) H&E staining and (b) CT scan analysis were used to evaluate the atherosclerotic plaques area.

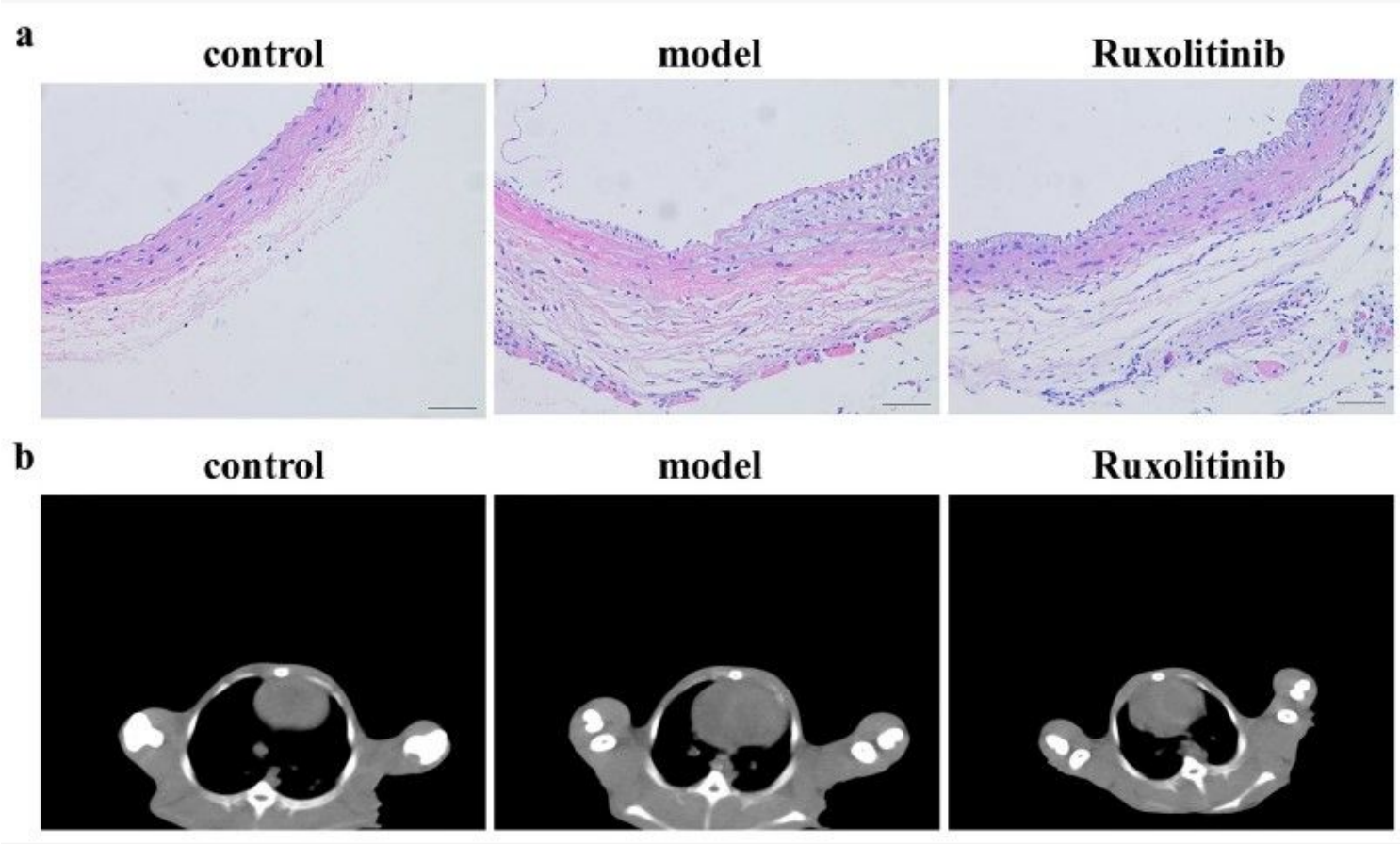

Figure 2

Ruxolitinib alleviated atherosclerosis progress in rabbits. (a) Representative images of H&E staining of abdominal aorta sections (100×). (b) Representative images of CT scan of abdominal aorta sections.

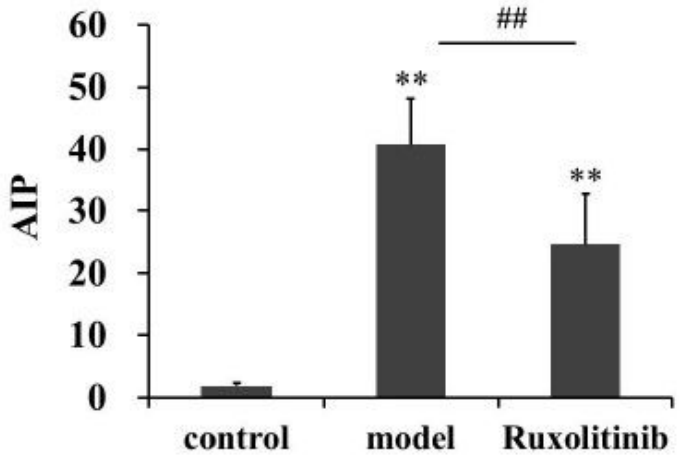

Figure 3

The levels of AIP were detected in ruxolitinib-treated rabbits with atherosclerosis. AIP was the measurement of the atherosclerotic lesion extent based on plasma lipids. **P < 0.01 versus control group; ##P < 0.01 versus model group. The results are expressed as the mean ± SD (n=3).

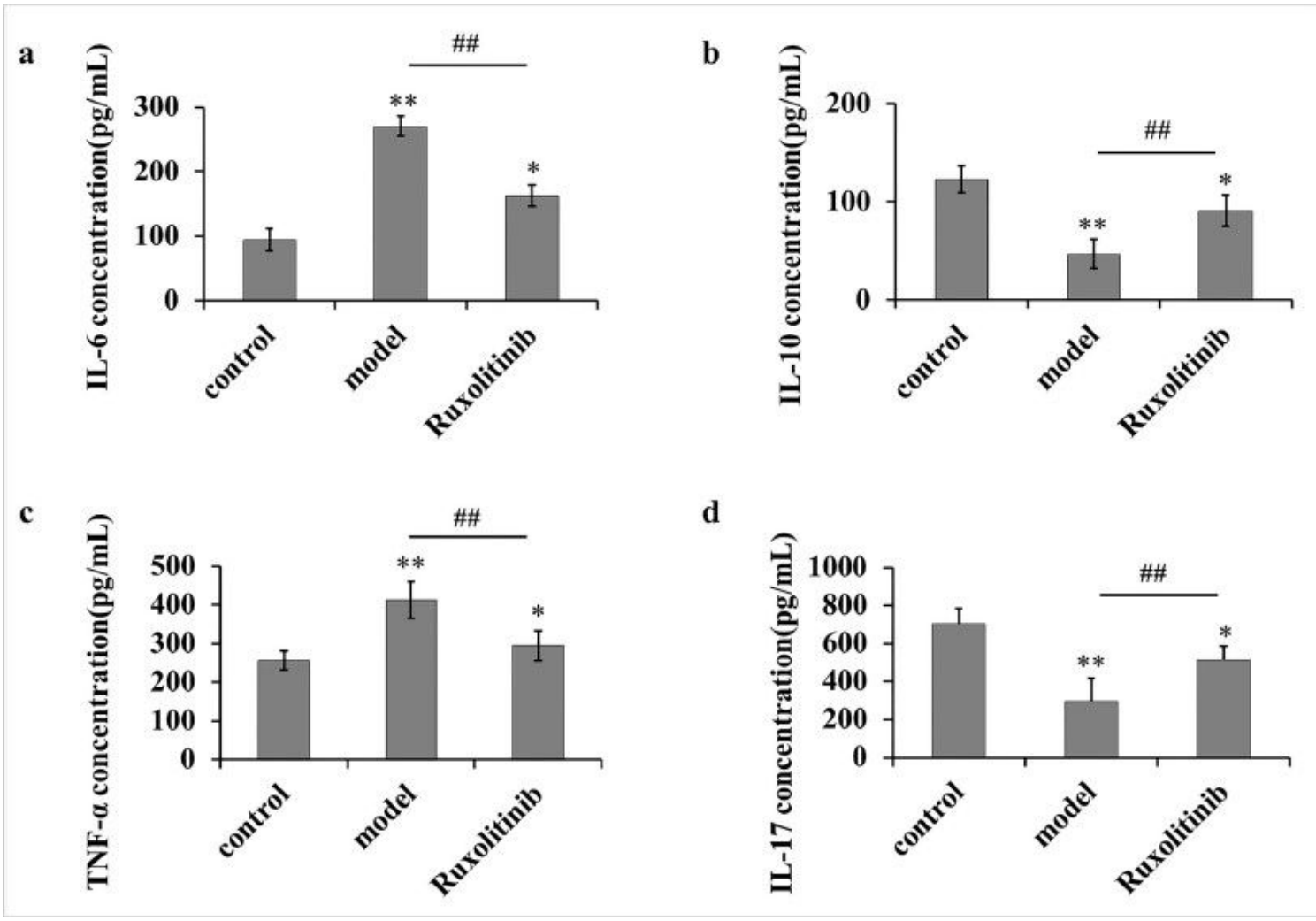

Figure 4

Administration of ruxolitinib decreased the contents of plasma IL-6, TNF-a, IL-10 and IL-17 in rabbits with atherosclerosis. (a~d) The levels of plasma IL-6, TNF-a, IL-10 and IL-17 were detected by enzyme-linked immunosorbent assay kits in rabbits. **P < 0.01 versus control group; ##P < 0.01 versus model group. The results are expressed as the mean ± SD (n=3).

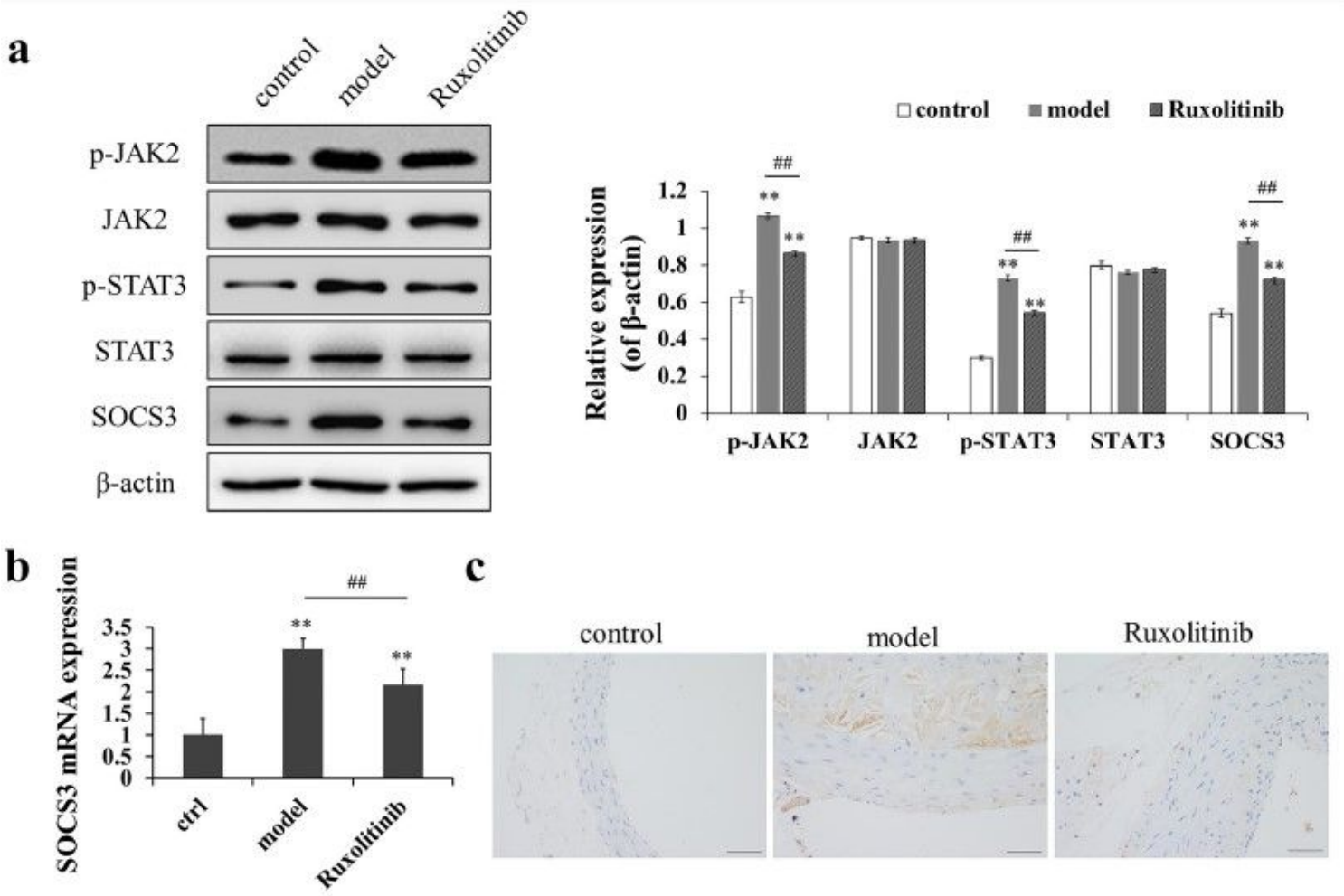

Figure 5

Administration of ruxolitinib inactivated JAK2/STAT3/SOCS3 pathway in rabbit vascular tissues. (a) Protein expression levels of p-JAK2, JAK2, p-STAT3, STAT3 and SOCS3 in rabbit vascular tissues by western blot analysis; quantification (right). (b) RT-PCR for SOCS3 expression at mRNA level in rabbit vascular tissues. (c) Immunohistochemical staining of SOCS3 in rabbit vascular tissues (Scale bar = 100 μm); **P < 0.01 versus control group; ##P < 0.01 versus model group. The results are expressed as the mean ± SD (n=3).

Supplementary Files

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