Bushen Kangshuai tablet inhibits progression of atherosclerosis by intervening in macrophage autophagy and polarization

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Abstract

OBJECTIVE: To investigate the efficacy of Bushen Kangshuai (BS-KS) tablet on autophagy and polarization in mouse macrophage RAW 264.7.

METHODS: Macrophage autophagy was induced by oxidized low-density lipoprotein (100 μg/mL). To detect the levels of autophagy, macrophage were transfected with double fluorescence LC3 autophagy adenovirus, then the numbers of autophagosomes and autophagic lysosomes were assessed by confocal microscopy. The autophagy related proteins expression of PI3K, Akt, phospho-mAkt (p-Akt) and mTOR, phospho-mTOR (p-mTOR), p62, microtubule-associated protein 1 (LC3-Ⅱ) were determined by western blotting. The macrophage polarization model was induced by lipopolysaccharide (1 μg/mL). The mRNA levels of iNOS, CD86 (M1 macrophages marker molecules), and CD206, Arg-1 (M2 macrophages marker molecules) were detected by real-time quantitative PCR. The concentration of cytokines TNF-α and IL-10 was determined by enzyme-linked immunosorbent assay. The protein expression of nuclear proteins PPAR-γ, NF-κB, and cytoplasmic protein IKB α was determined by western blotting.

RESULTS: The expression of the autophagy-related protein LC3-Ⅱ increased and the expression of p62 was decreased in the BS-KS intervention group. The protein expression of PI3K, p-Akt, and p-mTOR was also reduced. BS-KS also inhibited the mRNA expression of iNOS and CD86 on M2 macrophage, but promoted the expression of CD206 and Arg-1 on M2 macrophage. With respect to the regulation of inflammatory factors, BS-KS could inhibit the secretion of pro-inflammatory TNF-α and promote the secretion of anti-inflammatory IL-10. It also inhibited the protein expression of IKB-α and NF-κB, and promoted the expression of nuclear protein PPAR-γ.

CONCLUSION: We believe that BS-KS promotes macrophage autophagy by increasing the level of autophagy protein and inhibiting the PI3K/Akt/mTOR pathway.
mTOR signaling pathway. Furthermore, BS-KS seems to inhibit macrophage M1 polarization and promote M2 polarization via the PPAR gamma / NF-κB signaling pathway, thus playing an inhibitory role in atherosclerosis.

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Keywords: Atherosclerosis; Macrophages; Autophagy; Lipoproteins, LDL; Lipopolysaccharides; Signal transduction; Bushen Kangshuaitablets

INTRODUCTION

Atherosclerosis (AS) is a slow, complex disorder of lipid metabolism, which involves vasculitis. In the early stage of the disease, inflammation causes endothelial cell injury, monocyte adherence to the damaged endothelial surface and migration of endothelial cells to form macrophages. Macrophages engulf the modified lipids to form foam cells. As the disease continues to develop, apoptosis and necrosis of foam cells lead to necrosis of the lipid core, which activates inflammatory response, abnormal lipid metabolism, apoptosis, endoplasmic reticulum stress, and autophagy. Inflammatory reaction forms the basis of the whole scheme of AS development and progression, and it is closely related to the occurrence of acute cardio cerebrovascular events.1 Macrophages are the most representative inflammatory cells, and play an extremely important role in AS. At present, autophagy and polarization of macrophages play an indispensable role in the pathogenesis and development of AS. The protective cellular mechanism against oxidative stress and inflammation in AS plaque is basic or moderate autophagy, which is an important determinant of the stability of AS plaques. Insufficient autophagy or excessive autophagy may affect the course of plaque development. In the early phase of AS, macrophages may inhibit the formation and development of atherosclerotic plaques through autophagy to reduce the production of foam cells, while in the middle and late stages of AS, macrophages show reduced autophagy, pro-inflammatory effects dominate, cell matrix secretes increased metalloproteinases, and subsequently, the plaque area and necrosis of the core are enlarged. Monocytes and macrophages can differentiate into different cell subtypes under different microenvironment signals, which are classically activated M1 phenotype and alternative activated M2 phenotype.2 M1- and M2-type macrophages are present in AS plaques. In the unstable plaque tissue, M1 macrophages were the main type, and the proportion of type M2 macrophages was increased in the stable plaque tissue. The M1 and M2 type macrophages are in dynamic equilibrium in autophagy and polarization.3,4 Therefore, early intervention of macrophage autophagy from AS onset and intervention of macrophage polarization from AS in the middle and late stages may become a new target in AS treatment.

The Bushen Kangshuai (BS-KS) tablet, a Chinese patent medicine, is a prescription developed by Professor Ruan Shiyi. The batch number of the product is Z20070672. It has been proven as an effective clinical drug for the treatment of AS and cardiovascular disease in vivo and in vitro. Previous studies have shown that BS-KS has anti-inflammatory properties and can protect against nitrous, regulate lipid peroxidation damage and autophagy. And it inhibits the development of AS in the early stages, while in the middle and late stages of AS, stabilize the plaque, prevent plaque shedding, and inhibit disease progression.19 BS-KS has a stable dosage form, of which the main effective ingredients are tanshinone II A, salvianolic acid B, and emodin. Tanshinone II A and salvianolic acid B are the main bioactive components of Salvia miltiorrhiza with anti-inflammatory and anti-oxidant properties. Tanshinone II A can modulated the antioxidant system, inflammation, and endothelial dysfunction, inhibit the apoptosis of vascular smooth muscle cells and proliferation and migration of macrophages, and stabilize vulnerable plaques, thereby having anti-atherosclerotic effects.19-24 Salvianolic acid B is known to improve vascular endothelial function via suppression of endothelial cell apoptosis and inhibition of platelet-mediated inflammatory responses in vascular endothelial cells.19,20 Emodin inhibits homocysteine-induced CRP generation in vascular smooth muscle cells, playing the anti-inflammatory and anti-atherosclerotic effects.21 Oleo nolic acid inhibits ox-LDL-induced vascular smooth muscle cell (VSMC) proliferation and endothelial cell apoptosis.20,21 Fucoxanthin in seaweed is a potent antioxidant and has various pharmacological effects such as anti-tumor, anti-oxidative, and anti-hyperlipidemic. It has also been proposed that fucoxanthin can activate the Nrf2-ARE pathway and autophagy, which plays a protective effect in oxidative stress injury.22 Tuckahoe polysaccharide in Poria Cocos has anti-inflammatory and anti-lipid peroxidation properties. It could reduce the levels of low-density lipoprotein cholesterol, aids in removing free radicals in the body and contributes to the anti-aging effect. Studies have shown that the ethanol extract of Poria plays an anti-inflammatory role by inhibiting the production of nitric oxide (NO) and prostaglandin E2 (PGE2) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.23 Amomi Fructus extract has significant anti-inflammatory and analgesic effects. It has been proposed that various components of Amomi Fructus extract can attenuate the expression of pro-inflammatory cytokines like TNF-α and IL-6 in human mast cells.24 In this study, we aimed to evaluate the efficacy of BS-KS on autophagy and polarization in mouse macrophage RAW 264.7.
MATERIALS AND METHODS

Cells and materials

Mouse macrophage RAW 264.7 was obtained from the Chinese National experimental cell resource sharing platform. Oxidized low-density lipoprotein (ox-LDL) was purchased from Beijing Xinyuan Jiah Biological Technology Co., Ltd., Beijing, China; BS-KS tablets were obtained from First Teaching Hospital of Tianjin University of Traditional Chinese Medicine (Tianjin, China). LPS, GW9662, and p62 antibodies were purchased from Sigma (New York, NY, USA). All-in-One™ First-Strand cDNA Synthesis Kit and All-in-One™ qPCR Mix were purchased from GeneCopoeia (Rockville, MD, USA). PCR primers were biosynthesized from Sangon Biotech (Shanghai, China); mouse TNF-α enzyme-linked immunosorbent assay (ELISA) Kit and mouse IL-10 ELISA Kit were purchased from e Bioscience (Vienna, Austria); Cell Counting Kit-8 (CCK8) was purchased from Dojindo Laboratories (Kumamoto, Japan); mRFP-GFP-LC3 was purchased from Parkinson Biomedical Technology Co., Ltd., Shanghai, China; polyclonal antibodies against LC3 and PPAR-γ in rabbits were purchased from Abcam PLC (Cambridge, UK); monoclonal antibodies against PI3K, Akt, p-Akt, mTOR, p-mTOR, NF-kB p65, and IKBα were purchased from Cell Signaling Technology (Danvers, MA, USA); polyclonal antibodies against β-actin and PCNA were purchased from Proteintech Group (Wuhan, China); HRP-goat anti-rabbit IgG was purchased from Boster Biological Technology Co., Ltd., Wuhan, China; laser scanning confocal microscope was purchased from Carl Zeiss Oberkochen, Germany; PCR kit of fluorescent quantitation ABI7500, carbon dioxide incubator, and gel imaging system were purchased from Bio-Rad Laboratories (Hercules, CA, USA); 96-well plates was purchased from Corning Life Sciences (New York, NY, USA).

Screenings of drug-containing serum concentration

Taking the exponential growth phase RAW 264.7, the density of the cell suspension was adjusted to be 1 × 10⁶ / well cells. The cells were inoculated on 96-well plates, 100 µL for each well, 6 secondary Wells was set for each group. Cells were incubated with serum containing 5%, 10%, 15% and 20% kidney-tonifying and 15% kidney-tonifying and 10% CCK8 (CCK8: DMEM) was added to each well. After incubation at 37 °C for 24 h, the culture medium was replaced and the cells cultured for 36 h; fluorescence microscopy was used to observe the efficiency of adenovirus infection. RAW 264.7 cells were co-incubated with ox-LDL (60 mg/mL; 100 mg/mL) for 0, 6, 12, 24, and 48 h to establish the autophagy model and the ox-LDL intervention group as the control group. After treatment, the extent of autophagy in macrophages under different interventions was observed by laser scanning confocal microscopy. Dual fluorescent LC3 cell autophagy adenovirus reporter system, number of autophagosomes, and autolysosomes were measured by confocal microscopy to observe variation of autophagic flow intensity.

Western blot analysis

The nucleoprotein and plasma protein of macrophages in each group were extracted and lysed on ice for 30 min and centrifuged at 4 °C for 15 min. Protein concentrations were measured using BCA assay. SDS-PAGE electrophoresis was carried out at a constant current of 200 mA for 2 h at 4 °C and transferred onto PVDF membrane in 5% skimmed milk solution in TBST for 1 h. The membrane was incubated overnight at 4 °C refrigerator, and in secondary antibody (1 : 2000) for 1 h at room temperature, following detection with an ECL kit. ImageJ software was used for image gray-scale analysis, and the ratio of the gray value of all the target proteins to the gray value of the internal reference was calculated.

RT-PCR analysis

The total RNA was extracted by TRIZOL method. The concentration and purity of RNA were determined by UV spectrophotometer, and the content of RNA was 1.5-2.0 g/L, with OD₂₆₀/OD₂₈₀ being be-
The Reverse Transcription of RNA into cDNA was conducted according to All-in-One™ First-Strand cDNA Synthesis Kit; Fluorescence Quantitative PCR amplification was performed according to the instructions of the All-in-One™ qPCR Mix kit. For each amplification set, GAPDH was run as the internal reference control. The 2-ΔΔCt method was used to analyze the relative mRNA expression in the samples. Fluorescent Quantitative PCR primer sequences are shown in Table 2. The reaction conditions of PCR are shown in Table 2.

**ELISA analysis**

The ELISA plate wells were coated and stored in refrigerator at 4 °C. Before the experiment started, determine the number of microwell strips required and prepare Biotin-Conjugate. Wash microwell strips twice with Wash Buffer, then add 50 μL Sample Diluent in duplicate to all wells and 50 μL of externally diluted Standards in duplicate to standard wells. Diluted TNF-α and IL-10 in duplicate were added to the ELISA plate. Then 50 μL diluted Biotin-Conjugate was added to all wells and incubated 2 h at room temperature. Wash the plates with Wash Buffer, add 100 μL diluted Streptavidin-HRP was added to all wells and incubated for 1 h at room temperature. Wash microwell strips 6 times with Wash Buffer, add 100 μL of TMB Substrate Solution to all wells. After incubate the microwell strips for about 30 min at room temperature, 100 μL stop solution was added to all wells. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length. Blank the plate reader according to the manufacturer’s instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

**RESULTS**

**Ox-LDL improves the autophagosomes and autolysosomes of macrophage**

To explore the effect of Ox-LDL on macrophages autophagy, the macrophages were transfected with mRFP-GFP-LC3 adenovirus, mRFP was used for labeling and tracking of LC3, GFP showed green fluorescence. On microscopic imaging, yellow spots indicate autophagosomes and red spots indicate autolysosomes. We can detect the intensity of autophagy flow via counting spots of different colors. Using mRFP-GFP-LC3 adenovirus to monitor autophagic flow in real time, normal macrophages have a low level of autophagy, and only a little number of autophagosomes can be detected, while almost no autolysosomes can be detected. At 6, 12, 24 and 48 h, there was no statistical difference. It was found that both 60 μg/mL and 100 μg/mL ox-LDL could induce macrophage autophagy. At 6, 12, and 24 h, the autophagic activity increased with time and peaked at 24 h. Then, the autophagic lysosomes in the 60 μg/mL ox-LDL group continued to increase until 48 h, and it was impossible to judge when the autophagy intensity attenuated. At
48 h, the concentration of autophagy in the 100 μg/mL ox-LDL increased. Increases in the number of red autophagy lysosomes decreased, suggesting the fusion of autophagosomes, and lysosomes were blocked. Therefore, we selected 100 μg/mL ox-LDL intervention macrophages for 24 h as the best modeling conditions for this study (Figure 1).

Expression of autophagy-related protein
Beclin-1, LC3-Ⅱ, and p62 are specific marker proteins of autophagy at each stage. To detect the expression of autophagy-related protein, the cells were treated in groups, after which cell proteins were extracted, and LC3, LC3-Ⅱ, and p62 were detected by western blotting. β-actin was used as the loading control. Studies have shown that ox-LDL-stimulated macrophages can upregulate the expression of LC3-Ⅱ/LC3-Ⅰ proteins and down regulate the expression of p62 protein, and BS-KS can be further up regulate the expression of LC3-Ⅱ/LC3-Ⅰ protein and down regulate the expression of p62 protein (Figure 2).

Expression of PI3K, Akt, p-Akt, mTOR, and p-mTOR proteins
The PI3K/Akt/mTOR signaling pathway plays an important role in autophagy. BS-KS decreased expression levels of PI3K, p-Akt, mTOR, p-mTOR, while Akt expressions were not significantly different. There were no significant differences in the expressions of Akt, p-Akt, mTOR, and p-mTOR between the BS-KS and Rapamycin group. (Figure 3)

Expression of macrophages M1/M2 surface marker molecule mRNA
iNOS and CD86 are the surface marker molecules of M1, CD206, and Arg-1 are the surface marker molecules of M2. The results of this study showed that stimulation of macrophages with LPS 1 μg/mL for 3 h can significantly up-regulate the expression of mRNA of iNOS and CD86, inducing macrophage polarization into M1 type BS-KS can inhibit the mRNA expression of iNOS and CD86 while significantly up-regulating the expression of CD206 and Arg-1, inducing macrophage polarization into M2 type. GW9662 attenuated the M2 macrophage polarization induced by BS-KS (Figure 4).

TNF-α, IL-10 expression levels
Activation of M1 macrophages can induce the expression of pro-inflammatory. M2 macrophages can induce a high expression of inflammatory inhibitory factors. In our study, the cells were treated in groups. TNF-α and IL-10 concentration was detected by ELISA. The results showed that BS-KS could inhibit the secretion of the pro-inflammatory cytokine TNF-α, and significantly up regulate the secretion of anti-inflammatory cytokine IL-10. GW9662 group promotes the secretion of TNF-α and inhibits the secretion of IL-10, thus inhibiting the anti-inflammatory effect of BS-KS (Figure 5).

Expression of plasma protein IKBα and nuclear protein NF-κB, PPAR-γ
NF-κB plays a key role in the expression and release of inflammatory mediators during LPS-induced inflammation. While activation of PPAR-γ is a key factor in the polarization of M2 macrophages. To further verify the mechanism of macrophage polarization, the cells were treated in groups. The macrophages were stimulated with LPS 1 μg/mL for 24 h. Plasma protein IKBα, nuclear proteins NF-κB, and PPAR-γ were detected by western blotting, where β-actin and PCNA were used as the loading control. The results showed that BS-KS inhibited the expression of IKBα and nucleoprotein NF-κB, and promoted the expression of nucleoprotein PPAR-γ. GW9662 could eliminate the effect of BS-KS (Figure 6).

DISCUSSION
A variety of immune cells are involved in AS’s progress of this pathological process and affect the stability of plaques by secreting cytokines and chemokines. According to Traditional Chinese Medicine, the harmony of Yin and Yang in the human body is the key to maintaining good physical and mental health. Of various pathogenic factors that lead to AS, dysfunction or decrease of cardiomyocytes causes the disharmony of Yin and Yang in the heart. By eliminating endogenous pathogenic factors such as phlegm and blood stasis, new nutrients can be produced to achieve self-regulation and restore the dynamic balance between Yin and Yang. The M1 and M2 macrophages are also in the dynamic balance, consistent with the Yin and Yang balance theory.

There are some changes in autophagy level in the process of AS. The aortic AS plaques in human endarterectomy showed that there were autophagosomes in the ultrastructure of various cells. The number of autophagosome was the largest in lipid droplets in foam cells formed by macrophage phagocytosis of lipids. In addition, detecting early and advanced carotid artery AS plaques in humans (endarterectomy) using a quantitative immunohistochemical method showed that the expression of the autophagy-related gene ATG5 decreased significantly, while the expression of the autophagy receptor protein, p62 was significantly increased in the advanced plaque compared to the early one. The results showed that the autophagy level did not show a sustained rise with the progress of AS, rather began to decrease after reaching a certain peak.

In this study, macrophages were induced by ox-LDL to simulate the lipid environment of AS. By detecting the changes of autophagy related protein expression in macrophages, the regulation effect of BS-KS on autophagy...
Figure 1 Ox-LDL improves the autophagosomes and autolysosomes of macrophage
A1-A15: effect of the control group on autophagic flux, at 0, 6, 12, 24 and 48 h. B1-B15: effect of 60 μg/mL ox-LDL on autophagic flux, at 0, 6, 12, 24 and 48 h. C1-C15: effect of 60 μg/mL ox-LDL on LC3-labeled autophagosomes and autolysosomes, at 0, 6, 12, 24 and 48 h. A1-A5, B1-B5, C1-C5: GFP; A6-A10, B6-B10, C6-C10: mCherry; A11-A15, B11-B15, C11-C15: MERGE. A1, A6, A11, B1, B6, B11: 0 h; A2, A7, A12, B2, B7, B12, C2, C7, C12: 6 h; A3, A8, A13, B3, B8, B13: 12 h; A4, A9, A14, B4, B9, B14, C4, C9, C14: 24 h; A5, A10, A15, B5, B10, B15, C5, C10, C15: 48 h. OX-LDL: oxidized low density lipoprotein; LC3: light chain 3.
The importance of autophagy in macrophages was discussed. We chose the important autophagy related molecular LC3-II and p62 as detection index of autophagy happening. LC3 is the homologous gene of yeast autophagy marker gene Atg8. Cytoplasmic LC3 (LC3-I) can hydrolyze a small polypeptide and convert it into autophagy membrane type (LC3-II). LC3-II elevation represents the initiation of autophagy, and the degree of autophagy can be judged by detecting the ratio of LC3-II/I. p62 is an important gene associated with autophagy and the homologous gene of mammalian autophagy related gene Atg6. The degradation of p62 by autophagy can be used to detect the state of autophagy. The results showed that ox-LDL induced macrophages could up-regulate the expression of LC3-II/I protein, down-regulate the expression of p62 protein, and induce autophagy. Compared with the model group, the expression of LC3-II/I protein and p62 protein were
Akt/mTOR plays a key role in the regulation of autophagy. PI3K is activated to form PIP3 in the cell membrane as a second messenger to activate downstream proteins. PIP3 combines with signaling proteins Akt and PDK1 in cells containing pleckstrin up-regulated and down-regulated in BS-KS group and rapamycin intervention group, suggesting that BS-KS tablet could further promote autophagy. Signal pathway PI3K/Akt/mTOR plays a key role in the regulation of autophagy.

![Figure 4](image1.png)  
**Figure 4 Expression of macrophage surface marker molecules**  
A: iNOS mRNA expression of M1; B: CD86 mRNA expression of M1; C: CD206 mRNA expression of M2; D: Arg-1 mRNA expression of M2. Bushen Kangshuai group is 10% drug-containing serum, and GW9662 20 μmol/L intervention group respectively. Data were expressed as mean ± standard deviation of three independent experiments. iNOS: inducible nitric oxide synthase; CD86: CD86 molecule; CD206: CD206 molecule; Arg-1: Arginase 1. Compared with the control group, \( P < 0.01 \); compared with the model group, \( P < 0.05 \), \( P < 0.01 \); compared with the BS-KS group, \( P < 0.01 \).

![Figure 5](image2.png)  
**Figure 5 TNF-α, IL-10 concentration in cell supernatants**  
A: TNF-α concentration. B: IL-10 concentration. Bushen Kangshuai group is 10% drug-containing serum, and GW9662 20 μmol/L intervention group respectively. Data were represented as mean ± standard deviation. BS-KS: Bushen Kangshuai; TNF-α: tumor necrosis factor alpha; IL-10: Interleukin 10. Compared with the control group, \( P < 0.01 \); compared with the model group, \( P < 0.01 \); compared with the BS-KS group, \( P < 0.05 \), \( P < 0.01 \).

![Figure 6](image3.png)  
**Figure 6 PPAR-γ/NF-κB signaling pathways**  
A: relative protein expression of IKBa, and the relative protein expressions of NF-κB p65, PPAR-γ in nucleus; B: expression of the immunoreactive bands IKBa; C: expression of the immunoreactive bands NF-κB p65; D: expression of the immunoreactive bands PPAR-γ. Bushen Kangshuai group is 10% drug-containing serum, and GW9662 20 μmol/L intervention group respectively. IKBa: I kappa B-alpha; NF-κB: nuclear factor kappa B; PPARγ: peroxisome proliferator-activated receptor gamma. 1: control group; 2: Model group; 3: BS-KS group; 4: Rapamycin group. Data were expressed as mean ± standard deviation of three independent experiments. Compared with the control group, \( P < 0.05 \); compared with the model group, \( P < 0.05 \), \( P < 0.01 \); compared with the BS-KS group, \( P < 0.05 \).
homology domain, prompting PDK1 Akt protein phosphorylation and subsequently leading to the activation of Akt Ser308. Akt also can be activated by phosphorylation of Thr473 by PDK2. MTOR is the convergent point of many signal pathways, which is a key link to regulate autophagy through the upstream and downstream signal transduction to affect autophagy. It indicates that BS-KS can inhibit autophagy by inhibiting the activation of PI3K/Akt/mTOR signaling pathway and promoting autophagy in macrophages.

Inflammatory state resulting from the imbalance between the two may be a major factor affecting plaque stability. In the atherosclerotic vessels of rabbits, macrophages polarize the M1 phenotype and inhibit the M2 phenotypic polarization, and there is a positive correlation between the degree of M1 phenotypic polarization and the degree of AS. Activation of M1 macrophages can induce the expression of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β, which participate in the inflammatory response. M2 macrophages can induce a high expression of inflammatory inhibitory factors IL-10, TGF-β, and arginase, and low expression of IL-12 and other proinflammatory cytokines, thus inhibiting the inflammatory response, preventing the surrounding tissue to produce harmful immune response, and promoting the repair of damaged tissue. Macrophage polarization is regulated by transcription factors, and NF-κB is a key transcription factor that promotes the polarization of M1 type macrophages. Activation of NF-κB (especially the activation of its subunit p65) is a marker of macrophage activation. When NF-κB is activated by LPS, the free NF-κB rapidly translocates to the nucleus, activating the inflammatory genes and enhancing the synthesis and secretion of IL-1, IL-6, IL-12, and TNF-α, IL-23, and other inflammatory factors. The synthesis and secretion of bioactive molecules stimulate NF-κB persistently, thereby amplifying the inflammatory reaction. Activation of PPAR-γ is related to glycolipid metabolism and maturation of type M2 macrophages. Inactivation of PPAR-γ protein will block the transduction of alternative activation signal, thereby reducing M2 subtype macrophages activation. The functional marker of M2 macrophage named Arg-1 is suppressed in PPAR gene-knockout cells. PPAR-γ is activated when bind with its ligands, PPAR-γ-RXR heterodimers can combined with activation factor CBP and p300, thus inhibiting the activation of STAT1 and blocking the production of STAT1-related proinflammatory cytokines (IL-6, TNF-α). PPAR-γ promotes macrophage polarization to M2, exerting its anti-inflammatory function. PPAR-γ can also bind directly with NF-κB subunit p65/p50, form a transcriptional repression complex, and inhibit the expression of NF-κB.

The results of this study show that stimulation of macrophages with LPS can significantly up-regulate the expression of mRNA of iNOS and CD86 while significantly up-regulating the expression of CD206 and Arg-1, inducing macrophage polarization into M2 type. These results suggest that BS-KS may inhibit the polarization of M1 macrophages and induce the polarization of M2 macrophages. BS-KS could inhibit the secretion of the pro-inflammatory cytokine TNF-α, and significantly up regulate the secretion of anti-inflammatory cytokine IL-10. GW9662 group promotes the secretion of TNF-α and inhibits the secretion of IL-10, thus inhibiting the anti-inflammatory effect of BS-KS. These results suggest that BS-KS may play an anti-inflammatory role by promoting the secretion of anti-inflammatory cytokines and inhibiting the secretion of pro-inflammatory cytokines. The results showed that BS-KS inhibited the expression of IKKβ and nucleoprotein NF-κB, and promoted the expression of nucleoprotein PPAR-γ. These results suggest that BS-KS may inhibit the polarization of macrophages by activating PPAR-γ to inhibit NF-κB. However, the interaction between autophagy and polarization of macrophages and crosstalk between signaling pathways need to be further explored in our experiment.

In conclusion, moderate autophagy may reduce the inflammatory response and ensure the stability of an AS plaque; the transformation of M1 macrophages to M2 macrophages also enhances the stability of plaque and reduces the occurrence of clinical adverse events. BS-KS takes effect through effective targets and signaling pathways: in the prophase of AS, which possibly promotes macrophage autophagy by inhibiting PI3K/Akt/mTOR signaling pathway, while in the late stage of AS, BS-KS can inhibit M1 macrophage polarization and promote M2 macrophage polarization via the PPARγ/NF-κB signaling pathway.

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