

Vaspin Alleviates Osteoarthritis by Inhibiting NLRP3-mediated Inflammation

Xianjie Zhu

Qingdao Municipal Hospital Group

Shiyu Dai

Qingdao Municipal Hospital Group

Baohua Xia

Qingdao Municipal Hospital Group

Jianbao Gong

Qingdao Municipal Hospital Group

Bingzheng Ma (✉ 1018967905@qq.com)

Qingdao Municipal Hospital Group

Research Article

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Abstract

Background:

Osteoarthritis (OA) is a chronic degenerative joint bone disease characterized by cartilage degradation. Visceral adipose tissue-derived serine protease inhibitor (vaspin) is associated with the inflammatory and metabolic responses to OA. However, the underlying mechanisms of the pathological process of OA are not clear. The aim of the present study was to examine the protective effects of vaspin both *in vitro* and *in vivo*.

Methods:

Monosodium iodoacetate (MIA)-induced Wistar rat model of OA was used to assess the *in vivo* effects of vaspin administered for 12 weeks. The characteristics of OA were evaluated by haematoxylin and eosin (H&E) and safranin O/fast green staining. The anti-inflammatory effect of vaspin was assessed using immunohistochemical, qRT-PCR, and western blotting analysis. Parallel experiments to detect the molecular mechanism through which vaspin prevents OA were performed using LPS-treated chondrocytes.

Results:

Our results showed that the degeneration of cartilage and upregulated expression of matrix metalloproteinase (MMP)-1 and MMP-13 were ameliorated by vaspin. Additionally, vaspin suppressed the activation of TXNIP/NLRP3 and secretion of tumor necrosis factor α and interleukin-1 β *in vivo*. It was further confirmed that vaspin could also suppress LPS-induced NLRP3 inflammasome activation and reduce collagen formation in chondrocytes. Moreover, vaspin inhibited NLRP3 inflammasome activation by suppressing the ROS/TXNIP pathway.

Conclusions:

Vaspin inhibited OA by repressing TXNIP/NLRP3 activation in *in vitro* and *in vivo* models of OA, thus providing a novel therapeutic strategy for OA.

Background

Osteoarthritis (OA) is a highly prevalent disease and a leading cause of disability and chronic pain [1, 2]. OA is characterized by the progressive breakdown of articular cartilage and remodeling of the synovial joints, which can affect the knees, hips, spine, and fingers. In addition to increasing the personal and social burden of OA, the current treatment options lack disease mitigation abilities and are limited to pain relief to maintain joint function. Ultimately, the only treatment option is surgical joint replacement. Thus, exploring safe and effective therapeutic drugs for the early treatment of OA has become an urgent problem that needs to be addressed.

The past decade has seen a fundamental shift in our understanding of the mechanisms underlying OA. A previous study found that OA is a chronic inflammatory disease [3]. The chondrocytes and synovial cells in OA produce or overproduce various inflammatory mediators characteristic of inflammatory arthritis (such as IL1 β , TNF, and nitric oxide (NO)) [4–6]. The inflammasome is a crucial cytoplasmic multiprotein that relies on the maturation and secretion of caspase-1 to promote the release of IL-1 β and IL-18, thereby causing inflammation [7, 8]. The NLRP3 inflammasome, composed of NLRP3, an apoptosis-related speckle-like protein-containing acupaspase recruitment domain (ASC) and pro-caspase-1, is the most studied member of the NLR family [8]. In a recent study, the expression levels of the NLRP3 protein in the synovial membrane of patients with knee OA increased by 5.4 times compared with that in a control group [9]. This emphasizes the potential role of NLRP3 in OA as well as the possibility of its measurement as a biomarker for OA or its targeted inhibition.

The adipogen vaspin belongs to the family of serine protease inhibitors and is related to insulin resistance and metabolic syndrome [10]. It has been detected in cartilage, synovium, and osteophytes of OA patients undergoing joint surgery. Moreover, it was found that serum levels of vaspin in paired samples with high synovial fluid levels [11]. Vaspin is a new type of adipokine with anti-inflammatory properties, and the link between vaspin and arthritis has been demonstrated. Previous study has revealed that vaspin shows anti-inflammatory and anticatabolic effects in chondrocytes by inhibiting the NF- κ B signaling pathway [12]. However, the molecular mechanism of vaspin in OA and its relationship with the NLRP3 inflammasome is not yet fully understood. This study aimed to determine the effectiveness of vaspin in mitigating inflammation and the related mechanisms *in vitro* and *in vivo*.

Methods

Animals

Specific-pathogen-free Wistar rats (male, 280–320 g, 3-months old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). The rats were kept under standard laboratory conditions (temperature 24°C, 12 h light-dark cycles). All experimental procedures were conducted according to the Guiding Principles in the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 8023, revised 1978) and were approved by the Animal Ethics Committee of Qingdao University. After one week of acclimatisation, the OA model was prepared according to a previous study. A single intra-articular injection of 3 mg of monosodium iodoacetate (MIA; Sigma-Aldrich, MO, USA) was administered to the rats to induce OA. An equal volume of saline was injected into the control rats. The rats were divided randomly into three groups (n = 5 per group) as follows: (1) control group; (2) OA group: MIA injection; and (3) OA + vaspin group (vaspin 320 ng/kg in citrate buffer). Vaspin injection was given two weeks after MIA injection and administered for 45 days after MIA injection.

Histopathological and Immunohistochemistry (IHC)

Cartilage blocks were immersed in 10% neutral buffered formalin at 4°C for three days, followed by decalcification for 14 days in 30% formic acid solution and dehydration with ethanol in a conventional

gradient. The sample was embedded in paraffin and cut into 5- μ m sections.

The paraffin sections were dewaxed, hydrated with graded ethanol, stained with haematoxylin solution for 15 min, and counterstained with eosin solution for 5 min. After dehydration, transparency, and sealing with gradient alcohol, the pathological condition of articular cartilage was observed using the Image-Pro image analysis software.

For safranin O/fast green staining, the samples were stained with 0.5% Fast Green for 20 min and 0.5% Safranin O for 5 min, followed by gradient alcohol dehydration, transparent xylene, and sealed with neutral gum. The normal cartilage appeared red, and the background appeared green.

Immunohistochemical staining was performed according to the manufacturer's instructions (Solarbio, Beijing, China) and observed under a microscope (Olympus, Tokyo, Japan).

Isolation and culture of chondrocytes

We shaved the cartilage sections from the joint surfaces of the two knee joints of adult male Wistar rats. Cartilage samples were digested in .25% trypsin at 37°C for 1 h and then transferred to 0.3% collagenase II at 37°C for 6 h until the extracellular matrix was completely digested. The chondrocytes were then filtered through a mesh, and the resulting single-cell suspension was centrifuged at 1500 $\times g$ for 10 min. Next, we transferred the cells to a culture flask and incubated them with complete Dulbecco's Modified Eagle Medium (DMEM) in a 5% CO₂ incubator at 37°C. Chondrocytes were identified by collagen II immunohistochemical staining.

ELISA

According to the manufacturer's instructions, supernatants of the cell culture or rat sera were used to measure IL-1 β and tumour necrosis factor (TNF)- α (R&D Systems, Minneapolis, MN, USA).

Flow cytometric analysis

A peroxide-sensitive fluorescent probe 20.7 0-dichlorofluorescein diacetate (DCFH-DA) was used to detect the level of reactive oxygen species (ROS). After treatment, the chondrocytes were washed three times with PBS and incubated with DCFH-DA for 30 min in the dark. Fluorescence was detected with a flow cytometer (BD Biosciences, San Jose, CA, USA).

RNA extraction and quantitative real time-PCR

After incubation, total RNA was extracted from chondrocytes and cartilage tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse-transcribed to cDNA with RT Master Mix (Takara, Kyoto, Japan). Quantitative PCR was performed on a 7300 Real-Time PCR System using SYBR Green PCR Master Mix by denaturing at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 95°C for 5 s. The GAPDH fragment was amplified as an internal control. The primer sequences were as follows: NLRP3 forward (5'-GTAGGTGTGGAAGCAGGACT-3') and reverse (5'-

CTTGCTGACTGAGGACCTGA-3'), ASC forward (5'-AGTTTCACACCAGCCTGGAA-3') and reverse (5'-TTTTCAAGCTGGCTTTTCGT-3'), caspase-1 forward (5'-CCGAAGGTGATCATCATCCA-3') and reverse (5'-ATAGCATCATCCTCAAACCTTTCTG-3'), TXNIP forward (5'-GCTCAATCATGGTGATGTTCAAG-3') and reverse (5'-CTTCACACCACTTCCACTGTCAC-3'), vaspin forward (5'-GGG CAA GCTGAAGCACTTGGAG-3') and reverse (5'-CCCGTCATGTGGAGTCTGGGT-3'), and GAPDH forward (5'-CAAGTTCAACGGCACAG-3nc) and reverse (5'-CCAGTAGACTC CACGACAT-3').

Western blot analysis

Western blotting was performed as previously described [13]. The primary antibodies used were as follows: NLRP3, ASC, TXNIP, and caspase-1 (1:1000, Abcam, USA), MMP-1, MMP-13, and collagen II (1:2000, Abcam, Cambridge, MA, USA). Antigen-antibody complexes were visualized with the ECL western detection kit (Thermo Fisher Scientific, Waltham, MA, USA). The protein levels were normalized to the level of β -actin. ImageJ software was used to quantify the density of each band.

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD). Statistical significance between groups was analysed by Student's t-test between two groups or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons using GraphPad Prism version 6.0. Results with $P < 0.05$ was considered significant.

Results

Vaspin attenuates the histopathological features of OA in an MIA-induced rat model

We constructed an MIA-induced rat OA model to investigate the protective effects of vaspin on osteoarthritis *in vivo*. H&E and safranin O/fast green staining showed normal morphology of joints in the control group, whereas the knee joint in the OA group showed severe cartilage erosion. Treatment with vaspin alleviated the degeneration and erosion of articular cartilage (Fig. 1A-B).

Expression of vaspin in OA rat model

Serum vaspin expression was examined by ELISA. As shown in Fig. 2A, serum vaspin was decreased in the OA group compared to the control group. However, the difference was not notable ($P > 0.05$). The protein expression of vaspin was evaluated by IHC analysis. The results showed that the expression level of vaspin in the OA group was higher than that in the control group, even though the increase in expression level was not significant (Fig. 2B).

Vaspin alleviates rat OA by inhibiting NLRP3 activation

To further investigate the effect of vaspin on chondrocyte inflammation in the OA model, IHC staining was performed to detect the expression level of NLRP3. As shown in Fig. 3A, the rat OA model revealed a

marked increase in NLRP3 expression, while the vaspin treatment showed a marked reduction in the number of positive cells in the rat cartilage tissue. The expression of NLRP3 inflammasome in joint tissues was analysed by qPCR and western blotting. Our results showed that vaspin downregulated the gene expression of NLRP3, ASC, TXNIP, and caspase-1 in OA rats (Fig. 3B), which was consistent with the results of the protein analysis (Fig. 3C). Western blotting also revealed that the protein levels of MMP-1 and MMP-13 were suppressed, while the protein level of collagen II was increased after vaspin treatment (Fig. 3D). In addition, vaspin inhibited the levels of mature IL-1 β and TNF- α in OA rat serum (Fig. 3E-F). Overall, these results indicate that vaspin exerts a protective effect on OA by regulating the activation of the NLRP3 inflammasome.

Vaspin suppresses LPS-mediated TXNIP/NLRP3 activation in chondrocytes

The expression of proteins involved in collagen formation and NLRP3 inflammasome activation, were analysed by western blotting. Western blot analysis demonstrated that vaspin inhibited the LPS-induced upregulation of MMP-1, MMP13, NLRP3, ASC, and cleaved-caspase-1 while preventing LPS-reduced collagen II expression (Fig. 4A-B). The RT-qPCR results also indicated that vaspin reversed the LPS-induced increase in the expression of NLRP3, ASC, and caspase-1 (Fig. 4C). Moreover, ELISA results showed that vaspin significantly reduced the protein levels of IL-1 β and TNF- α in the supernatant of chondrocytes (Fig. 4D-E). Next, NLRP3 siRNA was used to further investigate the effect of vaspin on LPS-induced chondrocytes. Western blotting confirmed the successful transfection (Fig. 4F). As shown in Fig. 4G, NLRP3 knockdown significantly downregulated the expression of MMP-1 and MMP-13 but reversed the downregulation of collagen II in LPS-induced chondrocytes, which was comparable to the effect induced by vaspin. These findings demonstrate that vaspin can reverse the LPS-activated NLRP3 inflammasome and its downstream protein expression.

Vaspin suppresses NLRP3 inflammasome activation of rat chondrocytes through inhibition of ROS/TXNIP signalling

To investigate the regulatory mechanism underlying these effects, the ROS release was examined. chondrocytes were pre-treated with vaspin or NAC and then stimulated with LPS. The results showed that vaspin inhibited the LPS-induced generation of ROS, which was comparable to the effect induced by NAC (a known ROS scavenger) treatment (Fig. 5A). In addition, we found that NAC reduced TXNIP and NLRP3 inflammasome (NLRP3, ASC, and cleaved-caspase-1) expression. The above findings indicate that vaspin partially inhibits the TXNIP/NLRP3 pathway by reducing ROS production (Fig. 5B).

Discussion

OA is no longer regarded a typical degenerative disease but a multifactorial disease in which chronic inflammation plays a central role [13]. Current treatments for OA can only relieve pain, and no drugs have been approved by the FDA to prevent or slow the progression of the disease. Therefore, we urgently need to develop new drugs that selectively target inflamed joints to prevent damage to healthy tissues. Recent

studies have found that vaspin improves the inflammation of chondrocytes by inhibiting the NF- κ B pathway [12]. Our present research showed that vaspin reduces osteoarthritis in rats by inhibiting TXNIP/NLRP3 signalling and LPS-mediated activation of NLRP3 inflammasome in chondrocytes. Therefore, we discovered a new mechanism by which vaspin can prevent OA, which further proves the protective effect of vaspin against OA.

The chondrocytes in OA cannot maintain the balance between anabolic and catabolic activities in the tissue, which leads to disturbances in the synthesis and degradation of the extracellular matrix [14]. Various molecular components and mechanisms may turn joint trauma, chronic injury, or overuse into an inflammatory process [15, 16]. The pathogenesis of OA involves several cytokines such as TNF, IL-1 β , IL-15, and leukemia inhibitory factor. Among them, IL-1 β and TNF are involved in inducing cartilage catabolism and inhibiting anabolic processes that are essential for cartilage homeostasis [17, 18]. Due to the postulated role of IL-1 β in the pathology of OA and the main role of inflammasomes in the maturation of IL-1 β , the NLRP3 inflammasome has recently received extensive attention. Curcumin or estradiol inhibits the NLRP3 inflammasome and may downregulate inflammatory cytokines and prevent OA progression [19]. The ways to activate NLRP3 have been extensively explored, such as K⁺ efflux and ROS production [20]. Thioredoxin-interacting protein (TXNIP) inhibits cell apoptosis and inflammation by inhibiting thioredoxin (TRX), thereby promoting cell growth and reducing inflammation caused by oxidative stress [21–23]. The results of a study by Seong et al. showed that the ROS-TXNIP-NLRP3 inflammasome axis is crucial in the pathogenesis of inflammation [24]. In the present study, activation of the NLRP3 inflammasome was found in both OA models and LPS-induced chondrocytes, and the activation mechanism may be related to ROS/TXNIP. Activated NLRP3 is responsible for the pathogenic effects of OA, driving cartilage degeneration, and synovitis through the production of IL-1 β , IL-18, and matrix-degrading enzymes [25]. They can change the differentiation and function of chondrocytes and stimulate chondrocytes to release cartilage-degrading enzymes, such as metalloproteases (MMPs) and aggrecanases. These enzymes degrade type II collagen and aggrecan from the extracellular matrix, resulting in cartilage loss [26]. Therefore, regulating the NLRP3 inflammasome is crucial to maintaining adequate immune protection while preventing tissue damage caused by the overproduction of cytokines.

Many studies have proposed a link between vaspin and cardiovascular disease [27]. Moreover, vaspin expression in arthritis and its valuable role have been discovered in recent studies [28, 29]. Bao et al. found that the serum vaspin level in OA was higher than that in healthy individuals, and all joint tissues of OA patients, including the cartilage, synovium, meniscus, fat pad, and osteophytes, expressed the vaspin gene [11]. Our study also found that the expression level of vaspin in the serum of OA rats was significantly lower than that in the control group. Although at a low level, vaspin was expressed in the joint tissues, and the expression level in the joint tissues was higher for the OA group than for the control group. Recent studies indicate that Vaspin inhibits the expression of MMP-2 and MMP-9 induced by leptin and that it inhibits the production of NO and TNF- α induced by leptin, indicating that vaspin has anti-inflammatory and anticatabolic effects on chondrocytes [12]. Li et al. found that vaspin prevents diabetic cardiomyopathy (DCM) by inhibiting NLRP3 inflammasome activation [27]. Based on these results, we hypothesized that vaspin would improve the inflammatory response of OA models by

inhibiting the activation of the NLRP3 inflammasome. As expected, vaspin inhibited the activation of TXNIP/NLRP3 and the release of inflammatory factors such as IL-1 β both *in vitro* and *in vivo*. In addition, the knockdown of NLRP3 mimicked the negative regulatory effect of vaspin on the inhibition of NLRP3 inflammasome activation and IL-1 β and TNF- α secretion. *In vitro* experiments showed that vaspin and NAC (ROS inhibitors) significantly reduced ROS production and inhibited NLRP3 inflammasome activation.

Conclusion

In conclusion, the NLRP3 inflammasome promotes the pathogenesis of OA. Vaspin can inhibit the TXNIP/NLRP3 pathway, thereby reducing the damage to rat chondrocytes and the occurrence of OA. Vaspin has the potential to become a target drug for the treatment of OA. However, further research is needed to better understand its other effects.

Abbreviations

OA: osteoarthritis; **MIA:** monosodium iodoacetate; **H&E:** haematoxylin and eosin;

LPS: lipopolysaccharide; **MMP:** matrix metalloproteinase; **TXNIP:** thioredoxin-interacting protein

ROS: reactive oxygen species; **IL1 β :** interleukin-1 β ; **TNF:** tumor necrosis factor

NO: nitric oxide; **ASC:** acupaspase recruitment domain; **NF- κ B:** nuclear factor kappa-B

DMEM: Dulbecco's Modified Eagle Medium ; **DCFH-DA:** dichlorofluorescein diacetate

ROS: reactive oxygen species; **IHC:** Histopathological and Immunohistochemistry

NAC: N-Acetyl-L-cystein; **TRX:** thioredoxin; **DCM:** diabetic cardiomyopathy

Declarations

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of Qingdao University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

BM and XZ designed and carried out the study. SD,BX and JG participated in the experiments and statistical analysis. BM and XZ wrote the manuscript. BM revised the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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Figures

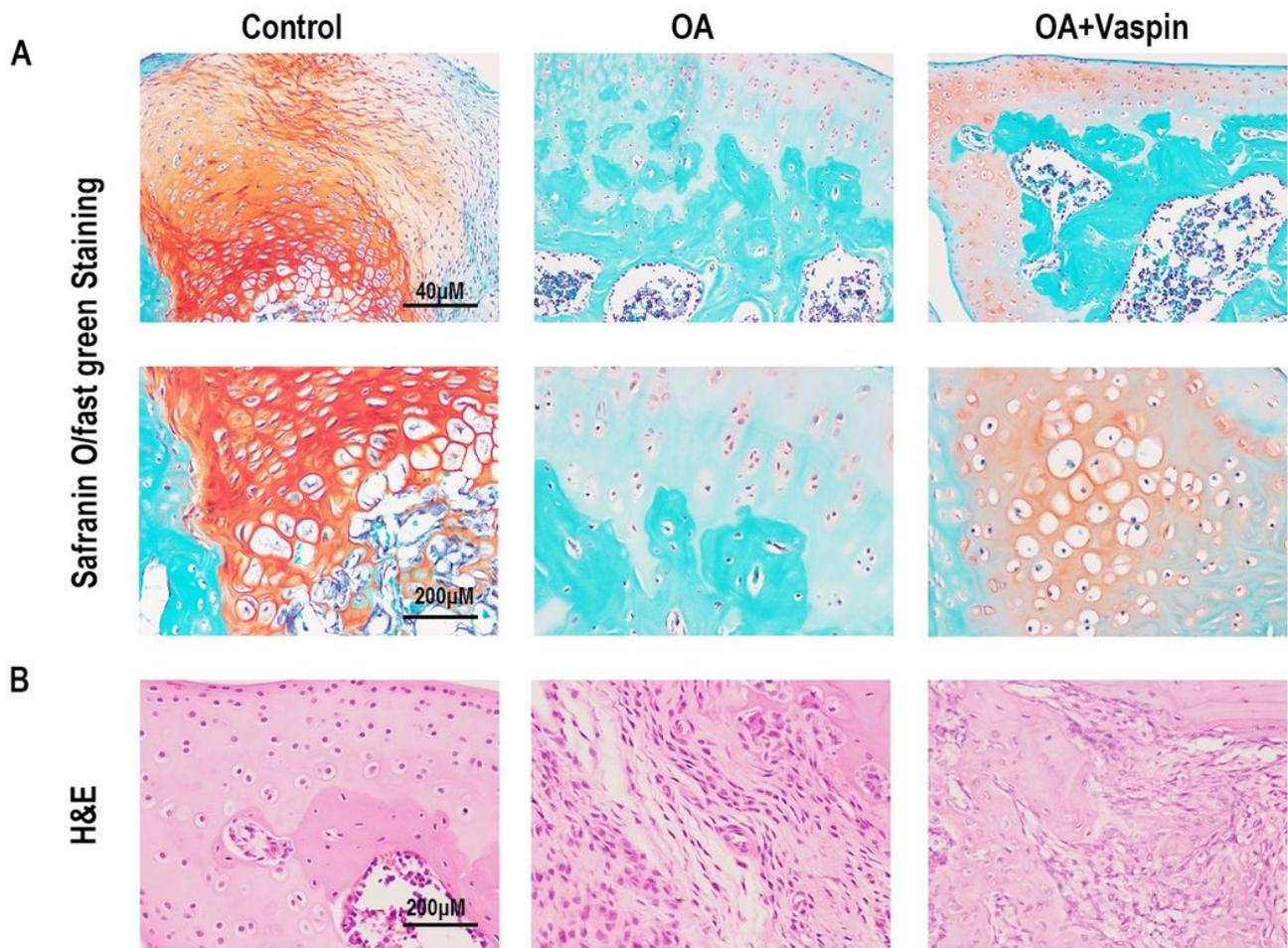


Figure 1

Vaspin alleviated articular cartilage destruction in OA rats. Safranin O/fast green staining (A) and H&E staining (B) were used to evaluate cartilage histopathology in OA rats. (In the upper figures, the bar indicates 40 μm . In the lower figures, the bar indicates 200 μm .)

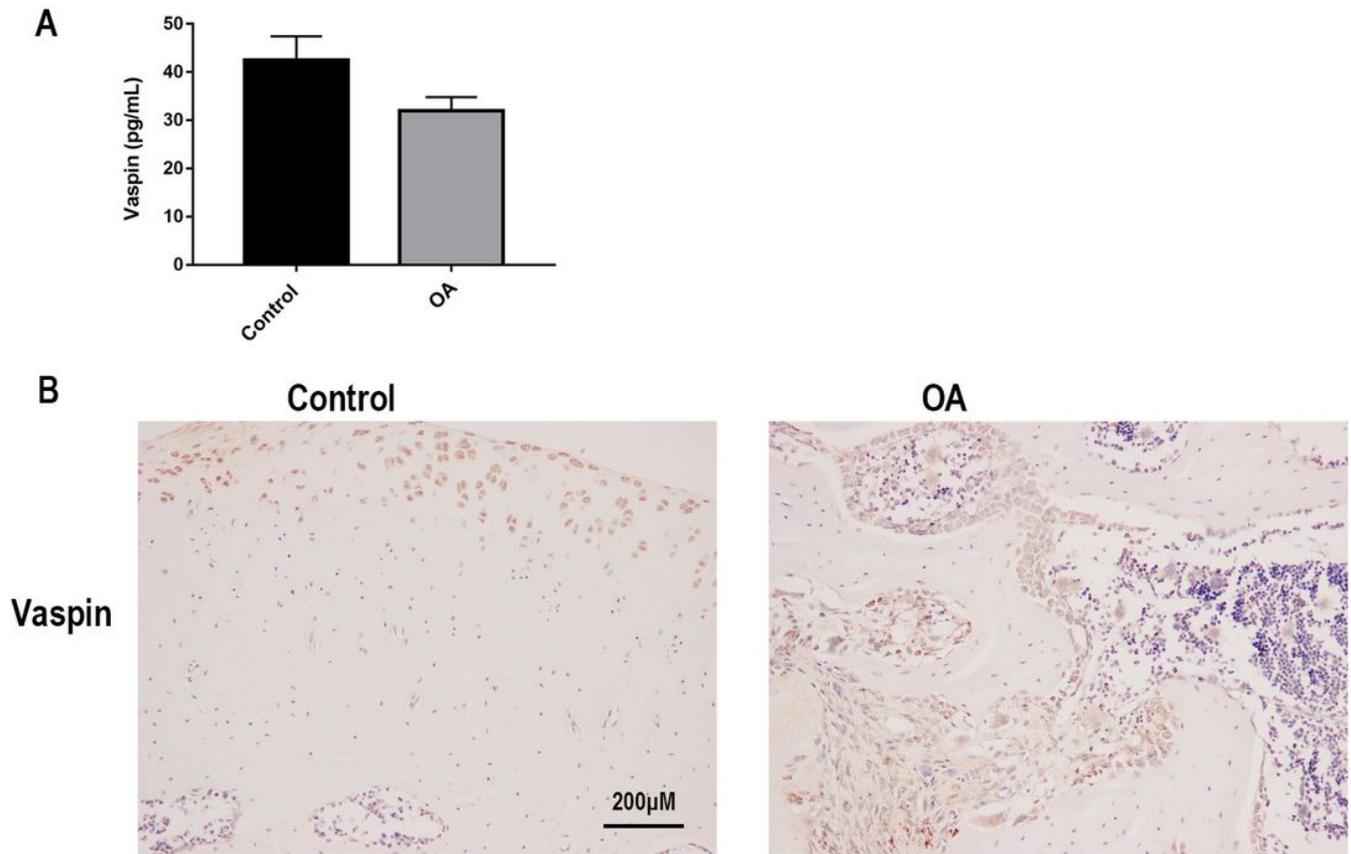


Figure 2

Expression of vaspin in serum and cartilage of OA rats. (A) The serum expression of vaspin was evaluated by ELISA. (B) The protein expression of vaspin was assessed by immunocytochemical staining.

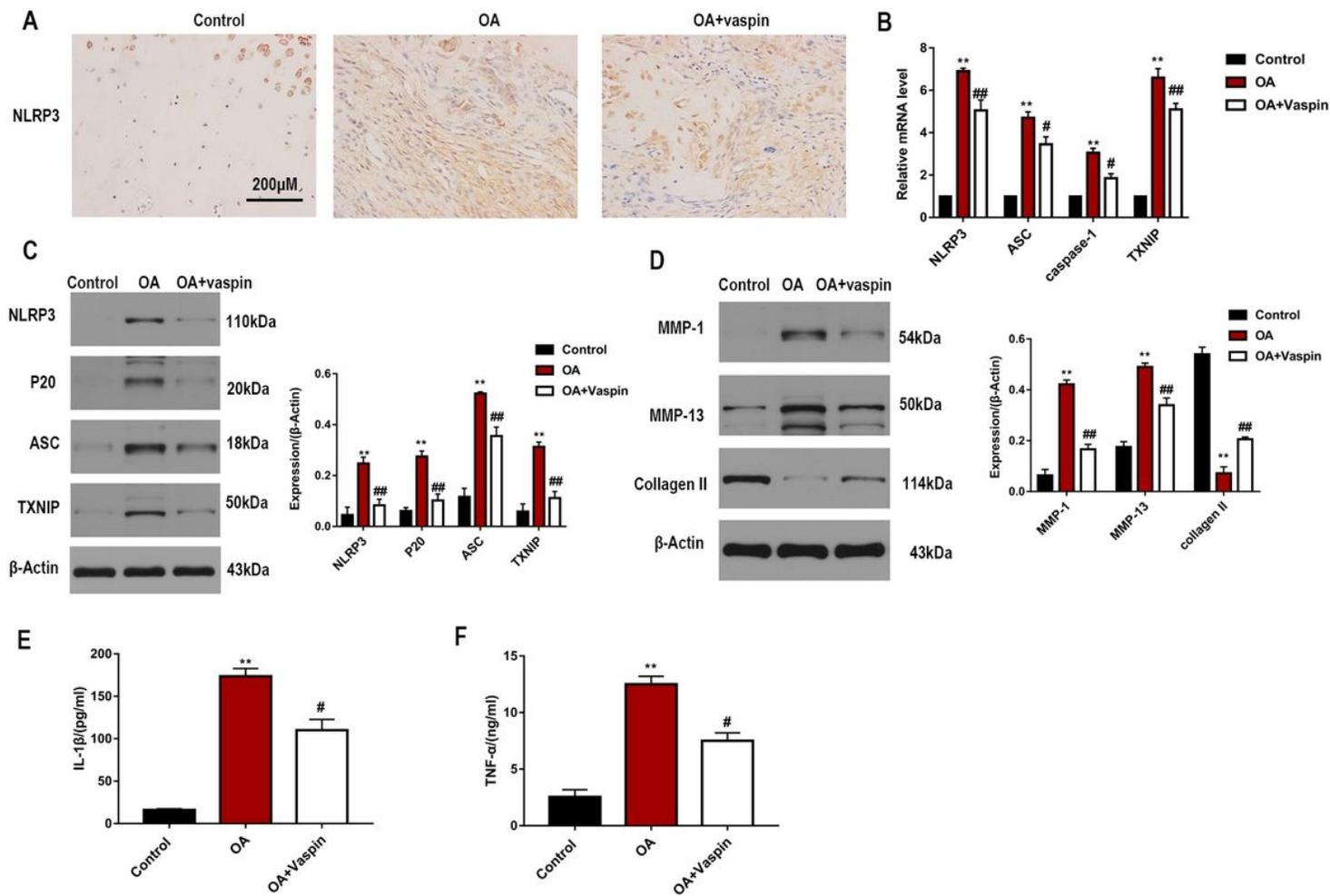


Figure 3

Vaspin attenuates NLRP3 inflammasome activation in OA rats. (A) Representative immunohistochemical staining for NLRP3 expression in OA rats. (B) mRNA levels of NLRP3, ASC, caspase-1, and TXNIP were assayed by RT-qPCR in joint tissues. (C) Expression of NLRP3, ASC, cleaved-caspase-1 (p20), and TXNIP in joint tissues assayed by western blotting. (D) The expression levels of MMP-1, Collagen II, and MMP-13 were evaluated by western blot and quantification analysis. ELISA results showing serum levels of IL-1β (E) and TNF-α (F) in the study groups. (n = 5-10 per group, *P < 0.05, **P < 0.01 vs. Control group; #P < 0.05, ##P < 0.01 vs. OA group).

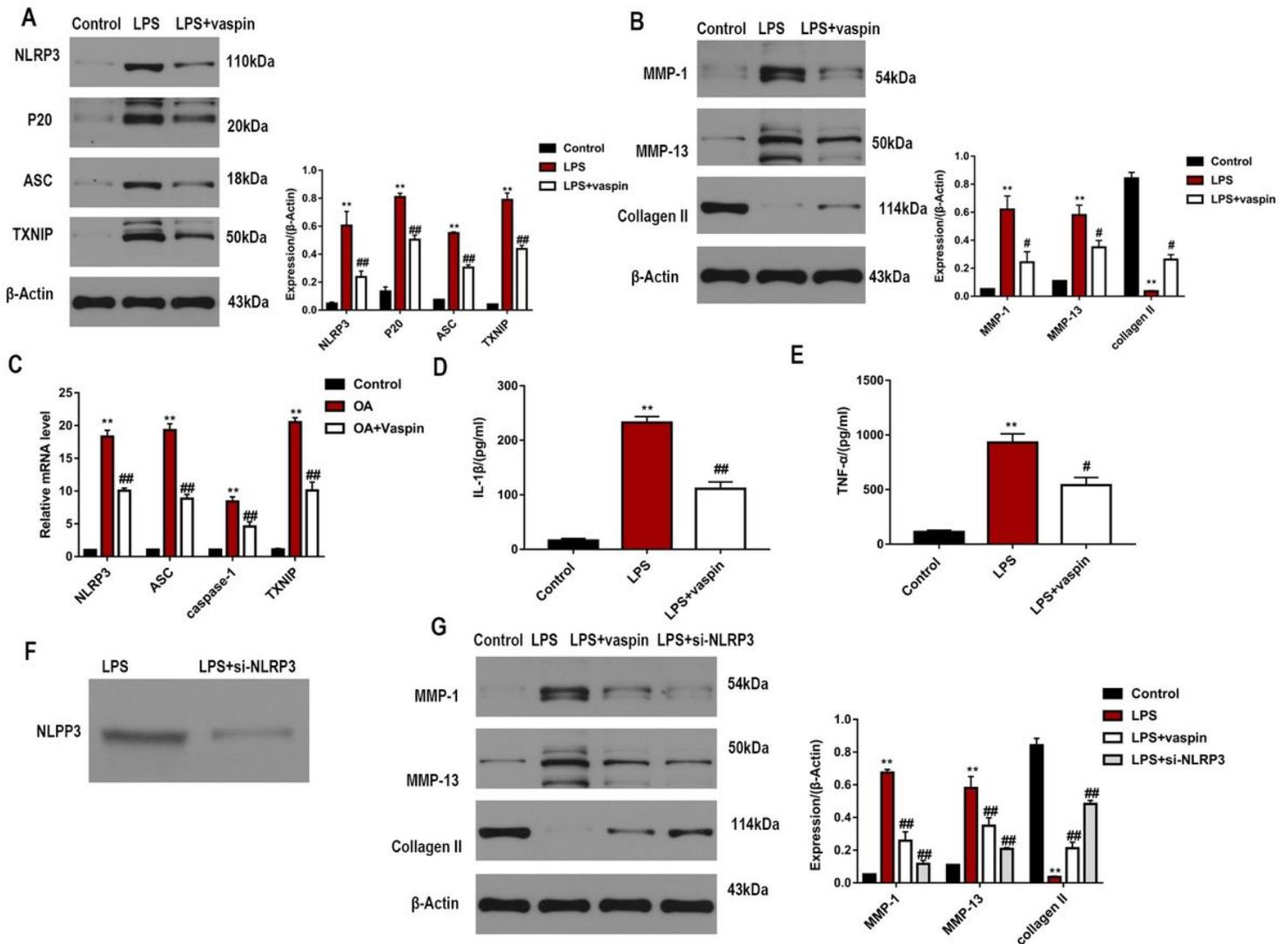


Figure 4

Vaspin inhibits NLRP3 inflammasome activation in LPS-treated Chondrocytes. (A) Relative protein levels of NLRP3, ASC, cleaved-caspase-1 (p20), and TXNIP in lysates of chondrocytes were determined by western blotting and normalized to β -actin. (B) The expression levels of MMP-1, Collagen II, and MMP-13 were evaluated by western blot and quantification analysis. (C) Levels of NLRP3, ASC, caspase-1, and TXNIP mRNA in chondrocytes were detected by RT-qPCR and normalized to those of GAPDH. Protein levels of IL-1 β (D) and TNF- α (E) in medium supernatants of chondrocytes were analyzed by ELISA. Chondrocytes were transfected with NLRP3 siRNA. Western blot analysis revealed the successful knockdown of NLRP3 (F). (G) Relative protein levels of MMP-1, Collagen II, and MMP-13 in lysates of chondrocytes were measured by western blot and normalized to those of β -actin. (n=3, *P < 0.05, **P < 0.01 vs. Control group; #P < 0.05, ##P < 0.01 vs. LPS group)

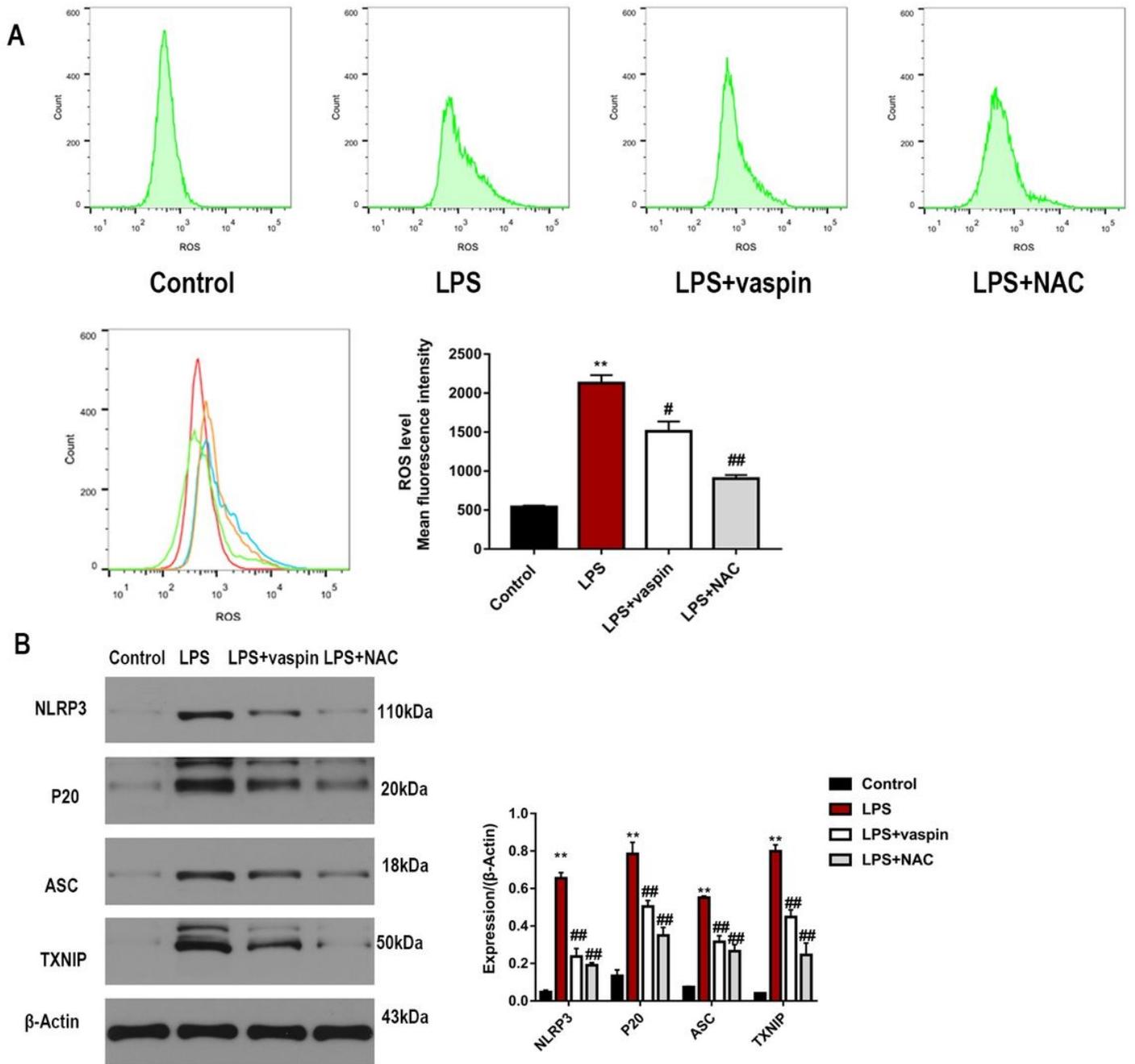


Figure 5

Vaspin inhibits LPS-induced reactive oxygen species (ROS) production in chondrocytes. (A) Flow cytometric histogram of chondrocytes in different groups and quantification analysis. (B) Expression of NLRP3, ASC, cleaved-caspase-1 (p20), and TXNIP in chondrocytes assayed by western blotting. (n=3, *P < 0.05, **P < 0.01 vs. Control group; #P < 0.05, ##P < 0.01 vs. LPS group)