

Paeoniflorin elevates the expression of SOCS3 in macrophages to prevent MIA-induced osteoarthritis in mice

Changlin Sun

Nanjing Medical University

Xiuting Qi

Nanjing Medical University

Xinmiao Qin

Nanjing Medical University

Wen Fan

Nanjing Medical University

Zijiao Tao

Nanjing Medical University

Liang Hu

Nanjing Medical University

Lai Jin

Nanjing Medical University

Chengyong Gu

the Affiliated Suzhou Hospital of Nanjing Medical University, Nanjing Medical University

Wentao Liu

Nanjing Medical University

Li Zhang

`drzhangli@njmu.edu.cn`

Children's Hospital of Nanjing Medical University

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Abstract

Osteoarthritis (OA) is a degenerative joint disease that occurs frequently in the middle aged and elderly population, limiting the joint function and disability with severe pain. However, there are limited treatments that can simultaneously relieve pain. Paeoniflorin is isolated from Chinese-medicine *Paeonia lactiflora* with anti-inflammation and immunomodulatory effects. Here we investigated the efficacy and possible mechanisms of paeoniflorin to attenuate pain, inflammation and cartilage degradation in an OA mouse model. The analgesic effect of paeoniflorin was studied by measuring allodynia with von Frey Hairs. H&E staining was used to detect structural integrity and inflammation of joint tissues. RAW264.7 was used to investigate the effect of paeoniflorin on related signaling pathway and on reflexivity of lipopolysaccharide (LPS)-induced inflammation through western blot. Paeoniflorin relieved mechanical allodynia and reduced cartilage degeneration *in vivo*. Meanwhile paeoniflorin could upregulate Gas6 expression, activate Axl receptors, upregulate suppressor of cytokine signaling3 (SOCS3) expression, inhibit MMP-9 expression and decrease p38 phosphorylation in joints. Cell experiments showed that paeoniflorin regulated Gas6-TAM pathway via ERK signal and decreased MMP-9 expression and M1 polarization *in vitro*. Paeoniflorin may inhibit joint inflammation and relieve pain via upregulating Gas6-TAM pathway and macrophage M2 polarization, which may be a potential treatment for osteoarthritis.

1. Introduction

Osteoarthritis is a disease characterized by progressive destruction of articular cartilage and chronic inflammation¹. It has affected more than 300 million people worldwide, and as a chronic progressive disease it can seriously reduce the quality of life in patients for over 50 years based on the average life span of patient and age of onset and result in great social costs². Various factors are related to the pathogenesis of osteoarthritis, including advanced age, metabolic syndrome, abnormal joint mechanics, and systemic inflammatory diseases³. Unfortunately, most of those clinical treatment now available for OA are only capable to reduce joint pain rather than to control the progression of the disease, leading to further loss for patients both physically and economically⁴.

Imaging evidence suggests that synovitis is present in considerable part of OA patients, and its degree is positively correlated with the severity of cartilage injury⁵. Macrophages play a crucial role in the development of synovial inflammatory responses in OA⁶. Inflammatory M1 polarization and activation of macrophages caused by stimulation of apoptotic chondrocyte debris can express and release a large number of inflammatory cytokines through the TLR4 signal pathway, which causes a vicious cycle of inflammatory response and tissue injury^{7,8}. Previous researchers find that TLR4 expression is increased in areas of cartilage injury in OA patients as an evidence, and TLR4 regulates MAPK and NF- κ B signals in a MyD88-dependent manner, activating downstream inflammation pathway then⁹. Among them, matrix metalloproteinase-9 (MMP-9) can degrade the cartilage extracellular matrix, destroy cartilage tissue and promote the expression and release of TNF- α and IL-1, which in turn leads to local inflammation and aggravated cartilage destruction^{10,11}.

Meanwhile, there is another possible role that has opposite contribution for macrophages, which is the M2 polarization of macrophage. Contrary to M1, M2 macrophages tend to activate inflammation resolving and tissue repairing program. Through efferocytosis, they clean up apoptotic cells and provide a pro-fixing environment¹². M2 polarization of macrophages would also lead to less M1 macrophages, which also means less severe inflammation.

The TAM receptors are a family of three receptor tyrosine kinases, including Tyro3, Axl, and MerTK. Activation of TAM receptors can mediate efferocytosis by macrophages and regulate the inflammatory cascade in rheumatoid arthritis to prevent the development of autoimmune reactions¹³. The activation of Toll-like receptors can cause an initial inflammatory peak and activate its downstream IFNAR/STAT1 signaling pathway in the local microenvironment of joint inflammation. Activated Axl receptors can form complexes with IFNAR, promote macrophage polarization switching from M1 to M2 and upregulates the transcript levels of SOCS3¹⁴. SOCS3 is an important endogenous anti-inflammatory factor that can inhibit TLR4, NF- κ B and MAPK signaling pathways¹⁵. Silencing of SOCS3 promotes IL-1 β induced expression of NF- κ B and COX2 in human OA cartilage¹⁶.

As the main ingredient of radix paeoniae alba and radix paeoniae rubra in the list of Chinese medicines that can be used in health food published by the National Health Commission of the People's Republic of China, paeoniflorin has long been used in the treatment of rheumatoid arthritis in China. A large number of studies have shown that paeoniflorin can play a protective role against apoptosis in rat chondrocytes stimulated by IL-1 β *in vitro*¹⁷. It can also inhibit the expression of MMP-3 and MMP-13 in human OA chondrocytes stimulated by IL-1 β ¹⁸. In addition, paeoniflorin can relieve postoperative pain by inhibiting microglial activation and reducing MMP-9 activity, and it was also confirmed that paeoniflorin was able to significantly inhibit the activation of p38 MAPK¹⁹.

Our previous studies have confirmed that paeoniflorin could significantly promote the expression of SOCS3 *in vivo* or *in vitro*. Paeoniflorin was shown in recent research to be able to upregulate Gas6 expression and Axl phosphorylation level *in vitro*²⁰. Therefore, we speculate that paeoniflorin may promote M2 polarization of macrophages and attenuate M1 macrophage inflammatory response by up-regulating the Gas6-TAM-SOCS3 pathway, inhibiting release of inflammatory cytokines and MMPs, and thus alleviate OA.

2. Materials and Methods

Animal care and husbandry were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the Nanjing Medical University Animal Care and Use Committee (NMJU-ACUC). During the experiments, animal pain and animal usage were minimized.

2.1 Mice and Induction of OA

Male ICR mice (6–8 weeks old) were purchased from Laboratory Animal Business Department of Shanghai Institute of Family Planning. All mice were maintained in a specific, pathogen-free facility and housed in microisolator cages containing sterilized food, bedding and water. All animal experiments were performed under protocols approved by the Nanjing Medical University Animal Care and Use Committee. Mice were randomly divided into four groups: control, monoiodoacetate (MIA) treatment alone, MIA plus paeoniflorin (90 mg/kg) treatment and paeoniflorin treatment alone. After anesthetizing with avertin (0.3mg/kg, intraperitoneally), we shaved the body hair near the knee joint of the mice, we used 30G syringe needle to complete intra- articular MIA (0.1 mg /10 μ l) injection through medial edge of the patellar ligament. Following injection, MIA absorption was accelerated by gently massaging the mouse knee joint site.

2.2 Treatment Protocol

To investigate whether paeoniflorin prevents osteoarthritis and suppresses joint destruction *in vivo*, we developed experimental protocol. Mice received intraperitoneal injections of 30 mg/kg paeoniflorin (MedChemExpress, USA, HY-N0293) once daily for 21 days, starting from the day of induced osteoarthritis.

At 21 days of feeding and paeoniflorin treatment after OA induction, mice were anesthetized and euthanized with pentobarbital sodium (150mg/kg, intraperitoneally). Tissue (knee joints) was then collected for further experiments.

2.3 Behavioral analysis

Animals were habituated to the testing environment at least 3 days before baseline testing. Mechanical sensitivity was detected using von Frey hairs. Animals were placed into boxes set on an elevated metal mesh floor and were allowed 30 min for habituation before testing. The plantar surface of each hind paw was stimulated with a series of von Frey hairs with log-arithmetical increments in stiffness, applied perpendicularly to the plantar surface. Each mouse was tested three times, and the average of the threshold was measured. Animals were considered allodynic when they displayed a response to 0.6 g or less. Normal responses fall within the 1–2 g range. The gait data were collected through the Gait Analysis and Processing System of ZhongShi Technology (ZS-BT/S) to evaluate the gait changes of mice. When collecting data, we ensured that the environment was kept dark enough for the machine to operate, with no interfering factors. After completing the measurement, the Walk Analysator was used to filter the required video clips, remove the impurities in the image that could affect the results, and correct the wrong footprints caused by system recognition.

2.4 Histopathology analysis

Mouse joint tissues were removed, fixed in 10% formalin, decalcified and embedded in paraffin. Sections were stained with hematoxylin and eosin to detect marginal erosion of the joint and articular architectural changes.

2.5 Cell culture

RAW264.7 cells (ATCC number: TIB-71) were maintained in Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) under humidified 5% CO₂ at 37°C. Before experiments, cells (10⁵ cells per well) were plated into six-well plates and cultured in DMEM containing 10% FBS for 24 h. The medium was replaced with serum-free medium for drug treatments.

2.6 Flow Cytometry

RAW264.7 cells were seeded in 6-well plates at a density of 4×10^5 . Before testing, cells were washed and re-suspended in PBS. By using the macrophage subpopulation markers CD16/32 (M1) and CD206 (M2), the cells were assessed to different phenotypes in flow cytometer. Cells were incubated with mouse CD16/32 PerCP/cy5.5 and mouse CD206 PE antibodies according to the manufacturer's instructions. Finally, they were analysed on a flow cytometer (BD FACSVerse). Data were analysed using Flowjo VX software.

2.7 Protein Extraction and Western blotting

Cultured cells were lysed with IP lysis buffer (Beyotime, China) containing protease and phosphatase inhibitors for whole protein extraction. Ground cartilage tissue were lysed using RIPA lysis supplemented with protease and Phosphatases inhibitors (Beyotime, China). Protein concentrations were determined by BCA protein assay (Thermo Fisher Scientific, Waltham, MA). Next, the proteins were fractionated by SDS- PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Subsequently, the membranes were blocked with skimmed milk for 2 h at room temperature. After blocking, the membranes were incubated with primary antibodies at 4 °C for at least 14 h and incubated with secondary antibodies at room temperature for 2 h. The protein bands were luminesced using Pierce™ ECL Western Blotting Substrate. Image J software (Rawak Software, Inc. Germany) was employed for densitometry analyses.

2.8 Statistical analysis

Statistical analyses were carried out using Prism 8.0 (Graph Pad). Unpaired Student's t-test was used to analyze differences between two groups. Alteration of protein expression and changes in behavioral responses were tested with one-way ANOVA. Error bars in all figures represent means $\pm$ SEM. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1 Abnormal expression of Axl and SOCS3 in OA mice

We first measured the mechanical thresholds of MIA treated mice through Von Frey test. As shown in Fig. 1.A, MIA decreased the mechanical thresholds of mice to less than 0.5g, indicating that MIA could induce allodynia in mice. We further picked SOCS3 and MMP9 to check at what degree the inflammation is that MIA could induce, since expression level of these proteins was regulated by inflammation and MMP9 was especially effective in causing allodynia and tissue injury. In Fig. 1.B,C, the results of Western Blot showed that anti-inflammation signal SOCS3 expression was downregulated and MMP9 was

upregulated, which supported the effect of MIA. Some research results showed that activated Axl and MerTK receptors can exert a protective role within the joint by reducing the production of proinflammatory cytokines, while Tyro3 is implicated in synovial hyperplasia, osteoclast differentiation and bone erosion during arthritis²¹. Moreover, activation of TAM receptors by Gas6 and Protein S could mediate the expression of SOCS3, so then we examined phosphorylation level of MerTK and Axl, two members of TAM receptors²². As shown in Fig. 1.D-F, their phosphorylated form level was decreased, indicating they were less activated after MIA. We also measured the expression level of their ligands, Gas6 and Protein S, and the Western Blot results showed that they were also downregulated as shown in Fig. 1.G,H, consist with the change of MerTK and Axl.

3.2 Paeoniflorin inhibited OA-induced mechanical allodynia and cartilage degradation in mice

We treated mice with paeoniflorin i.p. for 21 days as treatment, and performed Von Frey test. As shown in Fig. 2.A, paeoniflorin treatment upregulated mechanical thresholds significantly compared to MIA group, showing that paeoniflorin had a considerate effect in attenuating mechanical allodynia in MIA mice. And H&E staining of joints shown in Fig. 2.B figured out directly that paeoniflorin could attenuate MIA-induced cartilage degeneration.

Here we further adopted gait pattern examination as a further evidence for paeoniflorin treating effect as it could provide a comprehensive analysis of walking behavior of mice, as shown in Fig. 3.C. Here we focused on left hind, collected and analyzed several representative values of gaits. All the six values showed a decreasing trend after MIA treatment, indicating that MIA could significantly effect the walking behavior of mice due to the allodynia induced. And after paeoniflorin treatment, the changes of gait pattern were ameliorated, supporting the effect paeoniflorin had.

3.3 Paeoniflorin could enhance AXL level and reduce MMP9 expression

To further explore the underlying mechanism of paeoniflorin treating effect, we measured each proteins expression and activation level that were regulated by MIA mentioned above. The upregulation of SOCS3 and downregulation of MMP9(Figure.3A,B) showed that paeoniflorin could diminish inflammatory signals induced by MIA. Further, phosphorylation forms of Axl and MerTK were also upregulated as shown in Fig. 3. C,D. The downregulation of expression level of their ligands, Gas6 and Protein S, was also abolished by paeoniflorin(Figure.3E,F). These results together indicated that paeoniflorin could significantly reverse the inhibition of TAM receptors induced by MIA, leading to the resolving of downstream inflammation.

3.4 Paeoniflorin modulated macrophage inflammation and polarization in an Axl dependent manner

We sought to find evidence to prove that it was macrophages, the most common cells express TAM receptors, that were the key cells contributing to paeoniflorin treating effect, so we then focused more on the activities of macrophages after paeoniflorin treatment.

Here we first adopted Axl inhibitor, TP0903, as a supportive tool to explore whether paeoniflorin treatment relied on Axl activation. And the expression level of SOCS3 and MMP9, as shown in Figure.4A,C,D, supported our hypothesis. What's more, we also measured p38 level in the knee joints to evaluate the apoptotic level. As shown in Figure.4B, paeoniflorin could significantly reduce p38 expression, which could be also considered as that paeoniflorin could reduce apoptotic cells in knee joints. Such effect was then reversed by TP0903, indicating it was Axl-dependent.

Then we had a flow cytometry analysis to judge the trend of macrophage polarization with/without paeoniflorin treatment and with/without Axl inhibition. Here we first cultured Raw 264.7 and treated with LPS *in vitro* as a simulation of MIA in animal model to observe the macrophage polarization. We marked M1 macrophages with CD16/32 and M2 with CD206. In Figure.4E it turned out that LPS stimulation transformed most of macrophages to M1 phenotype. Paeoniflorin reversed such effect and we could find count M2 macrophages significantly increased, and TP0907 partly prevented it. We also verified if the change of inflammation and apoptosis *in vitro* would be consist with that *in vivo*. We measured the expression of SOCS3, MMP and p-p38 and Western Blot results showed same trend as *in vivo*, supporting the validation of cell experiments. These results together suggested that paeoniflorin took inflammation and pain resolving effect through inducing M2 but not M1 polarization of macrophages in a Axl-dependent way.

3.5 Paeoniflorin upregulated Gas6-Axl pathway through ERK1/2-STAT6 signal

We further explored whether there is an upstream signal of paeoniflorin regulation of TAM and macrophage polarization. Here we still used Raw 264.7 which was co-cultured with paeoniflorin for pathway investigation *in vitro*. The expression level of Gas6 and phosphorylation level of Axl were consist with the results *in vivo* as shown in Figure.5B-C, but p-MerTK level was otherwise unchanged after paeoniflorin treatment (Figure.5D).

It was reported that ERK was an upstream regulator of Gas6-TAM pathway and activation of STAT6 could mediate Gas6 expression in macrophages^{19,23}. Here we explored the potential of adjusting Gas6 expression of both molecule. Through Western Blot results we found that phosphorylation level of STAT6 was increased, and the ERK phosphorylation level grew with time after paeoniflorin treatment, suggesting that ERK and STAT6 were activated by paeoniflorin. To further confirm the competence of regulating Gas6-TAM pathway and their regulatory relation, we then adopted ERK inhibitor, KO947, to figure it out. The phosphorylation level of STAT6 was reduced to control level, confirming that STAT6 was activated by ERK signal. And SOCS3 upregulation was about to be completely abolished by KO947. This meant that what the inflammation resolving effect paeoniflorin had reached was basically relied on ERK signal.

3.6 Recombinant Gas6 protein showed similar effect to paeoniflorin

Finally, we adopted recombinant Gas6 as a positive control to confirm the key role of Gas6-TAM pathway of paeoniflorin treatment. In MIA mice, rGas showed a competence to partly raise mechanical threshold reduced by MIA, as shown in Figure.6A. And Western Blot results of p-p38, MMP9 and SOCS3 were all regulated by rGas6, showing a trend consist with paeoniflorin treatment. In confirmatory cell experiments *in vitro*, rGas6 also upregulated p38 phosphorylation level and SOCS3 expression level, and downregulated MMP9 expression, suggesting rGas6 was able to inhibit inflammation and apoptosis. To sum up, exogenous Gas6 supply could mimic the treating effect of paeoniflorin, which further demonstrated paeoniflorin protected MIA mice from allodynia, inhibited inflammation and apoptosis through Gas6 signal and downstream TAM activation.

4. Discussion

In China, there are a few Traditional Chinese Medicine showing effectiveness in attenuating allodynia and inflammation in experimental settings²⁴. It shows that Jingshu Keli attenuates cervical spinal nerve ligation-induced allodynia in rats through inhibition of spinal microglia activation. Analgesic alkaloids derived from Traditional Chinese Medicine do well in pain management²⁵. Danggui-shaoyao-san, a traditional Chinese medicine prescription basically composed of Chinese angelica and peony, alleviates the orthodontic pain and inhibits neuronal and microglia activation²⁶.

Growing evidence indicates that macrophages play an important role in the occurrence and development of osteoarthritis. During the inflammatory phase, activated M1 macrophages can produce high levels of proinflammatory cytokines and chemokines, such as TNF- α , IL-1, and IL-6, exacerbating the inflammatory response. And it can induce the destruction of cartilage layer by producing matrix metalloproteinases (MMP-2/9). During the resolution phase of inflammation, M2-type macrophages can inhibit the inflammatory process and promote tissue repair by releasing IL-10 and TGF- β . The results of clinical sample testing indicated that the number of M1 macrophages in synovial tissue and peripheral blood increased during OA development, along with a decrease in the number of M2 macrophages²⁷. In animal models of OA, inducing normalization of synovial macrophage polarization (increase ratio of M2 /M1 macrophages) can effectively alleviate symptoms of OA²⁸. Therefore, promoting macrophage M1-M2 transformation may be a key effective strategy for the treatment of OA.

SOCS3 is an important marker of M2 macrophages. SOCS3 can mediate a classical negative feedback loop to inhibit the function of NF- κ B and MAPK signaling pathways, and inhibit the downstream inflammatory signaling pathways of a variety of proinflammatory factors such as TNF- α , IFN- γ and IL-6, achieving inflammatory inhibition and maintenance of homeostasis²⁹. Previous studies showed that paeoniflorin could promote SOCS3 expression in BV-2 cells, while inhibiting LPS-induced TNF- α and IL-1 β production³⁰. The results of cell experiments also showed that paeoniflorin could significantly attenuate

incision-induced mechanical allodynia through this mechanism, and this effect can be eliminated by small interfering RNA targeting SOCS3¹⁹. We focused on the upstream and downstream pathways of SOCS3 and the mechanism by which paeoniflorin regulates SOCS3 expression and exerts therapeutic effects on OA.

We found that Gas6-TAM may be the core upstream signaling pathway of SOCS3, and TAM receptor family, especially Axl receptor-mediated signaling may play an important role in inhibiting the development of arthritis. The results of cell experiments showed that the ankle joints of Axl knockout mice showed more severe pathological characterization of arthritis³¹. Our results showed that there was insufficient expression of Gas6-TAM pathway-related proteins in the joint tissues of mice on the 21st day after unilateral knee joint injection of MIA. There were insufficient phosphorylation levels of Axl receptors and insufficient expression levels of Gas6. Insufficient SOCS3 expression levels and upregulated MMP-9 expression levels were also detected. While continuous systemic administration of paeoniflorin can partially reverse this condition, it has been shown that activation of the Tyro3 receptor mediates signaling of synovial hyperplasia, osteoclast differentiation, and bone erosion during arthritis development³². Therefore, in the present study, we did not examine changes of the phosphorylation level of the Tyro3 receptors in a mouse model of OA.

Outcomes of our cell experiments in Raw264.7 supported our conclusions about paeoniflorin's upregulating effect of Gas6-TAM pathway and downstream SOCS3 expression *in vitro*. However, paeoniflorin failed to affect the cellular MerTK receptor phosphorylation level and the content of protein S in the cellular supernatant, so we then focused on Gas6 and Axl receptors and adopted rGas6 and Axl inhibitor for further confirmatory experiments.

A study has illustrated that TNF- α stimulating gene-6 (TSG6) or IL-4 can promote the activation of upstream transcription factor STAT6 and the expression of Gas6 through the reprogramming of macrophages. In addition, it has been demonstrated that heme oxygenase 1 (HO-1) can regulate the expression of Gas6 through the synergy of ERK and affect the resistance of myeloma cells to the chemotherapeutic drug bortezomib *in vitro*³³. Our results similarly showed that paeoniflorin could regulate the phosphorylation level of transcription factor STAT6 and the expression level of Gas6 by affecting the activity of ERK in RAW264.7 cells. Therefore, we provided direct experimental evidence that paeoniflorin regulation of Gas6 is related to the signal of transcription factors ERK-STAT6.

The activation of the p38 MAPK signaling pathway and its mediated high expression of matrix metalloproteinases is closely related to the inflammation-mediated cartilage degeneration process during OA development³⁴. Our experimental results showed that paeoniflorin could inhibit LPS-induced high expression of p-p38 and MMP-9 in macrophages, and this process could be abolished by the Axl receptor inhibitor TP-0903. At the same time, paeoniflorin significantly increased the number of M2 macrophages, decreased the number of M1 macrophages, and this effect can also be abolished by Axl receptor inhibitors. Therefore, our results further confirm that it is feasible to use paeoniflorin to upregulate GAS6-Axl-SOCS3 signaling axis, inhibit MMP-9 expression and promote M2 macrophages

polarization, leading to the needed treatment effect such as analgesia, anti-inflammatory and tissue repair.

One limitation to our study is that we did not address the reason why paeoniflorin can upregulate Protein S *in vivo* without producing the same effect on macrophages, which could indicate it having targets other than macrophages *in vivo*. Considering Protein S is mainly produced by the liver, and a large number of studies have demonstrated that paeoniflorin has a significant hepatoprotective effect³⁵, the possibility of paeoniflorin affecting the expression of Protein S through the liver-bone axis cannot be excluded. The specific mechanism needs to be explored by our further study in the future.

In conclusion, our findings suggest that insufficient activation of TAM receptors and low expression levels of SOCS3 may be implicated in the pathogenesis of OA. Paeoniflorin may alleviate OA by inhibiting MMP-9 expression through activation of the ERK-Gas6-Axl-SOCS3 axis and promotion of M2 macrophage polarization.

Abbreviations

Osteoarthritis (OA), Monosodium Iodoacetate (MIA), TAM (Tyro3, Axl, MerTK), Paeoniflorin (PF), Suppressor of cytokine signaling3 (SOCS3), Signal transducer and activator of transcription6 (STAT6), Phosphorylated extracellular regulated protein kinases (p-p38), Interleukin-1 β (IL-1 β), Matrix metalloproteinase-9 (MMP-9), Lipopolysaccharide (LPS), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) Toll-Like receptor 4 (TLR4), Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Recombinant Growth Arrest Specific Protein 6 (rGAS6), Axl kinase inhibitor (TP-0903).

Declarations

Ethics statements

Studies involving animal subjects

Generated Statement: The animal study was reviewed and approved by Nanjing Medical University Animal Care and Use Committee (NMJU-ACUC-2410064). We checked and confirmed that the design, execution and data analysis of our experiments were in accordance with ARRIVE guidelines.

Studies involving human subjects

Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare no conflicts of interest or state specific conflicts.

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Authors contribution

Chengyong Gu, Wentao Liu and Li Zhang co-designed the overall study, Changlin Sun, Xiuting Qi, Xinmiao Qin and Wen Fan: Methodology, Software (Performing the experiments, analyzed the results). Changlin Sun, Xiuting Qi, Zijiao Tao and Liang Hu: Methodology (Carrying out the animal experiments and H&E staining). Changlin Sun, Xiuting Qi, Xinmiao Qin and Wen Fan: Methodology (Carrying out the western blotting analysis). Changlin Sun, Xiuting Qi, Xinmiao Qin and Wen Fan: Methodology (Helping carry out the cell cultures). Changlin Sun, Xiuting Qi, Xinmiao Qin and Lai Jin: Conceptualization, Methodology, Writing-Original Draft, Writing - Review& Editing.

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Figures

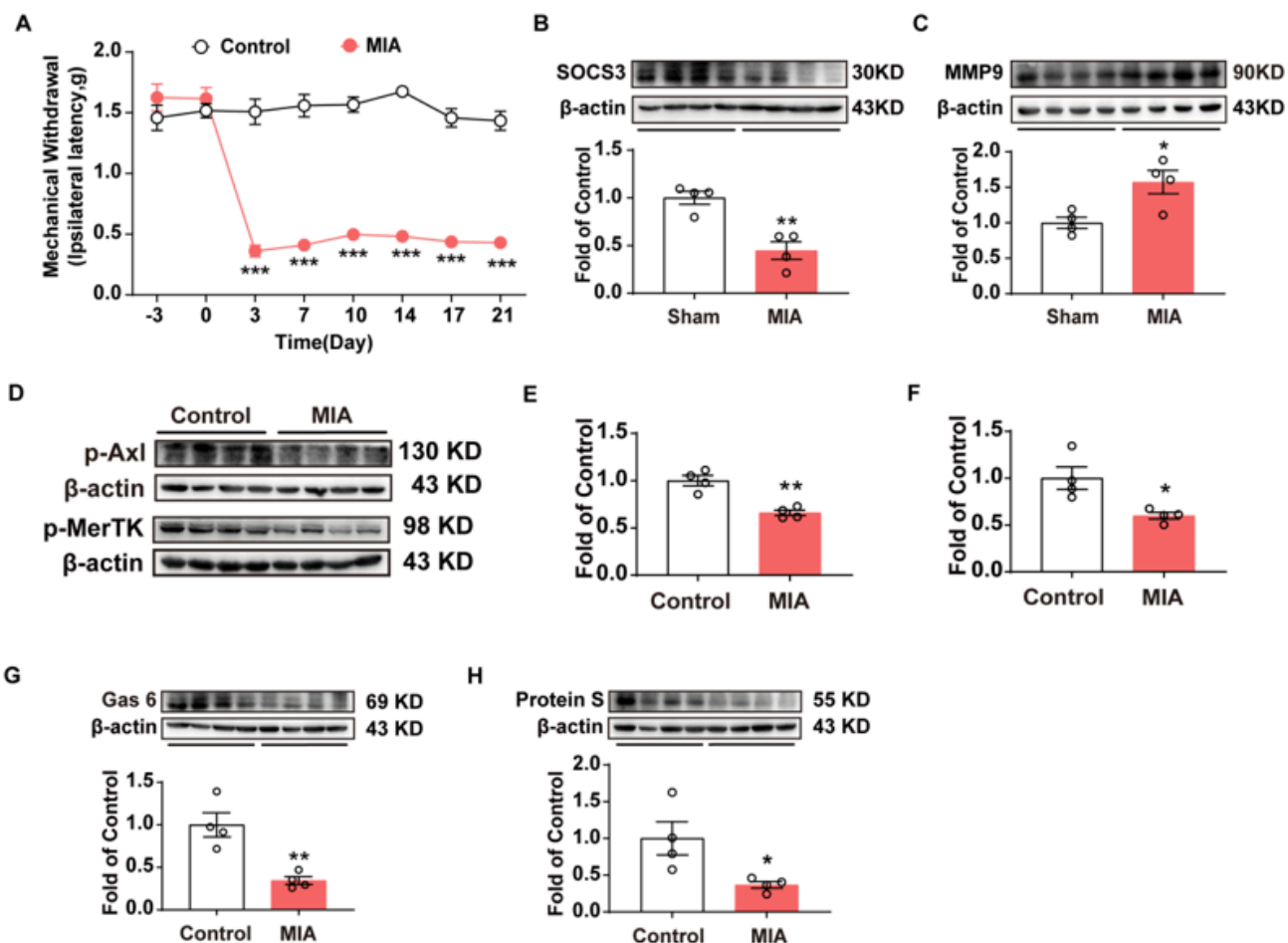


Figure 1

MIA induces the mechanical allodynia and abnormal expression of Axl, SOCS3, MMP-9 in Mice. (A) Mechanical withdrawal responses of ipsilateral hind paws were measured with Von Frey test at regular intervals up to 21d in the indicated groups of mice. (n= 6 per group). (B-C) SOCS3 and MMP-9 expression in mice knee joint were determined by western blotting on 21th day. (n= 4 per group). (D-H) p-Axl, p-

MerTK, Gas6 and Protein S expression in mice knee joint were determined by western blotting on 21th day after MIA injection. *P < 0.05, **P < 0.01, ***P < 0.001 vs Control group.

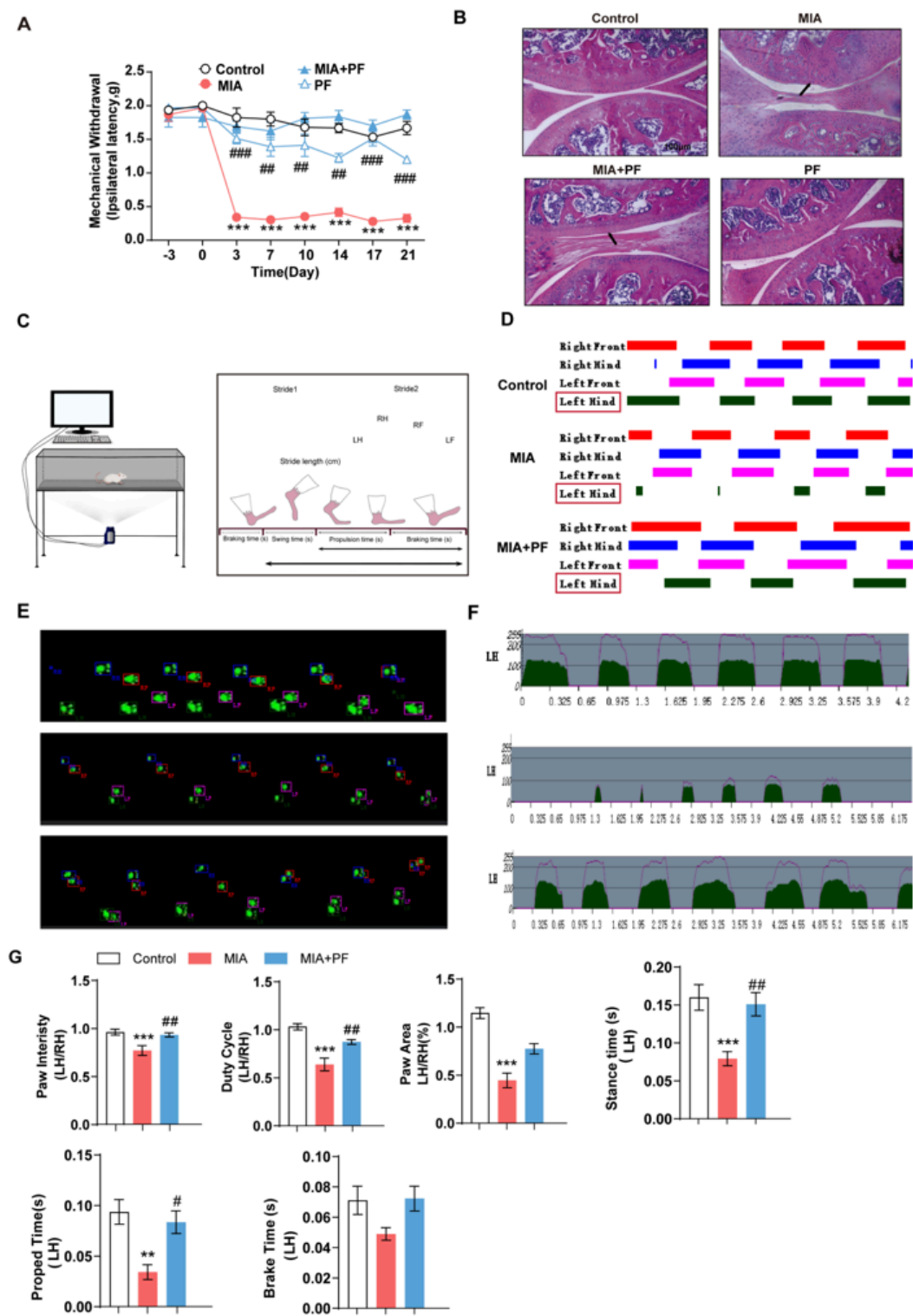


Figure 2

Paeoniflorin inhibited OA-induced mechanical allodynia and cartilage degradation in mice. Paeoniflorin (90 mg/kg, i.p.) was administrated to OA mice, mice of each group were sacrificed at 21th day. (A) Consecutive administration of paeoniflorin (90 mg/kg, i.p.) for 21 days significantly attenuated MIA-

induced mechanical allodynia in mice (n=6 per group). (B) Sections of knee joint tissues were separated and stained with H&E staining to observe the structure of articular cartilage (n= 3 per group). The photos were observed by microscope, 20×. (C) Diagrammatic drawing of Walk-Analysator system. (D-F) Representative footprint images were recorded during the treatment of Paeoniflorin. The colored bands represent standing time of each foot. (G) The paw mean intensity, duty cycle, stance time, propelled time and paw area were analyzed by WalkAnalysator system. LH, left hind. *P < 0.05, **P < 0.01 vs Control group; #P < 0.05, ##P < 0.01 vs MIA group.

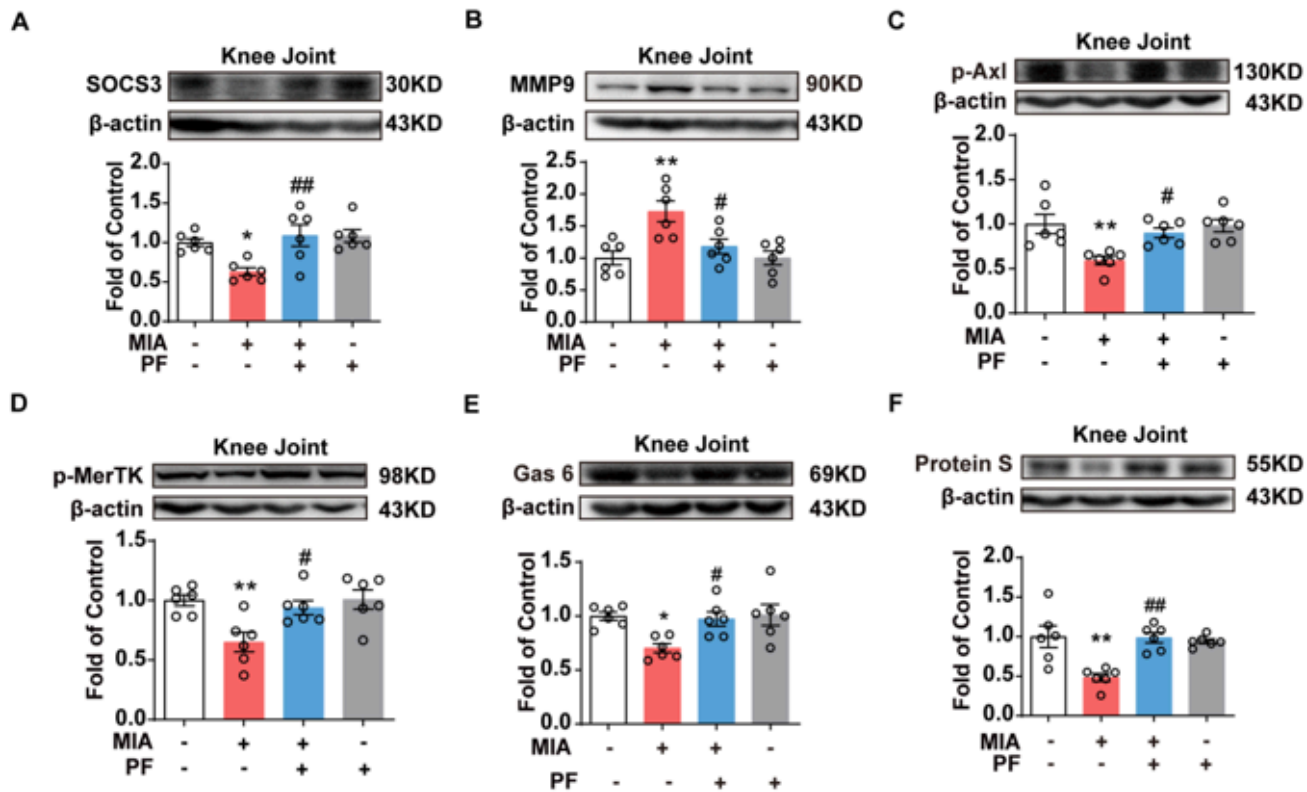


Figure 3

Paeoniflorin upregulated the activation level of Axl and MerTK receptors and reduce MMP9 expression in OA Mice. (A,B) SOCS3 and MMP-9 expression in mice knee joint were determined by western blotting on 21th day. (n= 6 per group). (C-F) Consecutive administration of paeoniflorin (90 mg/kg, i.p.) for 21 days significantly upregulated expression of p-Axl, p-MerTK, Gas6 and Protein S. (n= 6 per group). *P < 0.05, **P < 0.01, ***P < 0.001 vs Control group, #P < 0.05, ##P < 0.01, ###P < 0.001 vs PF group.

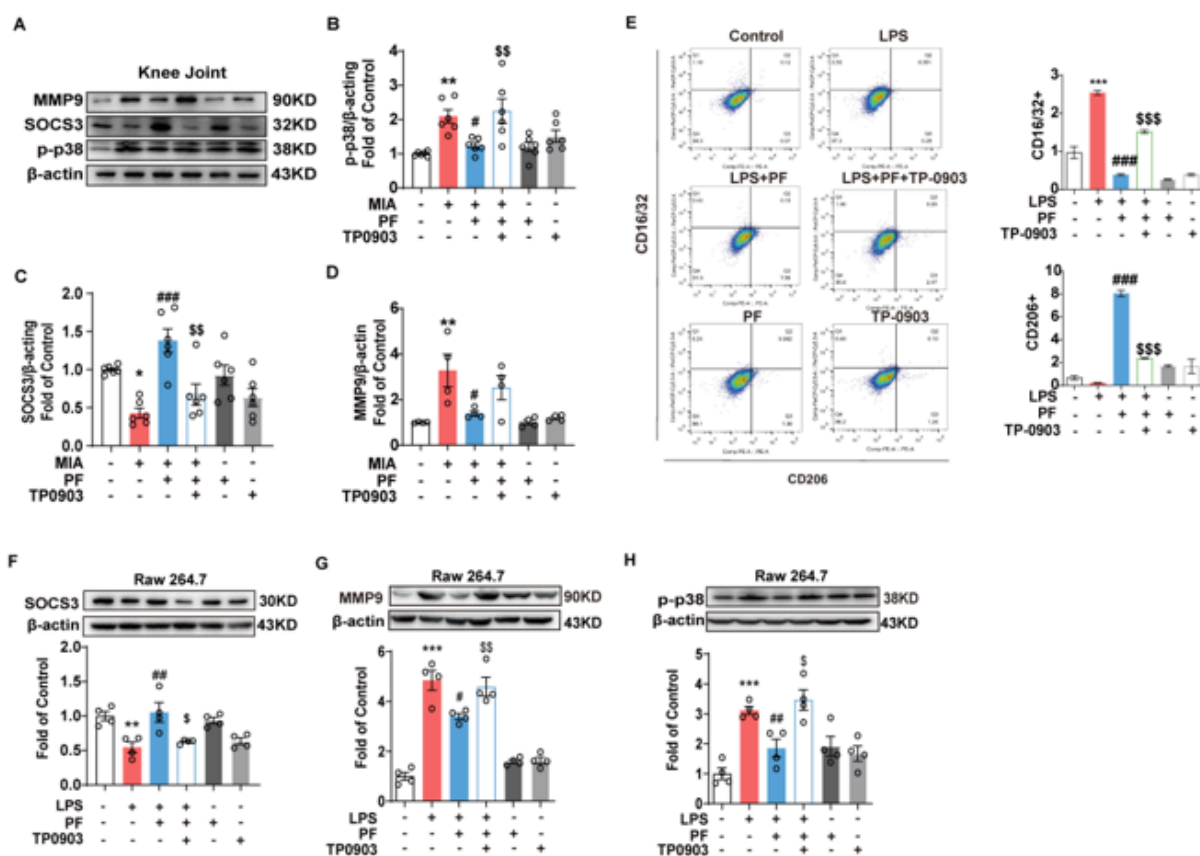


Figure 4

Paeoniflorin modulated macrophage inflammation and polarization in an Axl dependent manner. (A-D) Axl receptor inhibitor TP-0903(25 mg/kg) was injected intra-articular once every three days, MMP-9, SOCS3 and p-p38 expression in mice knee joint were determined by western blotting on 21th day. (E) Raw264.7 was cultured *in vitro*, TP-0903 (100 nM) was pre-administrated for 4 h, paeoniflorin (10^{-5} M) was pre-administrated for 4 h and then LPS (10 μ g/mL) was administrated for 20 h, the polarization of RAW264.7 cells was evaluated by flow cytometry. CD16/32: M1 macrophages, CD206: M2 macrophages. (F-H) The expression of MMP-9, SOCS3 and p-p38 were determined by western blotting. . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs Control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs MIA group; \$ $P < 0.05$, \$\$ $P < 0.01$ vs MIA+PF group.

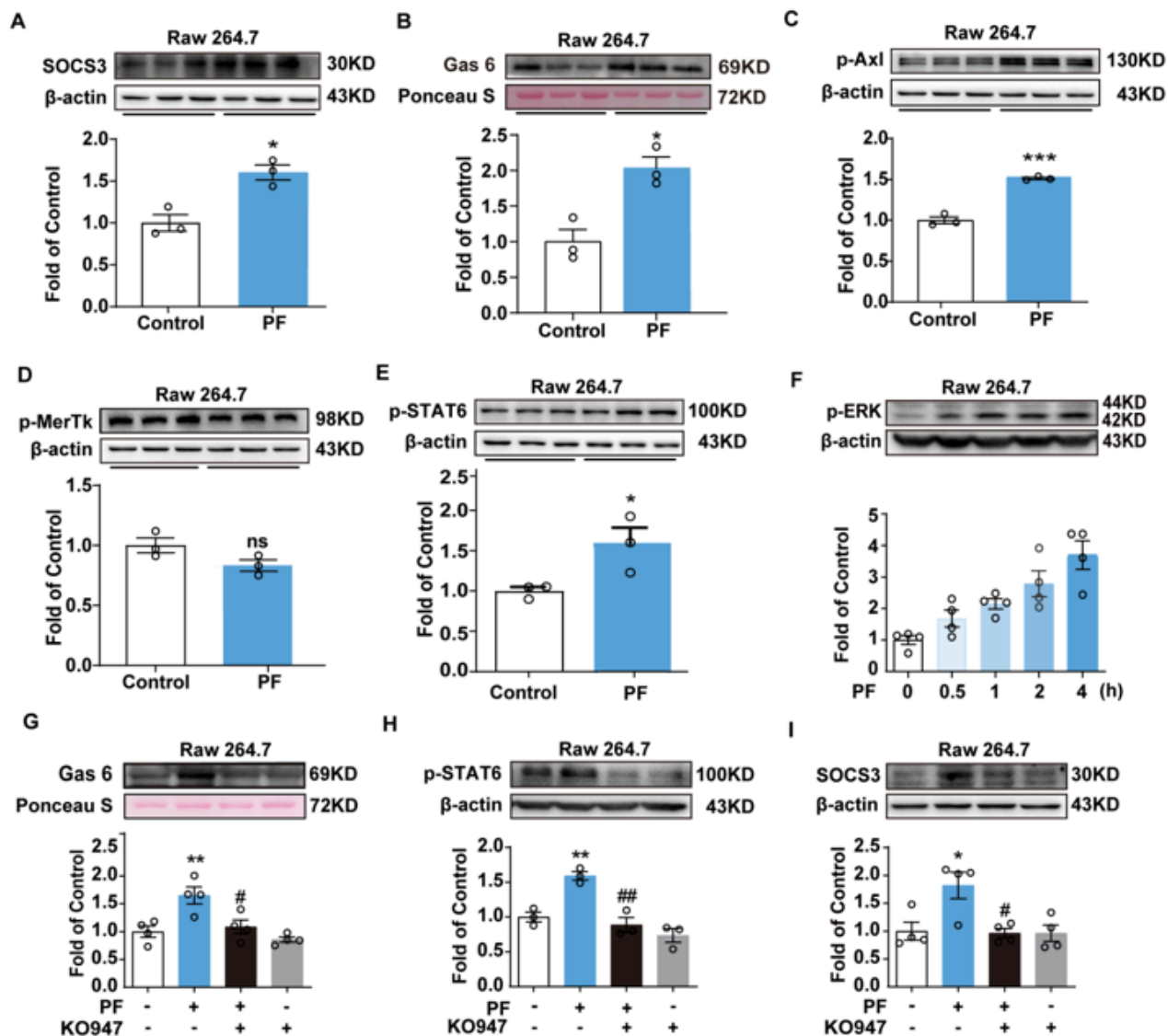


Figure 5

Paeoniflorin upregulated Gas6-Axl pathway through ERK1/2-STAT6 signal. (A) Paeoniflorin elevated SOCS3 expression in RAW264.7 cells. (B) Paeoniflorin elevated Gas6 level in Cell supernatant in RAW264.7 cells. (C) Paeoniflorin elevated phosphorylation level of Axl in RAW264.7 cells. (D) Paeoniflorin failed to elevate phosphorylation level of MerTK in RAW264.7 cells. (E) Paeoniflorin elevated phosphorylation level of STAT6 in RAW264.7 cells. (F) RAW264.7 cells were treated with paeoniflorin (10^{-5} M) for 0h, 0.5h, 1h, 2h, 4h, 6h, 8h, the phosphorylation of ERK was determined by western blotting. (G-I) KO-947 (10 nM) was pre-administrated for 4 h and then paeoniflorin (10^{-5} M) was administrated for 4 h, the level of Gas6, p-STAT6 and SOCS3 in cell supernatant was determined by western blotting. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs Control group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs PF group.

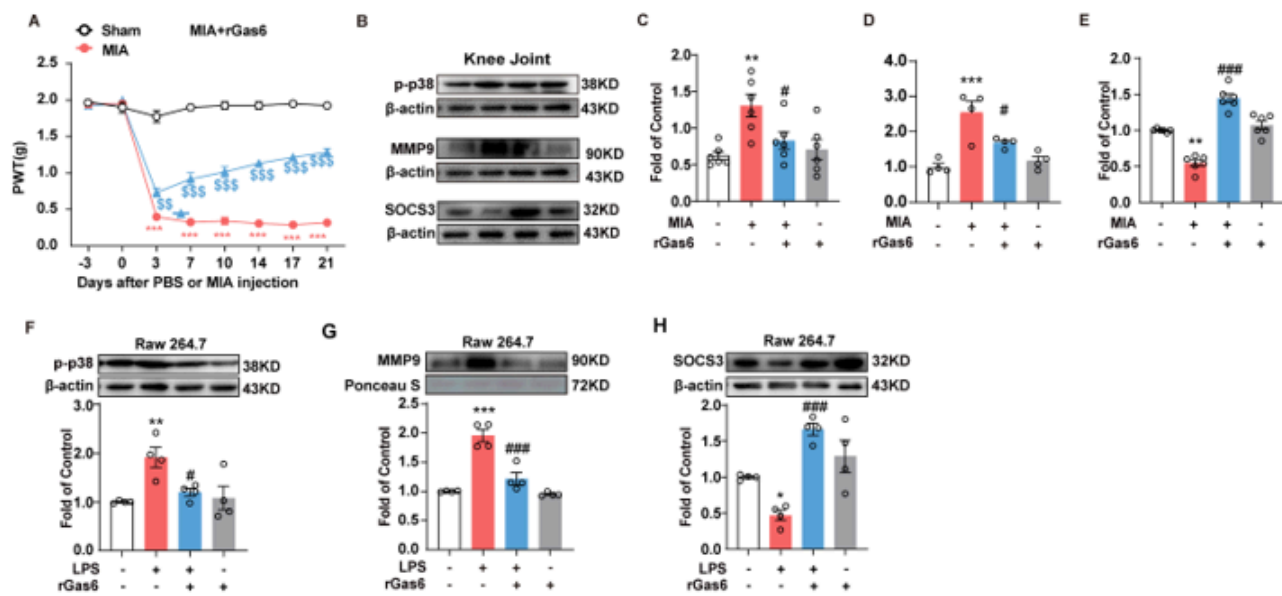


Figure 6

Recombinant Gas6 protein showed similar effect to paeoniflorin. (A) rGas6 (50 µg/kg) was injected intra-articular once every three days, the paw withdrawal threshold (pwt/g) was evaluated with Von Frey test (n=8). Mice (n=8) were treated with rGas6 (50 µg/kg, intra-articular-injection) 6h after the injection of MIA (0.1 mg/10 µl). (B-E) p-p38, MMP9 and SOCS3 expression in mice knee joint were determined by western blotting on 21th day. (F-H) rGas6 (400 ng/ml) was pre-administrated for 1h and then LPS (10 ug/ml) was administrated for 20h, the expression of p-p38, MMP-9 and SOCS3 were determined by western blotting. $P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs Control group; $\#P < 0.05$, $##P < 0.01$, $###P < 0.001$ vs MIA group.

Supplementary Files

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