RHEUMATOLOGY

Rheumatology 2017;56:1417–1427
doi:10.1093/rheumatology/kex142
Advance Access publication 18 April 2017

Original article

Leonurine attenuates fibroblast-like synoviocyte-mediated synovial inflammation and joint destruction in rheumatoid arthritis

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Abstract

Objective. To explore the role of leonurine in the regulation of synovial inflammation and joint destruction inRA.

Methods. Fibroblast-like synoviocytes were isolated from synovial tissue from RA patients. Pro-inflammatory cytokine and MMP expression was evaluated using real-time PCR and a cytometric bead array. Cell migration and invasion *in vitro* were measured using the Boyden chamber method and the scratch assay, respectively. Protein expression was measured by western blotting. Nuclear factor kappa B (NF-κB) nuclear translocation was detected by immunofluorescence. The *in vivo* effect of leonurine was evaluated in mice with CIA.

Results. Leonurine treatment significantly decreased the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNFα) and MMPs (MMP-1 and MMP-3) and suppressed the migration and invasion of RA fibroblast-like synoviocytes. The molecular analysis revealed that leonurine impaired TNFα-induced NF-κB signalling by inhibiting the phosphorylation and degradation of inhibitor of NF-κB alpha (IκBα) and subsequently preventing the nuclear translocation of the NF-κB p65 subunit. Leonurine also inhibited the p38 and Jun N-terminal kinase mitogen-activated protein kinases signalling pathways without affecting ERK signalling. Intraperitoneal injection of leonurine reduced synovial inflammation, joint destruction and the serum IL-1β, IL-6 and TNFα levels in mice with CIA.

Conclusion. Our findings show that leonurine reduces synovial inflammation and joint destruction in RA through the NF-κB and mitogen-activated protein kinases pathways. Leonurine has potential as a therapeutic agent for RA.

Key words: leonurine, rheumatoid arthritis, inflammation, fibroblast-like synoviocytes, immune disease

BASIC SCIENCE

Rheumatology key messages

- Leonurine contributes to fibroblast-like synoviocyte-mediated synovial inflammation in RA patients.
- Leonurine suppresses the activation of inflammatory pathways in RA fibroblast-like synoviocytes.
- Leonurine may represent a promising strategy to control RA severity.

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Submitted 27 August 2016; revised version accepted 3 March 2017

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Introduction

RA is a common chronic inflammatory joint disease that is characterized by synovial hyperplasia and erosion of bone and cartilage [1]. Increasing evidence indicates that activated RA fibroblast-like synoviocytes (FLSs) exhibit tumour-like characteristics [2, 3] and play a critical role in the development of the pannus by migrating into cartilage and bone [4–7]. Targeting proliferative fibroblasts could

facilitate the regeneration of synovial joints [8]. RA FLSs also regulate the secretion of inflammatory mediators and expression of MMP, which cause damage to cartilage and bone [9, 10]. Regulation of activated FLS-mediated inflammation and invasion may be a novel therapeutic strategy to target the destructive progress of RA.

Leonurine is an extract separated from *Leonurus cardiaca*, which is a traditional herbal medicine widely used in Asian countries, particularly India and China [11]. *L. cardiaca* shows therapeutic benefits for the treatment of internal disorders, such as cardiovascular disorders, amenorrhoea and skin inflammation [12]. Recent studies have shown the inhibitory effects of leonurine on inflammation and immunomodulation. Leonurine downregulated the pro-inflammatory cytokine levels in an LPS-induced mouse mastitis model by regulating the mitogen-activated protein kinases (MAPKs) [13]. Leonurine also reduced LPS-induced inflammatory responses in human umbilical vein endothelial cells via inhibition of reactive oxygen species and the nuclear factor kappa B (NF-κB) signalling pathway [14, 15]. However, the therapeutic benefits of leonurine in RA are unknown. Therefore, in the present study, we evaluated the role of leonurine in the regulation of synovial inflammation and joint destruction in RA and its underlying mechanisms. Our data showed that leonurine decreased pro-inflammatory cytokine and MMP production and the migration and invasion of RA FLSs by regulating the MAPK and NF-κB pathways. We also demonstrated that leonurine attenuated the severity of arthritis in mice with CIA. These results indicate that leonurine may possess therapeutic potential for RA.

Methods

Reagents and antibodies

Recombinant human TNFα was obtained from R&D Systems (Minneapolis, MN, USA). DMEM/F12, fetal bovine serum (FBS), antibiotics, PBS, trypsin-EDTA and other standard cell culture products were purchased from Invitrogen (Carlsbad, CA, USA). Leonurine and an anti-β-actin antibody were obtained from Sigma-Aldrich (St Louis, MO, USA). Anti-p38, anti-phospho-p38, anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK, anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK, anti-NF-κB, anti-phospho-NF-κB, anti-inhibitor of NF-κB alpha (IκBα), anti-phospho-IκBα, anti-inhibitor of nuclear factor kappa B kinase (IKK) and anti-phospho-IKK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). IL-1β, IL-6, IL-8, TNFα, MMP-1 and MMP-3 multiplex bead arrays were purchased from Bio-Rad (Hercules, CA, USA). SB203580, SP600125, pyrrolidinedithiocarbamate (PDTC) were purchased from Selleck (Houston, TX, USA).

Patients and controls

Twelve patients with RA (10 women and 2 men, aged 42–63 years) who had undergone synovectomy or joint replacement were enrolled in this study. RA was diagnosed according to the 1987 revised criteria of the ACR [16]. This study was approved by the Medical Ethical Committee of the First Affiliated Hospital, Guangzhou University of Chinese Medicine and was performed according to the recommendations of the Declaration of Helsinki. All patients gave informed consent before participating in the study.

Cell culture

Synovial tissues were cut into small pieces and digested with collagenase in DMEM/F12 medium for 2 h at 37 °C to isolate synoviocytes. The synoviocytes were cultured in DMEM/F12 medium containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in an incubator with 5% CO_2. These cells were used for all experiments. The cells were trypsinized at confluence and used between passages 3 and 5.

Cytokine assay

Total RNA was extracted using the TRIzol reagent (Invitrogen). The RNA was reverse-transcribed with the TaKaRa PrimeScript RT reagent kit according to the manufacturer's protocol (TaKaRa Bio, Shiga, Japan). Quantitative real-time PCR was performed using the Bio-Rad CFX96 system with the SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The instrument was programmed with the following PCR conditions: 40 cycles of a 5 s denaturation at 95 °C and a 34 s amplification at 60 °C. All reactions were run in triplicate and were normalized to the housekeeping gene *GAPDH*. The RT-PCR primers used in this study are listed in supplementary Table S1, available at *Rheumatology* Online.

The cytokine concentrations in the supernatant samples were quantitatively determined with the Human cytometric bead array (CBA) kit according to the instructions of the BD™ CBA Flex Set System (BD Biosciences, San Jose, CA, USA). Each BD CBA Flex Set contained one bead population with a distinct fluorescence intensity as well as the appropriate phycoerythrin detection reagent and standard. The tests were performed according to the manufacturer's recommendations, and samples were run in duplicate. The MMP (MMP-1 and MMP-3) concentrations in the supernatants were detected by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Cytotoxicity assay

RA FLSs were pre-treated for 48 h with leonurine at different concentrations (0, 5, 10 or 20 μM). The culture supernatants were removed, and the adherent cells were incubated for 30 min at 37 °C with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) salt solution (1 mg/ml in PBS). The dark blue formazan crystals were dissolved in acidified isopropanol, and formazan quantification was performed at a test wavelength of 570 nm and a reference wavelength of 620 nm.

FLS proliferation assays

RA FLSs were cultured for 24 h at a density of 1×10^4 cells/well in 96-well plates in serum-free medium. After serum starvation, the cells were incubated with TNFα (10 ng/ml) for 72 h and then with 10 mM 5-bromo-2′-deoxyuridine (BrdU) for 1 h. BrdU incorporation was assessed in triplicate using a cell proliferation ELISA kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

FLS apoptosis assays

RA FLSs were pre-treated with leonurine at different concentrations (0, 5, 10 or 20 μM) for 48 h. Then, the apoptosis of RA FLSs was analysed by staining the cells with annexin V–FITC and propidium iodide according to the manufacturer's protocol (BD Biosciences).

Wound migration

RA FLSs were plated to confluence in 35-mm culture dishes, wounded with pipette tips and then treated with various concentrations of leonurine (0, 5, 10 or 20 μM). After 48 h of incubation, migration was quantified by counting the cells that had moved beyond the reference line.

In vitro migration and invasion assays

The FLS chemotaxis assay was performed using the Boyden chamber method and a 6.5-mm-diameter filter with an 8.0-mm pore size (Corning Inc., Corning, NY, USA). The RA FLSs were pre-treated with leonurine at various concentrations (0, 5, 10 or 20 μM) for 24 h and then seeded into a Boyden chamber at a final concentration of 6×10^4 cells/ml. Serum-free DMEM/F12 was added to the upper chambers, and DMEM/F12 containing 10% FBS was added as a chemoattractant in the lower wells. The chamber was incubated at 37 °C in the presence of 5% CO_2 for 24 h. After incubation, the non-migrating cells were removed from the upper surface of the filter using a cotton swab. The filters were fixed in methanol for 15 min and stained with 0.1% crystal violet for 15 min. Chemotaxis was quantified by counting the stained cells that migrated to the lower side of the filter using an optical microscope. The stained cells were counted as the mean number of cells per 10 random fields for each assay. For the *in vitro* invasion assay, similar experiments were performed using inserts coated with a Matrigel basement membrane matrix (BD Biosciences).

IF staining

RA FLSs were grown on glass coverslips to 90% confluence. A sterilized pipette tip was used to generate wounds across the cell monolayer, which was washed three times with starvation medium to remove the detached cells. RA FLSs were pre-treated with 1% (v/v) dimethyl sulfoxide (DMSO) or leonurine (20 μM) for 24 h, stimulated with or without TNFα for 30 min, fixed with 4% paraformaldehyde and then permeated with 0.1% Triton X-100 in PBS. The cells were incubated with an anti-p65 NF-κB antibody (diluted 1:200) for 1 h at room temperature and then with an FITC-conjugated secondary Ab (Santa Cruz Biotechnology, Dallas, TX, USA). To detect the pseudopodia organization, the cells were incubated with Alexa Fluor-546 rhodamine-phalloidin (Invitrogen, Eugene, OR, USA), and the nuclei were visualized using 0.25 mg/ml 4′,6-diamidino-2-phenylindole. The coverslips were mounted onto glass slides with anti-fade mounting medium and examined using fluorescence microscopy.

Western blotting analysis

For each experiment, a total of 5×10^5 cells were seeded; when subconfluence (70%) was reached, the cells were made quiescent for 24 h in DMEM/F12 medium containing 0.5% FBS and then treated with various agents. To detect the expression of signal proteins, we rinsed the cells twice with ice-cold PBS and added 0.5 ml of ice-cold lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 100 mg/ml phenylmethylsulfonyl fluoride, 0.1% SDS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 10 mg/ml aprotinin, 2 mg/ml leupeptin and 10 mM EDTA]. The cells were incubated for 20 min on ice and then scraped and centrifuged. The protein concentrations were measured using the Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of protein were solubilized in Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.00625% bromophenol blue), boiled for 5 min, separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary Abs as indicated in Tris-buffered saline–Tween 20 containing 5% non-fat milk at 4 °C overnight. The membranes were incubated with the appropriate secondary Abs for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ, USA). Each blot was representative of at least three similar independent experiments.

Determination of the effect of leonurine on CIA

CIA was induced in DBA1 mice by intradermal injection of 200 mg of bovine type II collagen emulsified at a 1:1 ratio (v/v) in Freund's complete adjuvant. The mice were boosted 3 weeks later using bovine type II collagen emulsified at a 1:1 ratio (v/v) in incomplete Freund's adjuvant. Two groups (n = 8 mice each) were treated with once-daily intraperitoneal injections of either DMSO (vehicle) or leonurine (30 mg/kg/day) for 14 days beginning on the day of arthritis onset. The arthritis severity was graded on a scale of 0–4 for each paw in a blinded fashion as follows: 0, no change; 1, significant swelling and redness of one digit; 2, mild swelling and erythema of the limb or swelling of more than two digits; 3, marked swelling and erythema of the limb; and 4, maximal swelling and redness of the limb followed by ankylosis. The total score was recorded as the sum of the scores in the four limbs. After 14 days of treatment, the hind limbs were removed and fixed in 10% neutral buffered formalin. The tissue was decalcified in 8% formic acid and embedded in paraffin. The sections were stained with haematoxylin and eosin. An inflammation, hyperplasia, bone damage score was obtained using the following scoring system: 0, normal; 1, mild; 3, moderate; and 4, severe.

To determine the toxic effect of leonurine on mice with CIA, a colorimetric enzymatic method was used for the biochemical analysis of serum samples. The enzymatic activities of aspartate aminotransferase and alanine aminotransferase were detected to investigate liver function alterations. Serum creatinine and blood urea nitrogen (BUN) were detected as renal parameters. These parameters were analysed using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Measurement of serum cytokine production in mice

Mouse plasma was obtained by centrifugation of blood in serum separator Microtainer tubes after cardiac puncture. The serum levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) in the mice were examined by ELISA according to the manufacturer's instructions (R&D Systems).

Statistical analysis

The data are expressed as the mean (S.E.M.). Analysis of variance with the Student–Newman–Keuls test was used to evaluate differences between the experimental groups. The histological results were analysed using the Kruskal–Wallis non-parametric test. $P < 0.05$ was considered significant.

Results

Inhibitory effect of leonurine on the TNFα-induced pro-inflammatory cytokine and MMP production by RA FLSs

Pro-inflammatory cytokines and MMPs play important roles in synovial inflammation and joint destruction. As shown in Fig. 1A and B, the IL-1β, IL-6, IL-8, TNFα, MMP-1 and MMP-3 mRNA and protein expression levels in TNFα-induced RA FLSs were decreased by leonurine treatment at concentrations ranging from 5 to 20 μM.

Effects of leonurine on RA FLS viability

The MTT assay was performed to evaluate the toxic effects of leonurine on RA FLSs. As shown in Fig. 1C, the RA FLSs were incubated with varying concentrations (0, 5, 10 or 20 μM) of leonurine for 48 h. Based on these results, leonurine did not affect cell viability, even at concentrations as high as 20 μM.

Effects of leonurine on the proliferation and apoptosis of RA FLSs

A previous report indicated that leonurine participated in the regulation of tumour cell proliferation and apoptosis [17]. Therefore, we examined the role of leonurine in the proliferation and apoptosis of RA FLSs. Our results showed that leonurine was not involved in the proliferation or apoptosis of RA FLSs (Fig. 1D and E).

Inhibitory effect of leonurine on the migration and invasion of RA FLSs

To determine the role of leonurine in RA FLS migration, we examined the chemotactic migration of FLSs using a Boyden chamber assay. As shown in Fig. 2A, leonurine treatment markedly suppressed the migratory capacity of RA FLSs in a concentration-dependent manner. To evaluate the effect of leonurine on FLS migration, we used a monolayer wound scratch assay. Consistent with previous results, leonurine treatment resulted in a significant reduction in cell migration (Fig. 2B).

The ability to invade cartilage is a critical behaviour in RA pathogenesis. The *in vitro* invasion potential of RA FLSs is related to the rate of joint destruction in RA patients [18]. A thin layer of reconstituted extracellular matrix (Matrigel) was used to evaluate the role of leonurine in the invasive behaviour of RA FLSs *in vitro*. As shown in Fig. 2C, leonurine treatment caused a significant reduction in RA FLS invasiveness.

Leonurine impairs lamellipodia formation in RA FLSs

Dynamic reorganization of the actin cytoskeleton is critical for optimal cell migration. To confirm the role of leonurine in the regulation of actin reorganization in RA FLSs, we used fluorescent phalloidin staining to visualize polymerized actin in leonurine (20 μM) or 1% (v/v) DMSO-treated migrating cells shortly after wounding. As shown in Fig. 2D, the FLSs in the DMSO group displayed flat or ruffling lamellipodia at their leading edge, whereas the cells treated with leonurine exhibited significantly suppressed lamellipodia formation.

Inhibitory effect of leonurine on NF-κB activation by TNFα-induced RA FLSs

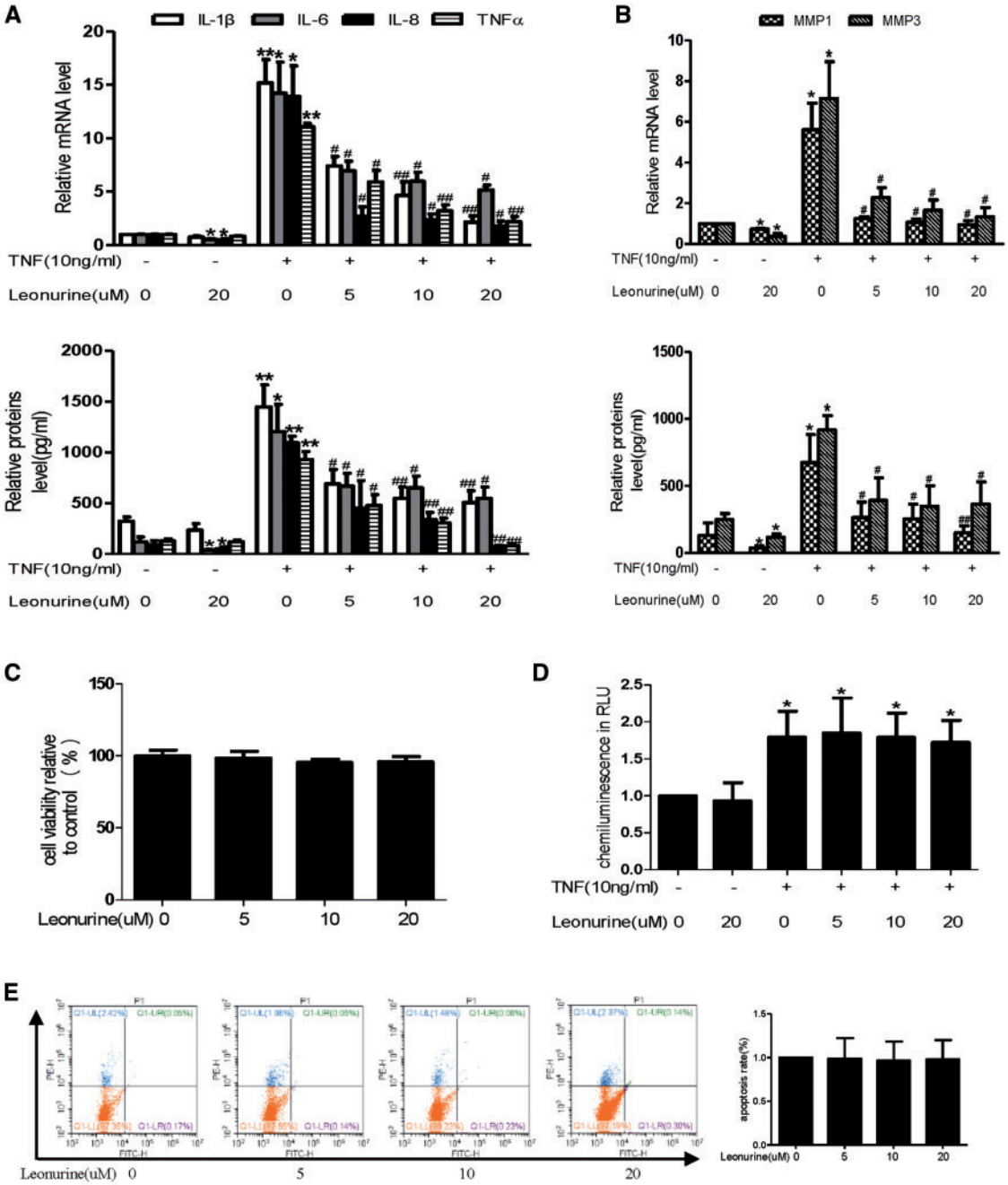
NF-κB plays a critical role in regulating the production of synovial inflammation and joint destruction. Therefore, we evaluated the effect of leonurine on NF-κB activation. We found a significant decrease in phosphorylated NF-κB and IκBα following treatment of TNFα-stimulated RA FLSs with leonurine, but leonurine treatment had no effect on the expression of phosphorylated IKK (Fig. 3A). As shown in Fig. 3B, we also performed IF staining using an anti-p65 antibody targeting a major subunit of NF-κB. We observed a significant reduction in p65 nuclear accumulation in the RA FLSs treated with leonurine.

Inhibitory effect of leonurine on the phosphorylation of MAPK pathway components in TNFα-induced RA FLSs

MAPKs also play important roles in regulating synovial inflammation and the invasion of RA FLSs. To determine the role of leonurine in the MAPK pathways, we measured the expression of candidate signalling molecules following leonurine treatment. As shown in Fig. 3C, leonurine treatment decreased the TNFα-induced phosphorylation of the phospho-JNK and p38 MAPKs but had no effect on the expression of phospho-extracellular signal regulating kinase 1/2 (ERK1/2).

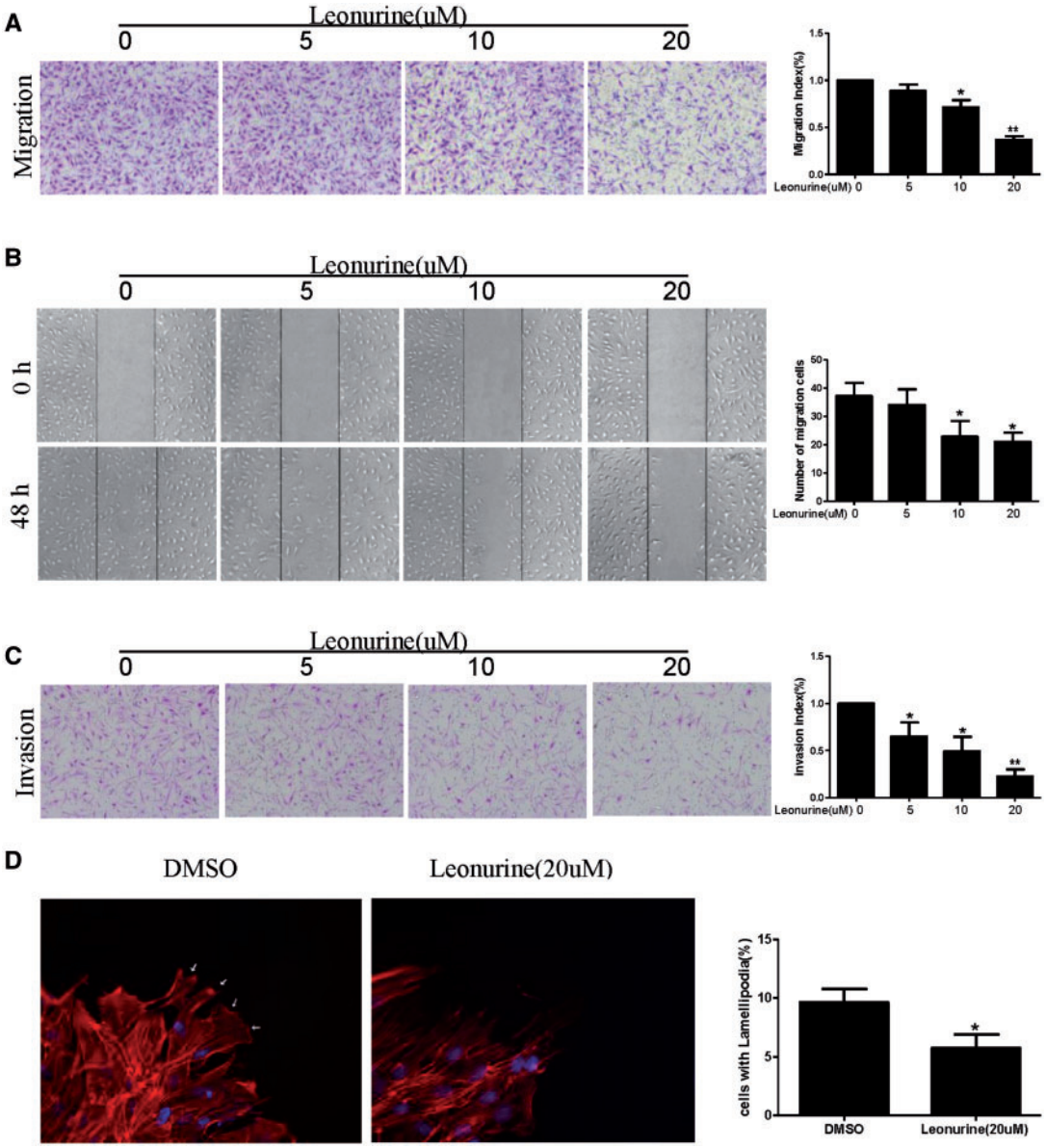
Since the MAPK pathway is considered a critical upstream factor for NF-κB activation [19, 20], we determined the effect of p38 and JNK chemical inhibitors on

Fig. 1 Effect of leonurine on the expression of pro-inflammatory cytokines and MMPs in RA fibroblast-like synoviocytes



(**A**) Effect of leonurine on the production of pro-inflammatory cytokines. The IL-1β, IL-6, IL-8 and TNFα mRNA and protein expression levels were measured by quantitative real-time PCR and cytometric bead array assay. (**B**) The MMP-1 and MMP-3 mRNA and protein expression levels were measured by quantitative real-time PCR and ELISA. (**C**) Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay in RA fibroblast-like synoviocytes (FLSs) after 48 h of leonurine treatment. (**D**) Effect of leonurine on RA FLS proliferation measured by 5-bromo-2′-deoxyuridine incorporation into the cellular DNA. (**E**) Effect of leonurine on RA FLS apoptosis evaluated by flow cytometry. The data are representative of six independent experiments [mean (S.E.M.)] from six RA patients. *$P < 0.05$ and **$P < 0.01$ *vs* dimethyl sulfoxide (control); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs* treatment with TNFα. RLU: relative light unit.

FIG. 2 Effect of leonurine on RA fibroblast-like synoviocyte migration and invasion



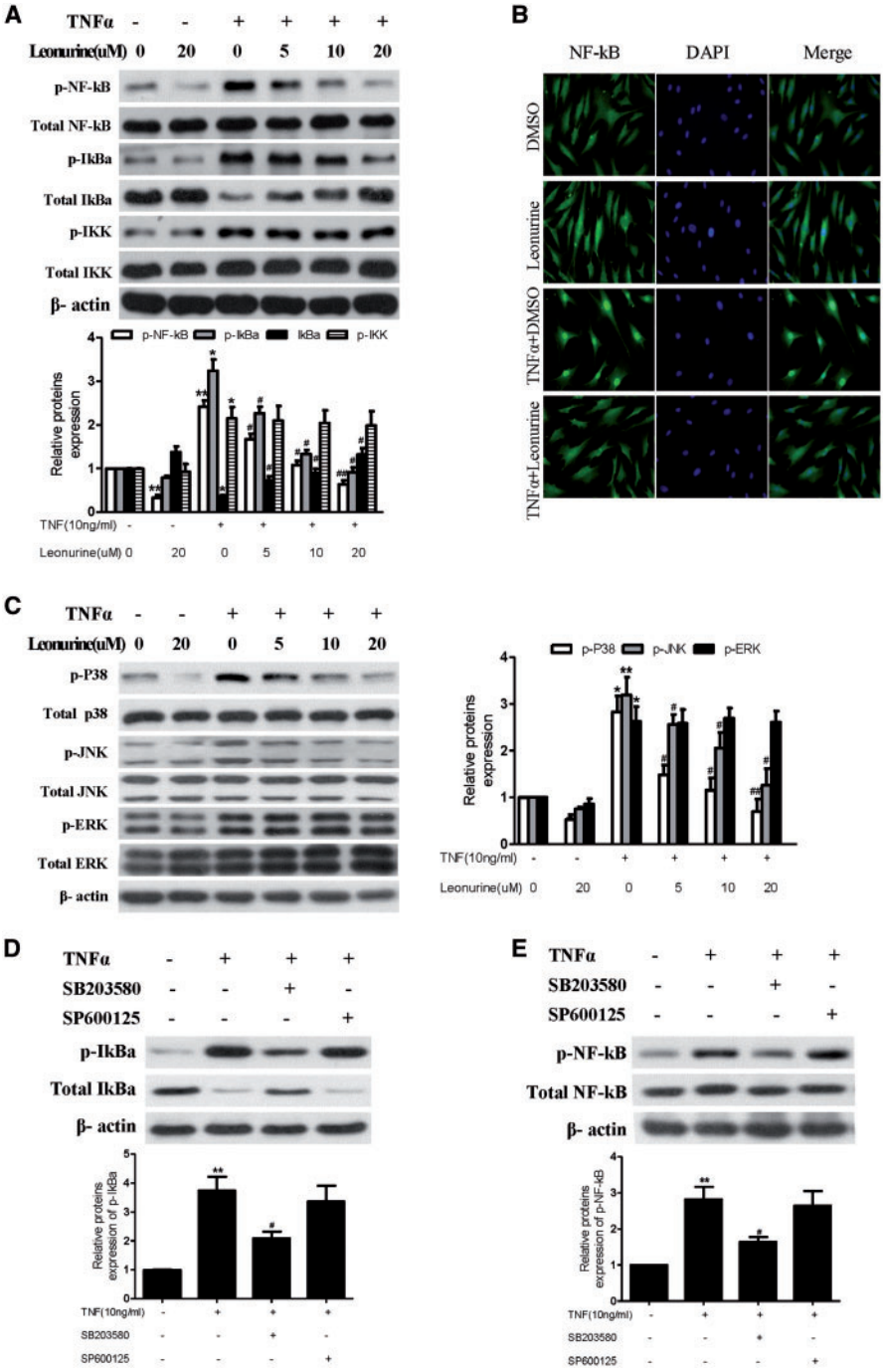
(**A**) Effect of leonurine on RA fibroblast-like synoviocyte (FLS) migration was evaluated in a Boyden chamber, and chemotaxis was quantified by counting the migration index (100x). (**B**) Effect of leonurine on the wound migration of RA FLSs (50x). (**C**) Effect of leonurine on RA FLSs invasion was performed using inserts coated with a Matrigel basement membrane matrix in Boyden chambers, and chemotaxis was quantified by counting the invasion index (100x). (**D**) Effect of leonurine on RA FLS lamellipodia formation. Cells were fixed and stained with fluorescent phalloidin after treated with dimethyl sulfoxide or leonurine for 24 h and imaged using fluorescence microscopy (200x). Arrows indicate lamellipodia formation. The data are representative of independent experiments [mean (S.E.M.)] from six RA patients. *$P < 0.05$ and **$P < 0.01$ *vs* dimethyl sulfoxide (control).

NF-κB activation to evaluate whether p38 or JNK mediated the leonurine-induced inactivation of NF-κB. The p38 chemical inhibitor decreased activation of the NF-κB pathway, whereas the JNK chemical inhibitor did not affect NF-κB activation (Fig. 3D and E). These data suggest that p38 mediates TNFα-induced NF-κB activation in RA FLSs.

Effect of p38, JNK and NF-κB chemical inhibitors on pro-inflammatory cytokine and MMP expression and the migration and invasion of RA FLSs

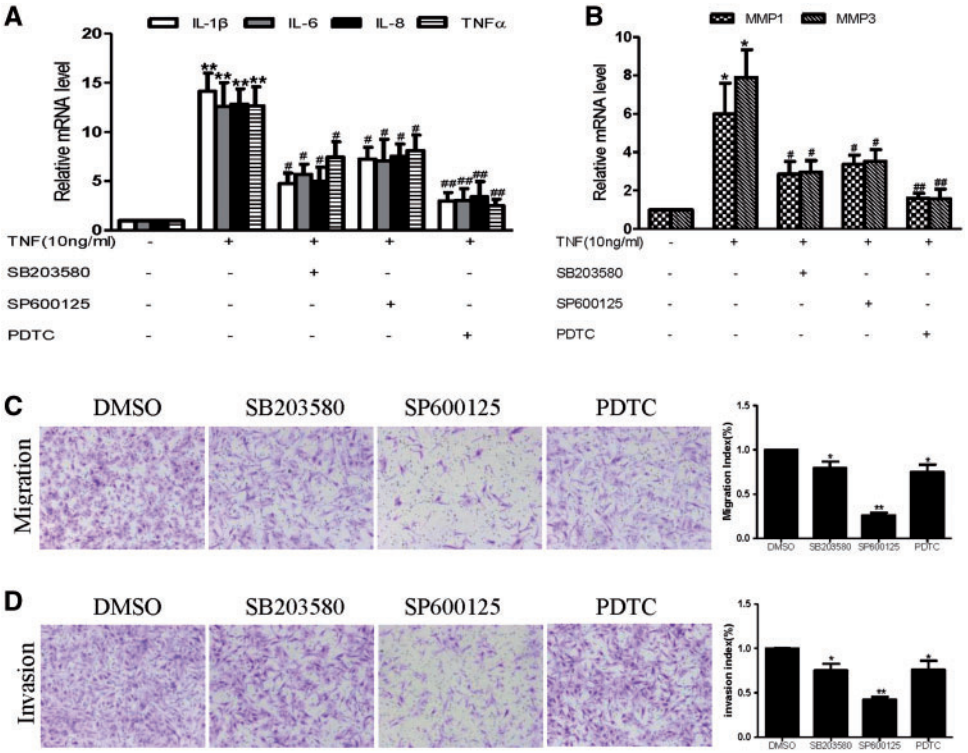
We demonstrated that treatment with chemical inhibitors of p38, JNK and NF-κB reduced the TNFα-induced increase in IL-1β, IL-6, IL-8, TNFα, MMP-1 and MMP-3 expression in RA FLSs (Fig. 4A and B). We also observed an

FIG. 3 Effect of leonurine on the TNFα-induced nuclear factor kappa B and mitogen-activated protein kinase activation in RA fibroblast-like synoviocytes



(**A**) Effect of leonurine on the TNFα-induced p65, inhibitor of nuclear factor kappa B alpha (IκBα) and inhibitor of nuclear factor kappa B kinase (IKK) phosphorylation. (**B**) Effect of leonurine on TNFα-induced nuclear translocation of p65 (green stain) with nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI; blue stain) (200x). (**C**) Effect of leonurine on TNFα-induced mitogen-activated protein kinase phosphorylation. ERK, extracellular signal-regulated kinase. (**D** and **E**) Effect of the p38 or c-Jun N-terminal kinase (JNK) chemical inhibitor on nuclear factor kappa B (NF-κB) activation. IκBa (**D**) and NF-κB p65 (**E**) phosphorylation was assessed by western blotting analysis after treatment with the p38 chemical inhibitor SB203580 or JNK chemical inhibitor SP600125. The data are representative of independent experiments [mean (S.E.M.)] from eight RA patients. *P $<$ 0.05 and **P $<$ 0.01 *vs* dimethyl sulfoxide (control); $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs* treatment with TNFα alone.

FIG. 4 Pathway inhibitors effect pro-inflammatory cytokine and MMPs expression, migration and invasion of RA fibroblast-like synoviocytes



(**A**) Effect of the p38, c-Jun N-terminal kinase (JNK) or nuclear factor kappa B (NF-κB) inhibitor on pro-inflammatory cytokine mRNA expression. The IL-1β, IL-6, IL-8 and TNFα mRNA expression levels were measured by quantitative real-time PCR after treated with the p38 inhibitor SB203580, JNK inhibitor SP600125 or NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). (**B**) Effect of the p38, JNK or NF-κB inhibitor on the MMP-1 and MMP-3 mRNA expression. (**C**) Migration was evaluated using a Boyden chamber migration assay (100x). (**D**) Invasions were performed using inserts coated with a Matrigel basement membrane matrix in Boyden chambers (100x). The data are representative of independent experiments [mean (S.E.M.)] from six RA patients. *$P < 0.05$ and **$P < 0.01$ *vs* dimethyl sulfoxide (control); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs* treatment with TNFα.

inhibitory effect of these chemical inhibitors on the migration and invasion of RA FLSs (Fig. 4C and D).

Attenuation of the severity of arthritis in mice with CIA via leonurine administration

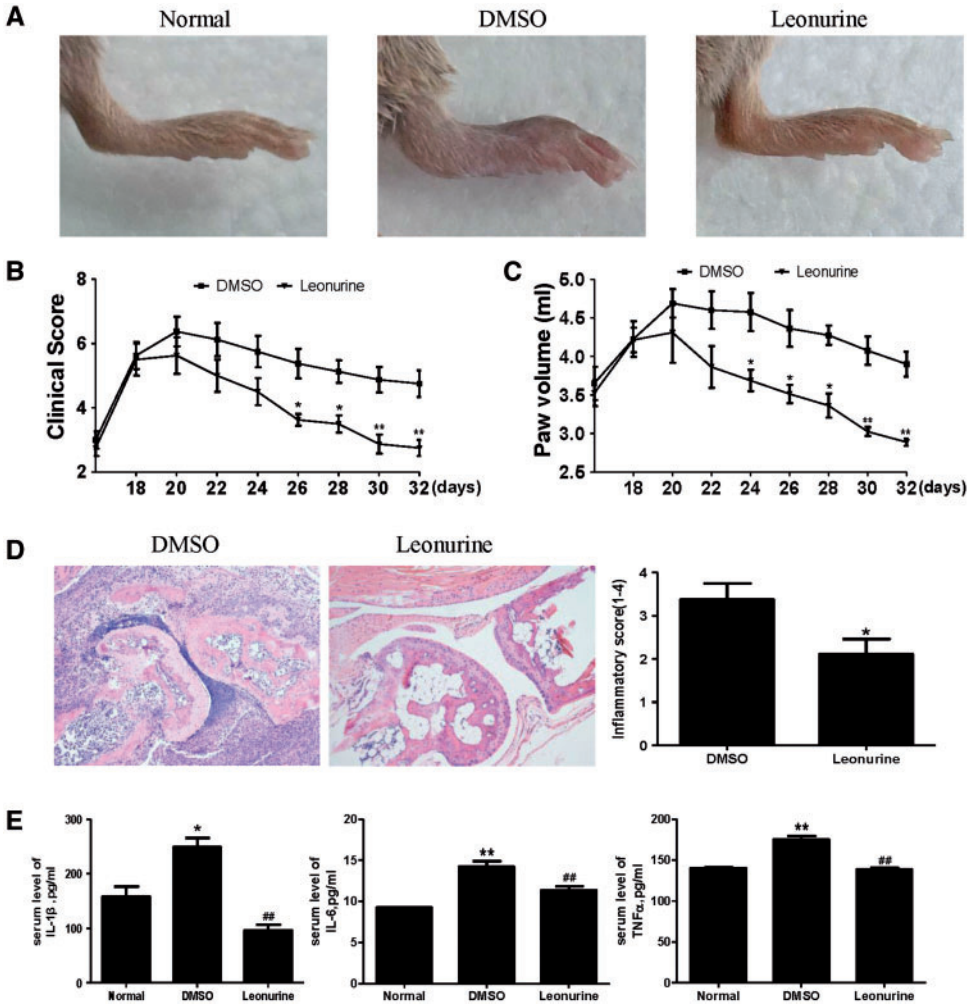
Given these observations in cultured RA FLSs, the *in vivo* effect of leonurine on the synovial inflammation of RA was evaluated in mice with CIA. Intraperitoneal injection of leonurine suppressed the increase in the clinical score compared with mice treated with DMSO (Fig. 5A–C). We also conducted histological examinations to evaluate the joint pathology in mice. The leonurine-treated mice showed a significant reduction in the pathological disease severity and a corresponding reduction in the macroscopic inflammation score compared with the DMSO-treated mice (Fig. 5D). Additionally, we observed a significant reduction in the serum IL-1β, IL-6 and TNFα levels in the mice with CIA treated with leonurine (Fig. 5E).

Moreover, we found no significant gain in body weight between the leonurine and DMSO groups over the course of the experiment. We found no significant changes in the liver (aspartate aminotransferase and alanine aminotransferase levels) or renal (serum creatinine and BUN levels) parameters in mice treated with leonurine (supplementary Table S2, available at *Rheumatology* Online). These data indicate that leonurine treatment is safe in CIA mice.

Discussion

In the present study, we demonstrated that leonurine treatment suppressed pro-inflammatory cytokine and MMP production and the migration and invasion of RA FLSs. Leonurine also inhibited the activation of the NF-κB, p38 and JNK MAPK pathways. Intraperitoneal administration of leonurine attenuated synovial inflammation and joint destruction in mice with CIA. Our findings suggest that leonurine has potential as a novel therapeutic agent for RA.

FIG. 5 Leonurine attenuated synovial inflammation in mice with CIA



Mice were treated with once-daily intraperitoneal injections of either dimethyl sulfoxide (DMSO; vehicle) or leonurine (30 mg/kg/day). All mice were sacrificed after either the initial DMSO injection or 14 days after the leonurine injection. (**A**–**C**) Effects of leonurine on the articular index and (**B**) paw swelling (change in volume) (**C**) in the CIA mice (100x). (**D**) Histological findings. The specimens with removed arthritic paws were stained with haematoxylin and eosin (100x). (**E**) Serum IL-1β, IL-6 and TNFα levels in the mice. Values are the mean (S.E.M.) in eight leonurine-treated mice and DMSO-treated mice and six normal mice. *$P < 0.05$ and **$P < 0.01$ *vs* normal mice; $^{\#\#}P < 0.01$ *vs* DMSO-treated CIA mice.

Leonurine, which is derived from *Leonurus cardiaca*, has been implicated in a large range of pharmacological actions [21]. Several studies have shown that it exhibits a wide range of biological activities, including analgesic [22], anti-inflammatory [23], anti-tumour [17] and bone protectant activities [24]. These observations suggest benefits of leonurine for the treatment of inflammatory disorders and tumours. However, whether leonurine has the potential for RA treatment is unknown. In this work, we found that leonurine downregulated not only IL-1β, IL-6, IL-8 and TNFα production but also MMP-1 and MMP-3 expression in TNFα-stimulated RA FLSs in a concentration-dependent manner, which suggested a role for leonurine in the regulation of FLS-mediated inflammation. The results from the *in vivo* experiments in mice with CIA further support the hypothesis that leonurine may have potential as an agent to control synovial inflammation in RA.

Increasing evidence suggests that the migration of FLSs to cartilage and bone plays a key role in cartilage destruction in RA. Once they arrive at cartilage or bone, FLSs can activate osteoclasts to enhance bone erosion and destruction [25, 26]. Thus, regulation of FLS migration may be a new therapeutic strategy for the destructive progress of RA. Therefore, we determined the effect of leonurine on the migration and invasion of RA FLSs in this study. We demonstrated that leonurine suppressed the migration and invasion of FLSs from RA patients and reduced

formation of lamellipodia, which is a critical step that controls cell migration [27, 28]. Consistent with our findings, leonurine also inhibited LPS-induced inflammatory responses in human umbilical vein endothelial cells and the proliferation of H292 in lung cancer cells [15, 23]. These findings suggest that leonurine might be beneficial for the prevention of joint destruction in RA patients.

Multiple signal transduction pathways participate in the regulation of RA pathogenesis, including NF-κB and MAPK, which are considered key signalling factors in the control of synovial inflammation and joint destruction [29, 30]. To investigate the signalling mechanisms affected by leonurine in the regulation of synovial inflammation, we evaluated the effect of leonurine on the NF-κB and MAPK pathways. In the present study, leonurine suppressed the TNFα-induced phosphorylation of IκBα and inhibited the translocation of nuclear NF-κB, indicating that leonurine regulated the NF-κB pathway through interference with the cytoplasmic IκBα signal. Consistent with our data, recent studies have shown that leonurine can regulate the activation of NF-κB in human umbilical vein endothelial cells [15], RAW 264.7 macrophages [31] and mouse bone marrow monocytes [13].

Recent studies have shown that leonurine inhibits MAPK pathways in TNFα-induced human umbilical vein endothelial cells and an LPS-induced mouse mastitis model [13, 14]. In this work, leonurine inhibited TNFα-induced p38 and JNK phosphorylation but did not affect the phosphorylation of the ERK MAPKs in RA FLSs. Furthermore, we demonstrated that chemical inhibitors of JNK, p38 and NF-κB significantly reduced pro-inflammatory cytokine and MMP expression and the migration and invasion of TNFα-induced RA FLSs. These data suggest that the JNK, p38 and NF-κB pathways are involved in the regulation of leonurine in TNFα-induced RA FLSs.

The MAPK pathway has been considered a crucial upstream factor for the activation of the NF-κB signalling complex [19, 20]. Several reports have indicated the involvement of MAPKs in the regulation of NF-κB activation through IκBα phosphorylation [32, 33]. In the current study, we found that the p38 chemical inhibitor exhibited inhibitory effects on IκBα phosphorylation. Taken together, our findings suggest that leonurine regulates the pathological behaviours of RA FLSs through the JNK MAPK and p38-mediated NF-κB pathways.

In summary, we demonstrate for the first time that leonurine ameliorates joint inflammation by regulating the JNK MAPK and p38-mediated NF-κB pathways. Our findings suggest that leonurine may be a potential therapeutic agent for inflammatory arthritis.

Funding: This work was supported by grants from the National Natural Science Foundation of China (Grant number 81573930) and the Guangdong Natural Science Foundation (Grant number 2014A030313403).

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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