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RESEARCH PAPER

Methyl salicylate lactoside inhibits inflammatory response of fibroblast-like synoviocytes and joint destruction in collagen-induced arthritis in mice

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BACKGROUND AND PURPOSE
Methyl salicylate 2-*O*-β-D-lactoside (MSL), whose chemical structure is similar to that of salicylic acid, is a natural product derivative isolated from a traditional Chinese herb. The aim of this study was to investigate the therapeutic effect of MSL in mice with collagen-induced arthritis (CIA) and explore its underlying mechanism.

EXPERIMENTAL APPROACH
The anti-arthritic effects of MSL were evaluated on human rheumatoid fibroblast-like synoviocytes (FLS) *in vitro* and CIA in mice *in vivo* by obtaining clinical scores, measuring hind paw thickness and inflammatory cytokine levels, radiographic evaluations and histopathological assessments.

KEY RESULTS
Treatment with MSL after the onset of arthritis significantly prevented the progression and development of rheumatoid arthritis (RA) in CIA mice without megascopic gastric mucosa damage. In addition, MSL inhibited the production of pro-inflammatory mediators, the phosphorylation and translocation of NF-κB, and cell proliferation induced by TNF-α in FLS. MSL non-selectively inhibited the activity of COX *in vitro,* but was a more potent inhibitor of COX-2 than COX-1. MSL also inhibited the phosphorylation of inhibitor of NF-κB kinase, IκBα and p65, thus blocking the nuclear translocation of NF-κB in TNF-α-stimulated FLS.

CONCLUSION AND IMPLICATIONS
MSL exerts therapeutic effects on CIA mice, suppressing the inflammatory response and joint destruction by non-selectively inhibiting the activity of COX and suppressing activation of the NF-κB signalling pathway, but without damaging the gastric mucosa. Therefore, MSL has great potential to be developed into a novel therapeutic agent for the treatment of RA.

Abbreviations

CIA, collagen-induced arthritis; CII, chicken collagen type II; CMC-Na, sodium carboxyl methyl cellulose; FLS, fibroblast-like synoviocytes; *G. yunnanensis*, *Gaultheria yunnanensis* (Franch.) Rehder; GI, gastrointestinal; IKK, IκB kinase; IκB, inhibtor of NF-κB; MSL, methyl salicylate 2-*O*-β-D-lactoside; MTT, methylthiazoltetrazolium; MTX, methotrexate; NSAIDs, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; SI, selectivity index

Introduction

Rheumatoid arthritis (RA) is a chronic disease causing progressive cartilage and bone destruction, as well as polyarticular inflammatory autoimmune disease, which is characterized by the proliferation of fibroblast-like synoviocytes (FLS) along with subintimal infiltration of inflammatory cells (Huber *et al.*, 2006). The function of FLS is to control synovial fluid volume and joint homeostasis. Hence, the proliferation of FLS plays a crucial role in pathogenesis by regulating the secretion of inflammatory mediators, such as TNF-α, IL-6 and PGE_2 (Mor *et al.*, 2005; Tran *et al.*, 2005). TNF-α is considered to be one of the major pro-inflammatory mediators essential for the initiation of synovial inflammation and joint destruction in RA patients (Feldmann and Maini, 2001).

NF-κB is a ubiquitously expressed pro-inflammatory transcription factor that regulates the expression of various cytokines involved in cellular transformation, survival, proliferation and inflammation (Perkins, 2007; Xu *et al.*, 2007). The regulation of NF-κB depends on its translocation and subsequent DNA binding. In resting cells, it resides in an inactive state in the cytoplasm existing as a heterotrimer consisting of p50, p65 and IκBα subunits. In response to a variety of stimuli including pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β as well as bacterial products such as LPS, inhibitory IκBα undergoes phosphorylation, ubiquitination and degradation. This allows the p65 : p50 heterodimer to be translocated into the nucleus, and bind to B sites on enhancer elements of the target genes inducing transcription (di Meglio *et al.*, 2005). Inhibiting phosphorylation of the kinase or blocking translocation of NF-κB has been shown to suppress inflammatory arthritis (Okazaki *et al.*, 2005). NF–κB-inducing kinase is a key mediator existing as an alternative arm of the NF-κB pathway, and mice lacking this kinase were shown to be resistant to collagen-induced arthritis (CIA; Aya *et al.*, 2005). We have also shown that a novel salicylate analogue inhibited NF-κB activation by blocking p65 translocation into the nucleus in RAW264.7 cells (Xin *et al.*, 2013). Therefore, NF-κB, by regulating the proliferation of FLS induced by pro-inflammatory stimuli, plays an important role in the pathogenesis of RA (Okamoto, 2006; Lee *et al.*, 2011).

RA is varied and difficult to treat, so its treatment is still the biggest problem we are facing. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used agents for treating arthritis because they are generally effective for the relief of pain and inflammation, but they can induce serious gastrointestinal (GI) side effects. Although now, several biological agents have proven to be an effective and promising treatment for RA patients, the risks that are associated with their use have to be taken into account (Moreland, 1998; Cohen *et al.*, 2002; Keystone *et al.*, 2004; Smolen *et al.*, 2008). The most significant risk associated with treating patients with biological therapy is infection. Thus, there is a real need to develop novel therapeutic agents, especially small molecules or natural products.

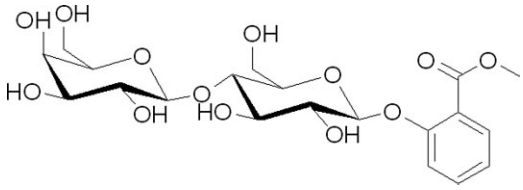

Figure 1
The chemical structure of MSL.

Gaultheria yunnanensis (Franch.) Rehder (*G. yunnanensis*) is a traditional Chinese medicine that is widely used to treat the inflammation, pain and chronic tracheitis associated with RA (Ma *et al.*, 2001). Methyl salicylate 2-*O*-β-D-lactoside (MSL; Figure 1) is a natural product derivative isolated from *G. yunnanensis*. It is interesting to note that the chemical structure of MSL is related to salicylic acid. In previous studies, we found that MSL showed anti-inflammatory and analgesic properties in a mouse model of croton oil-induced ear swelling and acetic acid-induced writhing response (Wang *et al.*, 2011). This compound exerts anti-inflammatory effects by suppressing the production of pro-inflammatory cytokines and inhibiting the activation of the NF-κB signalling pathway in LPS-treated macrophage cells (Zhang *et al.*, 2012). Here, we investigated the effects of MSL on CIA in mice *in vivo* and on human rheumatoid FLS *in vitro*, and explored the underlying mechanisms of these effects. We demonstrated that MSL significantly suppressed the development and progression of CIA by non-selectively inhibiting the activity of COX (for nomenclature see Alexander *et al.*, 2013) and suppressing activation of the NF-κB signalling pathway, but without evoking gastric mucosa damage. MSL has the potential to become a novel therapeutic agent for human RA.

Methods

Animals

A total of 70 male DBA/1 mice (18–22 g) and a total of 15 male Wistar rats (220–250 g) were purchased from Beijing vital river experimental animal technology company (Beijing, China). Mice and rats were housed four to five per cage, under specific pathogen-free conditions, at a constant temperature of 22 ?4°C, humidity 60%~65%. They were fed standard laboratory chow and water *ad libitum*, and kept under a 12 h dark/light cycle. The experimental protocol was

approved by Experimental Animal Care and Use Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College. All efforts were made to minimize the number of animals used and their suffering. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Induction of CIA

Male DBA/1 mice (8–10 weeks old) were immunized (s.c.) at the base of the tail with 100 μg native chicken collagen type II (CII, Sigma, St. Louis, MO, USA) and an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO, USA) denoted as day 0. The mice were then given a s.c booster injection of an equal amount of CII emulsified in Freund's incomplete adjuvant on day 21. The control mice were injected in the same way with saline. Mice with CIA were divided into four groups (n = 5 per group), a model group and three MSL-treated groups. On day 20 (1 day before the second immunization on day 21), three dose of MSL (200, 400 and 800 mg·kg^{-1} body weight in 100 μL of 1% sodium carboxyl methyl cellulose, CMC-Na, respectively) or vehicle (CMC-Na) were administered p.o. to the CIA mice every day until day 24. Mice were killed and joint tissues were harvested to detect the phosphorylation of the NF-κB signalling pathway and relative proteins. The timeline for the development of CIA and treatment is shown in Figure 2A.

To examine the therapeutic effects of MSL, CII-treated mice were divided into five groups (n = 10 mice per group) including the model group, MSL-treated group and MTX-treated group on day 26 after the first immunization. The MSL-treated groups were treated p.o. with MSL (200, 400 and 800 mg·kg^{-1} body weight in 100 μL of 1% CMC-Na, respectively) every day, and the MTX-treated group was treated p.o. with MTX (2 mg·kg^{-1} body weight 100 μL of 1% CMC-Na) every 3 days (according to clinical usage) as a positive agent until day 53 after primary immunization; and the control group (injected with saline) and model group (injected with CII) were treated p.o. with the vehicle (an equal volume solvent of 1% CMC-Na) every day. The timeline for the development of CIA and treatment is shown in Figure 2B. From day 31 after the first immunization, clinical arthritis scores were evaluated using a scoring system of 0–4 for each limb: 0, normal; 1, definite redness and swelling of the ankle or one digits; 2, two joints involved; 3, more than two joints involved; 4, severe arthritis of the entire paw and all digits. Paw thickness was measured with a Vernier caliper. Arthritis scoring and paw thickness measurements were performed by two independent observers. On day 53, the mice were killed and joint tissues were harvested from each animal for end point assessment of histology and photography, and detection of pro-inflammatory substances.

Radiographic evaluation

Plain radiographs of the paws were obtained using a mammographic imager, based on a direct-detection flat-panel array design using exposure settings of 30 kVp and 90 mA. The radiological analysis was evaluated according to a previously described method, with a scoring system to assess the

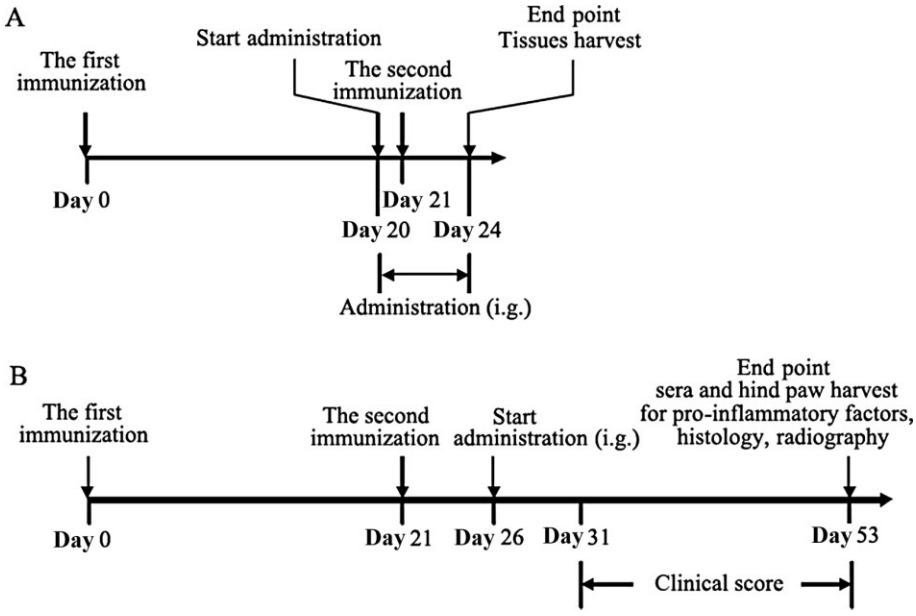

Figure 2

Timeline for the process of CIA development and treatment. (A) DBA/1 mice were immunized on day 0 and 21 respectively. On day 20 (1 day before second immunization), mice were allocated into groups (n = 5 mice per group) and treated with MSL until day 24 after the first immunization. Mice were killed and joint tissues were collected to detect the phosphorylation of proteins involved in the NF-κB signalling pathway. (B) To examine the therapeutic effects of MSL, DBA/1 mice were immunized on day 0 and 21 respectively. On day 26 after the first immunization, mice were allocated into groups and treated with MSL or MTX until day 53 (n = 10 mice per group). Meanwhile, clinical scores were evaluated and hind paw thickness were measured from day 31. At the end point of day 53, mice were killed to conduct other evaluations.

degree of joint destruction and bone erosion (Joosten *et al.*, 1999). The radiological analysis was scored by independent observers in a blinded manner.

Histopathological assessment

For histological analysis, mice were killed and paws were skinned and fixed in 4% buffered formaldehyde and then decalcified in 12% disodium EDTA for 3 weeks. Tissues were then paraffin embedded, sectioned and stained with haematoxylin and eosin. Histopathological assessment of the joints of the arthritic mice were carried out as previously described for bone erosion and inflammation evaluation (Guma *et al.*, 2009). The histopathological changes were evaluated by independent observers in a blinded manner.

Gastric mucosa toxicity assay

On day 53 after the first immunization, CIA mice were killed and the stomachs were inflated, by injecting 10 mL of 1.5% formalin for 10 min to fix the tissue walls, and opened along the greater curvature. Gastric mucosa injury was evaluated after long-time administration of MSL or MTX.

Male Wistar rats (10–12 weeks old) were deprived of food for 16 h before the experiment, but were allowed access to water. Animals were administered 0.75 mL 100 g^{-1} of aspirin (200 mM; 270 mg·kg, $n = 6$) or MSL (200 mM; 940 mg·kg^{-1}, $n = 6$), control rats ($n = 3$) received an equal volume of the solvent of CMC-Na. After 4 h, the stomachs of the rats were removed, collected and processed in a similar manner to that of the CIA mice.

Preparation of serum and joint tissues

On day 53, after the first immunization, the blood was collected from the hearts of CIA mice and kept at room temperature. Two hours later, the blood was centrifuged at 5000× *g* for 15 min at 4°C and the supernatant was stored at –70°C before the detection of pro-inflammatory factors.

After the mice were killed, the ankle joints of the hind paws of five mice were randomly chosen from each group, removed and snap frozen in liquid nitrogen, then suspended in 1 mL of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 3 μg·mL^{-1} aprotinin, 3 μg·mL^{-1} pepstatin A, 2 μg·mL^{-1} leupeptin, 0.1 mM benzamidine, 1 mM dithiothreitol) containing a protease and phosphatase inhibitor cocktail, and homogenized. The homogenates were incubated for 2 h on ice to obtain full lysis, and then centrifuged at 15 000× *g* for 15 min at 4°C, the supernatants were transferred in aliquots to new tubes. The samples were stored at –70°C before the levels of pro-inflammatory cytokines were assessed by ELISA and protein expression by Western blot analysis. All of the extraction procedures were performed on ice.

Culture of human rheumatoid FLS

Human RA FLS (gifted by Prof. Jia Wang, Institute of Rheumatology and Immunology, the Second Xiangya Hospital, Central South University, Hunan, PR China) were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2. Only cells from four to nine passages were used in the experiment. All treatments were performed in serum-free medium.

Cell viability assay and TNF–α-induced FLS proliferation

Cell viability was measured by the MTT assay, as previously described (Zhang *et al.*, 2012). FLS were seeded at a density of 10^4 cells per well in a 96-well plate to determine any potential cytotoxic effects. Cells were deprived of serum for 12 h and then treated with MSL (0–500 μM) for a further 48 h, and then incubated in 0.5 mg·mL^{-1} MTT solution. After 4 h incubation, the dark blue formazan crystals formed in intact cells were dissolved in DMSO, and the absorbance at 570 nm was read using a microplate reader.

The 5-Bromo-2-deoxyUridine (BrdU) assay was used to examine the effect of MSL on the proliferation of FLS, this test is based on measuring BrdU incorporation during DNA synthesis. Briefly, the cells were pretreated with MSL (1, 10 and 100 μM) for 2 h and then incubated with or without TNF-α (10 ng·mL^{-1}) in the medium for another 48 h. Eight hours before termination of the experiments, 10 μM BrdU was added to each well and the incorporation of BrdU into newly synthesized DNA was measured using an ELISA kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacture's protocol.

Cytokine analysis

Human RA FLS were plated in 96-well plates at a density of 2 × 10^4 cells per well, then pretreated with MSL (1, 10 and 100 μM) for 1 h, and then 10 ng·mL^{-1} of TNF-α was added before incubation for another 48 h. The culture supernatants were collected and centrifuged at 10 000× *g* for 5 min to remove the particulate matter before measurement of pro-inflammatory cytokines.

The cytokine levels in the culture supernatants of FLS or the diluted serum and protein extracts of joint tissue homogenates of mice were determined using cytokine-specific ELISA kits for TNF-α, IL-1β, IL-6 and PGE_2. The assays were performed according to the manufactures' instructions and the absorbance value of each sample was standardized to individual protein concentrations.

Immunofluorescence staining

Approximately 10^4 FLS were seeded in 96-well plates and pretreated with MSL (1, 10 and 100 μM) for 2 h before incubation with TNF-α (10 ng·mL^{-1}) for 30 min. The cells were then washed and fixed with a 4% paraformaldehyde for 30 min at 37°C, permeabilized with 0.3% Triton X-100 for 10 min, and blocked with 3% BSA for 30 min at room temperature. Next, cells were incubated with primary anti-NF-κB p65 antibody (1:200, Cell Signaling Technology, Beverly, MA, USA) over night at 4°C, and Alexa Fluor 488-conjugated goat anti-rabbit IgG as a secondary antibody (1:500, Invitrogen, Eugene, OR, USA) at room temperature for 2 h. After being washed with PBS, cells were incubated in DAPI for 10 min in the dark to dye the nucleus. The p65 protein and nuclei are displayed as fluorescent green and blue respectively. The translocation of p65 was detected as previously described (Liu *et al.*, 2012). Cell imaging was simultaneously viewed on the Cellomics ArrayScan V^{TI} HCS Reader (Thermo Fisher Scientific

Cellomics, Pittsburgh, PA, USA) to analyse the translocation of p65. The translocation of p65 was determined by the value of Mean_CircRingAvgIntenDiff (Bertelsen, 2006).

Western blot analysis

Human RA FLS were seeded in 60 mm culture dishes at a density of 5×10^6 cells per dish, and pretreated with MSL 1, 10, and 100 μM for 1 h, then exposed to TNF-α (10 ng·mL^{-1}) for 30 min or 48 h. The extraction of cell protein was performed as previously described (Zhang *et al.*, 2012).

Analysis of the proteins extracted from FLS or joint tissues by Western blot was performed using standard methods. Equivalent amounts of protein (20 μg) from each sample were mixed with gel loading buffer in a ratio of 1:1, heated at 95°C for 5 min, electrophoresed in a 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). The nitrocellulose membranes were blocked with 5% BSA. The blots were probed with antibodies against the phospho-inhibitor of NF-κB (IKK) β, phospho-IκBα, phospho-p65, IKKβ, IκBα, p65, GAPDH (all from Cell Signaling Technology), COX-1 and COX-2 (all from Abcam, Cambridge, UK). HRP-conjugated anti-IgG (Santa Cruz Biotechnology, Beverly, MA, USA) was used as a secondary antibody. Membranes were visualized with a chemiluminescence system (ChemiDoc™XRS, Bio-Rad, Richmond, PA, USA). The relative intensities of the bands were quantified by Quantity One Software (Bio-Rad).

Assessment of COX activities

To elucidate the direct effect of MSL on COX, the inhibitory effects of MSL on ovine COX-1 and recombinant COX-2 activities were investigated using a fluorescence inhibitor assay (catalogue number 700 100, Cayman Chemical, Ann Arbor, MI, USA). The assay was performed according to the manufacturer's assay protocol. MSL were assayed in concentrations ranging from 5 mM to 0.5 μM, and the IC_{50} values were calculated.

Statistical analysis

All data are expressed as mean ± SEM. Statistical significance was evaluated by one-way ANOVA with Bonferroni's *post hoc* correction used for multiple comparisons of clinical score and hind paw thickness. The significance of differences between groups was determined using Student's unpaired *t*-test. A *P*-value less than 0.05 was considered significant.

Reagents

MSL was synthesized by the Institute of Materia Medica, Chinese Academy of Medical Sciences with improved stability and better water-soluble characteristics (Wang *et al.*, 2011). Methotrexate (MTX) was obtained from Shanghai Sine Pharmaceutical Co., Ltd. (Shanghai, China). Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM, FBS and the antibiotic–antimycotic solution were purchased from Gibco (Gibco BRL, Grand Island, NY, USA). All ELISA kits were purchased from Jiamei Biotech Co. (Beijing, China).

Results

Suppression of synovial inflammation and bone destruction in mice with CIA by treatment with MSL

To test the effect of MSL on CIA development after disease onset, MSL was given, 6 days after the second booster immunization, every day until day 53. The clinical score was used to evaluate the progression of arthritis development. Macroscopic evidence of arthritis such as erythema or swelling was markedly evident in vehicle-treated mice. In contrast, in CIA mice treated with MSL, the progression and development of CIA were blocked in a dose-dependent manner (Figure 3A). Consistent with the clinical scoring, measurements of paw swelling in CIA mice also showed MSL to be highly effective (Figure 3B). Compared with 200 mg·kg^{-1} of MSL, which had no significant therapeutic effect, dosages of 400 and 800 mg·kg^{-1} MSL exerted a significant anti-inflammatory effect, and dramatically attenuated arthritis severity in CIA mice, according to mean arthritis score and paw thickness measurement. As a positive control treatment, MTX also mediated an anti-inflammatory effect, inhibiting the arthritis development in CIA mice.

We also investigated the effect of MSL on CIA mice by X-ray imaging. A representative example of the bone destruction of CIA mice paw is shown in Figure 3C by plain radiograph. Severe bone erosions were observed in vehicle-treated CIA mice, with articular destruction, joint displacement and irregular bony proliferation covering the entire ankle region. However, the bone destruction was significantly attenuated in groups receiving 400 and 800 mg·kg^{-1} of MSL or MTX. The mean radiographic scores indicated that destruction of the joints was significantly suppressed in the MSL-treated or MTX-treated mice compared with the vehicle-treated mice (Figure 3D).

To further assess the effect of MSL on arthritis development, the hind paw joint was evaluated histologically and then semi quantitatively graded for synovial proliferation, cartilage damage, inflammatory cell infiltration and bone erosion. The results show that MSL dose-dependently inhibited the inflammatory infiltration and bone destruction in groups receiving 400 and 800 mg·kg^{-1} doses. As shown in Figure 3E and F, both cartilage and bone destruction as well as inflammation were remarkably alleviated by MSL or MTX as compared with vehicle-treated mice.

Negligible toxic effects of MSL on gastric mucosa

The stomachs of rats were collected and processed to expose the gastric mucosa after single and mega dose administration of MSL or aspirin; obvious macroscopic gastric mucosa bleeding anabrosis was observed after a single administration of aspirin. In contrast, hardly any gastric mucosa damage was observed in MSL-treated rats (Figure 3G, panels a–c). Consistent with these results obtained after single or mega dose administration of MSL, bleeding or ulcers were not observed in gastric mucosa of CIA mice receiving chronic oral administration of MSL or MTX (Figure 3G, panels d–f).

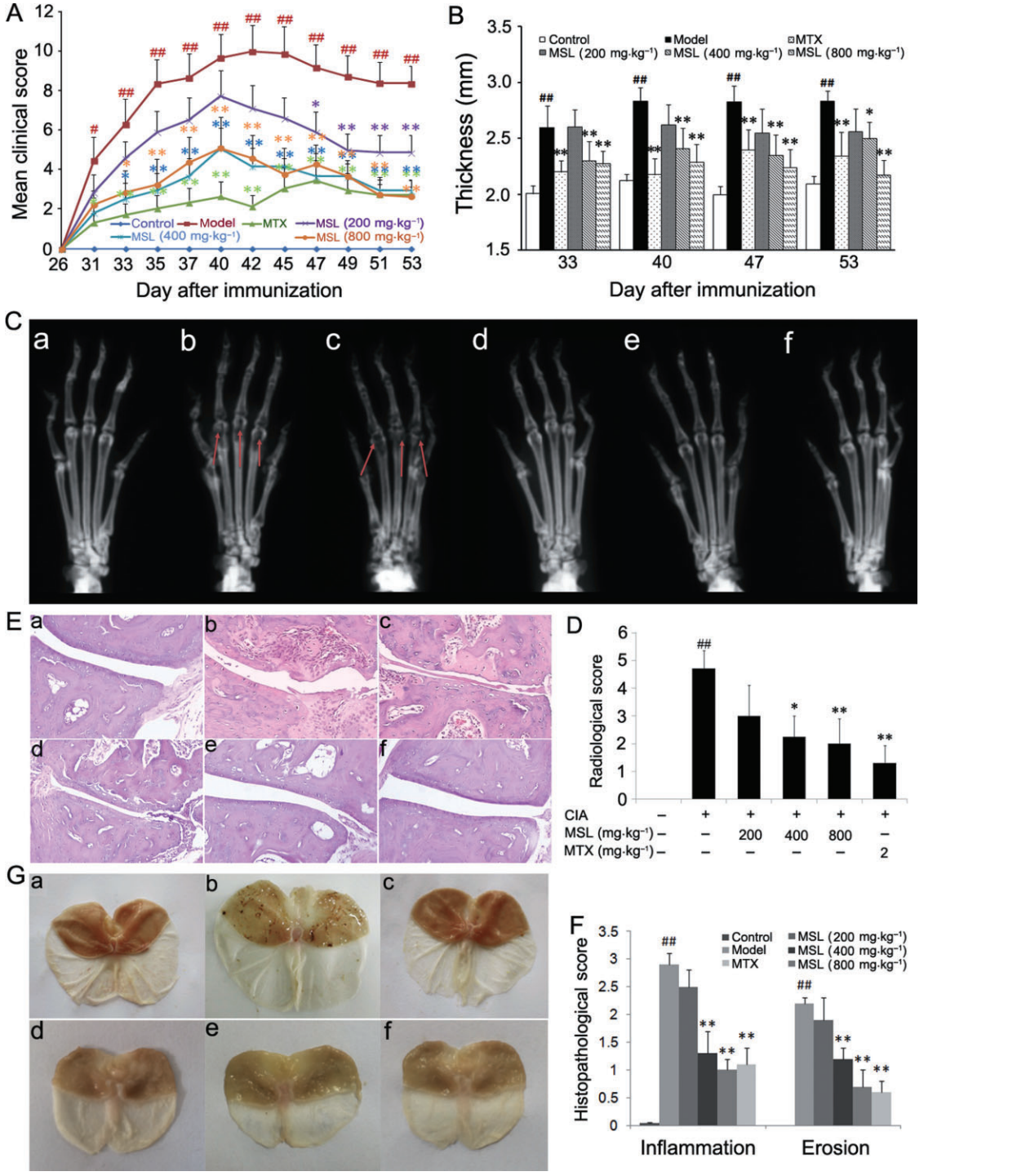

Figure 3

Treatment with MSL significantly blocks the progression of CIA. Mice were administered MSL, MTX or vehicle, p.o., after disease onset. Mean clinical scores (A), hind paw thickness (B) were determined on the indicated days after the first immunization (The control is non-immunization by CII and vehicle-treated DBA/1 mice, the model is vehicle-treated CIA mice.). (C) Representative radiographs of the hind paws of CIA mice were shown to depict the bone destruction in different groups (a, control; b, model; c, 200 mg·kg^{-1} MSL treatment; d, 400 mg·kg^{-1} MSL treatment; e, 800 mg·kg^{-1} MSL treatment; f, MTX treatment) on day 53, and radiological scores were determined as described in Methods (D). Representative haematoxylin and eosin-stained sections of ankles from mice of different groups (as C) on day 53 is shown in (E), original magnification 100×. (F) Inflammatory and lesion scores were determined as described in the Methods section. Representative macroscopic gastric mucosa are shown as (G) after mega dose and single administration of MSL (a, 0 mM, $n = 3$; b, 200 mM, $n = 6$) or aspirin (c, 200 mM, $n = 6$) in rat, and chronic administration of MSL (d, 0 mg·kg^{-1}; e, 800 mg·kg^{-1}) or MTX (f, 2 mg·kg^{-1}) in CIA mice until day 53($n = 10$). Values in (A)–(F) are the mean ± SEM ($n = 10$ mice per group), $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus control (vehicle-treated DBA/1 mice), $^{*}P < 0.05$ and $^{**}P < 0.01$ versus model (vehicle-treated CIA mice).

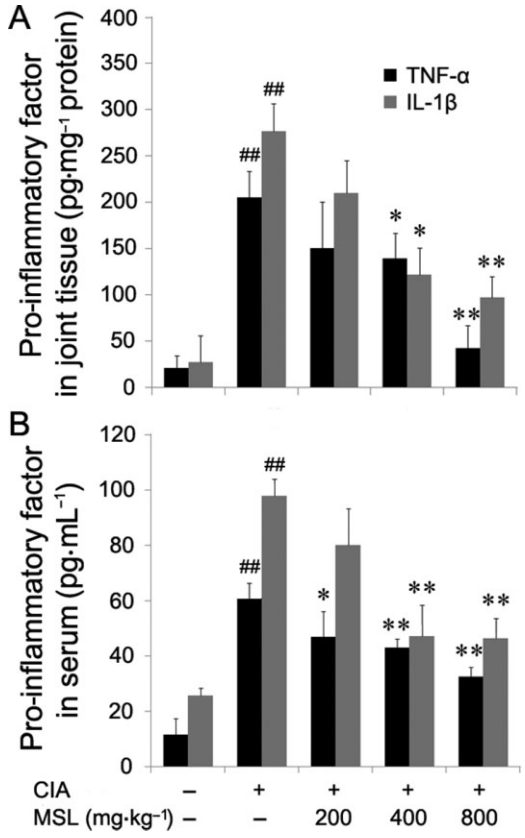

Figure 4

Inhibition of the production of pro-inflammatory cytokines in joint tissues and serum from mice with CIA and treated with MSL. Hind paws and serum were collected on day 53 from mice with CIA as well as from normal mice. Cytokines in joint homogenates (A) and serum (B) were measured by ELISA. Values are the mean ± SEM of three independent experiments ($n = 10$ mice per group). $^{\#\#}P < 0.01$ versus vehicle-treated DBA/1 mice, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle-treated CIA mice.

Treatment with MSL significantly suppresses inflammatory cytokine production in CIA mice

On day 53 after immunization, joint tissues and serum of CIA mice were collected and levels of TNF-α and IL-1β were measured by ELISA. Compared with vehicle-treated CIA mice, those treated with MSL had significantly decreased production of TNF-α and IL-1β in the joint tissues (Figure 4A). Consistent with the joint tissue results, treatment with MSL significantly reduced the systemic level of TNF-α and IL-1β (Figure 4B) in the serum. These results suggest that MSL might provide beneficial effects by down-regulating the synthesis of inflammatory cytokines.

Effects of MSL on the proliferation of FLS

MSL (0–500 μM) did not show any significant effects on the proliferation of normal cultures of FLS in the 48 h incubation time period (Figure 5A). In the subsequent experiments, concentrations of MSL did not exceed 100 μM.

The effect of MSL on proliferation of FLS was evaluated using the BrdU assay in the presence or absence of TNF-α. Treatment with TNF-α alone for 48 h significantly increased the proliferative potential of FLS ($P < 0.01$) and MSL significantly inhibited TNF–α-induced FLS proliferation (Figure 5B). Incubation with MSL in the absence of TNF-α did not affect the proliferation of FLS.

Effects of MSL on TNF-α-induced production of pro-inflammatory mediators by FLS

To examine the anti-inflammatory efficacy of MSL, FLS were incubated with various concentrations of MSL (0–100 μM) in culture supernatants, followed by stimulation with 10 ng·mL^{-1} TNF-α for 48 h. The addition of TNF-α significantly increased the production of IL-1β and IL-6 compared with that of control. However, pretreatment with MSL for 2 h greatly inhibited the production of IL-1β and IL-6 in a concentration-dependent manner (Figure 5C and D).

Effects of MSL on PGE_2 production and COX-1/2 expression

To better understand the anti-inflammatory effects of MSL, we investigated the effects of MSL on PGE_2 production and COX expression *in vivo* and *in vitro*. PGE_2 secretion was significantly inhibited in MSL-treated CIA mice compared with vehicle-treated CIA mice (Figure 6A). Furthermore, MSL inhibited the expression of COX-1 and -2 in the joint tissues of CIA mice in a dose-dependent manner (Figure 6C and E).

A further study was performed on FLS for PGE_2 production and COX expression using the same method mentioned earlier. Incubation of FLS with TNF-α resulted in a 17-fold increase in the production of PGE_2 in culture supernatants (Figure 6B). Pretreatment with MSL significantly decreased this level of PGE_2 in a concentration-dependent manner. TNF-α 10 ng·mL^{-1} markedly up-regulated the COX-2 expression levels, but only caused a slight increase in COX-1 after 48 h stimulation. However, both COX-1 and -2 expression levels were concentration-dependently reduced to a different extent by pretreatment with MSL (Figure 6D and F).

MSL inhibited NF-κB signal activation in CIA mice

As shown in Figure 7A, C and E, the protein expression of IKKβ, IκBα and p65 did not show significant difference in MSL-treated CIA mice, vehicle-treated CIA mice or control mice. However, the expression levels of phosphorylated IKKβ, IκBα and p65 were significantly increased in vehicle-treated CIA mice compared with control mice, and the 5 day pretreatment with MSL markedly inhibited the phosphorylation of IKKβ, IκBα and p65. These results suggest that MSL may mediate a protective effect in mice with CIA by suppressing activation of the NF-κB signalling pathway.

Effects of MSL on TNF-α-induced IKKβ phosphorylation and NF-κB translocation in human RA FLS

The expression and phosphorylation of NF-κB was investigated in RA FLS pretreated with MSL. TNF-α stimulation for 30 min not only caused significant phosphorylation of IKKβ

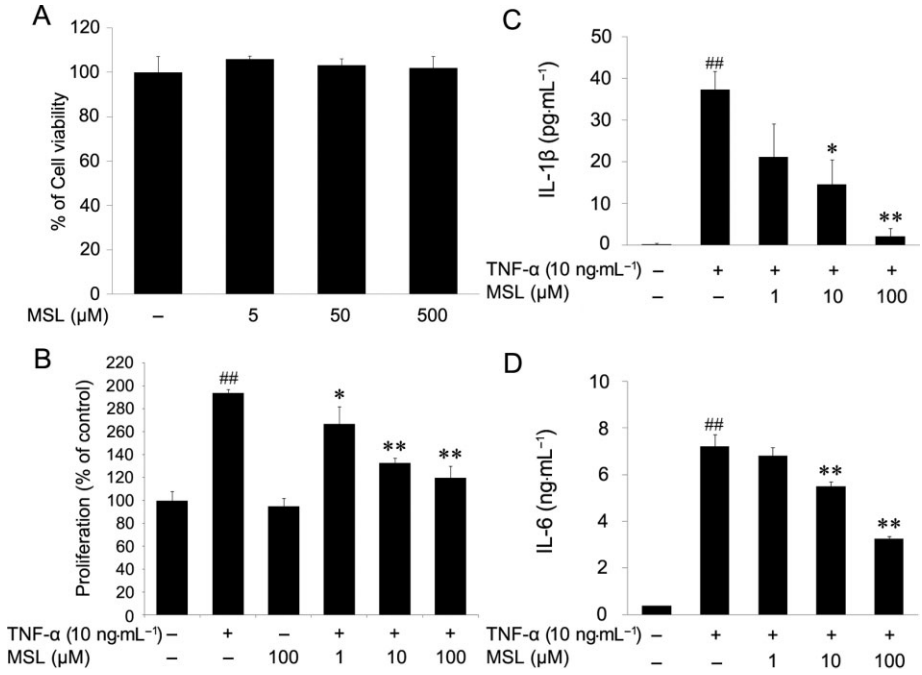

Figure 5

The effects of MSL on the production of pro-inflammatory factors by TNF–α-stimulated FLS. (A) Cell viability was assessed by MTT assays. (B) MSL inhibits the TNF–α-induced proliferation of FLS, shown using the BrdU incorporation assay. FLS were treated with 1, 10 and 100 μM MSL for 2 h and continuously incubated with TNF-α (10 ng·mL^{-1}) for 48 h, and the production of IL-1β (C) and IL-6 (D) was measured by ELISA. The assays were performed as described in the Methods. Values are the means ± SEM of three independent experiments. $^{\#\#}P < 0.01$ versus untreated cells, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus TNF–α-treated cells.

and IκBα, but also increased the phosphorylation of p65. However, MSL pretreatment markedly inhibited the phosphorylation of all three in a concentration-dependent manner (Figure 7B, D and F).

To further investigate the effect of MSL on the NF-κB signalling pathway, the translocation of cytosolic p65 into the nucleus was evaluated by immunofluorescence assay via image and quantification. As shown in Figure 8A and B, without TNF-α stimulation, basal NF-κB p65 was distributed in the cytoplasm with green fluorescence and nucleus displayed as blue fluorescence by DAPI. TNF-α 10 ng·mL^{-1} induced nuclear translocation of NF-κB p65 in FLS; merged images indicate a cyan fluorescence in the nucleus. Mean_CircRingAvgIntenDiff values drastically increased in TNF–α-stimulated cells compared to control cells. However, in the presence of MSL, p65 nuclear translocation induced by TNF-α was significantly inhibited in a concentration-dependent manner.

Direct inhibitory effect of MSL on COX

The effect of MSL on COX activity was detected by a direct inhibition *in vitro* assay. The IC_{50} value of MSL was found to be 22.7 μM for COX-1 and 5.58 μM for COX-2, indicating that MSL is a non-selective inhibitor of COX isozyme activity. Meanwhile, MSL showed a stronger inhibition of COX-2 than COX-1, with a selectivity index (SI; SI = IC_{50} COX-1/IC_{50} COX-2) of 5.

Discussion and conclusion

In this study, we demonstrated that MSL effectively suppresses the inflammatory response and joint tissue destruction in both *in vivo* and *in vitro* assays. Our results indicate that MSL has a therapeutic effect in CIA mice by inhibiting the production of inflammatory cytokines and blocking the activation of the NF-κB signalling pathway. In addition, MSL non-selectively suppressed the activity of COX. Another most important characteristic of MSL is that it has almost no gastric mucosa toxicity. No obvious macroscopic bleeding or anabrosis of the gastric mucosa was observed in either CIA mice receiving long-term oral administration or a mega dose of MSL, or in rats given a single oral dose of MSL. This indicates that MSL will provide a better treatment for RA compared with other NSAIDs.

The joint tissues of patients with RA are characterized by infiltration of innate immune cells into the synovium, leading to chronic inflammation, pannus formation and subsequent irreversible joint and cartilage damage (Simmonds and Foxwell, 2008). RA synovial fluid contains a wide range of effector molecules including pro-inflammatory cytokines (such as TNF-α, IL-1β and IL-6) and some metabolic proteins (COX-1 and -2). These molecules interact with one another forming a vicious cycle, which can perpetuate itself resulting in chronic and persistent inflammation (Muller-Ladner *et al.*, 2005). Pro-inflammatory cytokines are known to play a critical role in mediating

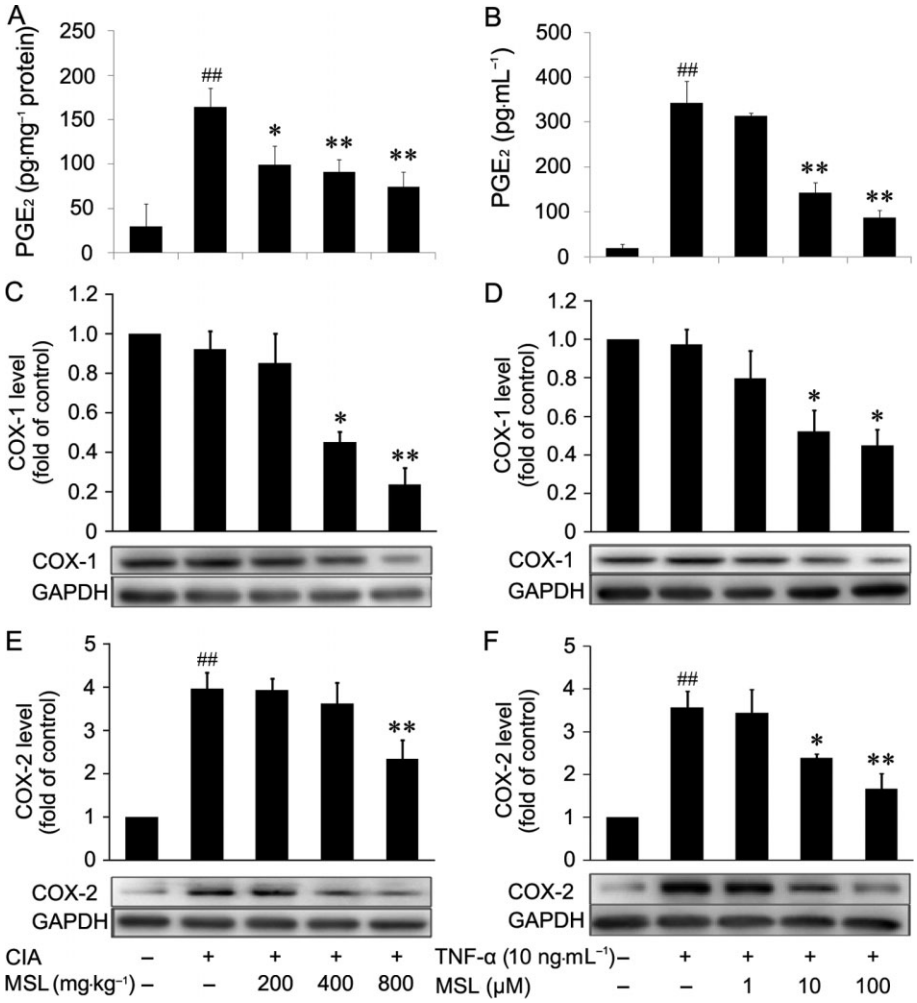

Figure 6

The effect of MSL on PGE_2 production and COX-1 or -2 expression in CIA tissue and human RA FLS. DBA/1 mice (n = 10 mice per group) were treated with vehicle or MSL on day 26, hind paws were collected on day 53. PGE_2 (A) was measured by ELISA, and the expression of COX-1 (C) or COX-2 (E) was determined by Western blots in joint tissue homogenates. Cells were pretreated with different concentrations of MSL for 2 h, and then stimulated with TNF-α (10 ng·mL^{-1}) for 48 h, PGE_2 (B) was measured by ELISA in the culture medium, and the expression of COX-1 (D) or COX-2 (F) was assayed by Western blots. Values are the mean ± SEM of three independent experiments. $^{\#\#}P < 0.01$ versus vehicle-treated DBA/1 mice or untreated cells, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle-treated CIA mice or TNF–α-treated cells.

synovitis, pannus formation, and erosion of cartilage and bone in RA (Smolen and Steiner, 2003; McInnes and Schett, 2007). The proliferation of FLS following the production of pro-inflammatory cytokines and activation of NF-κB in FLS is a very important step in RA development. Here, we found that pretreatment with MSL significantly inhibited the TNF–α-triggered production of IL-1β and IL-6 from FLS. Consistently, treatment of CIA mice with MSL markedly reduced joint tissue and serum levels of TNF-α, IL-1β and PGE_2. Moreover, we demonstrated that MSL significantly reduced PGE_2 production and inhibited the expression of COX-1/2 in both TNF–α-stimulated FLS and joint tissues of mice with CIA. Taken together, these results demonstrate that MSL has a broad anti-inflammatory effect in macrophages and FLS.

The proliferation of FLS leads to the production of pro-inflammatory factors via activation of NF-κB, which plays a crucial role in the pathogenesis of inflammatory arthritis (Okamoto, 2006). The transcription factor, NF-κB, regulates numerous pro-inflammatory genes that can contribute to joint inflammation, especially TNF–α-stimulated inflammation. In this study, we observed activation of the NF-κB signalling in cultured FLS and joint tissue of CIA mice by detecting the phosphorylation levels of relative proteins and p65 translocation in the presence and absence of MSL. We found that phosphorylation of IKKβ, IκBα and p65 was triggered in the joint tissue of CIA mice, and that this phosphorylation was suppressed by prophylactic doses of MSL, and these phenomena were confirmed in the *in vitro* culture system. Pretreatment with MSL markedly prevented the translocation of NF-κB by inhibiting the phosphorylation of IKKβ, IκBα and p65 in TNF–α-induced human RA FLS. Our findings suggest that MSL inhibits activation of the NF-κB signalling pathway by affecting the phosphorylation of IKKβ,

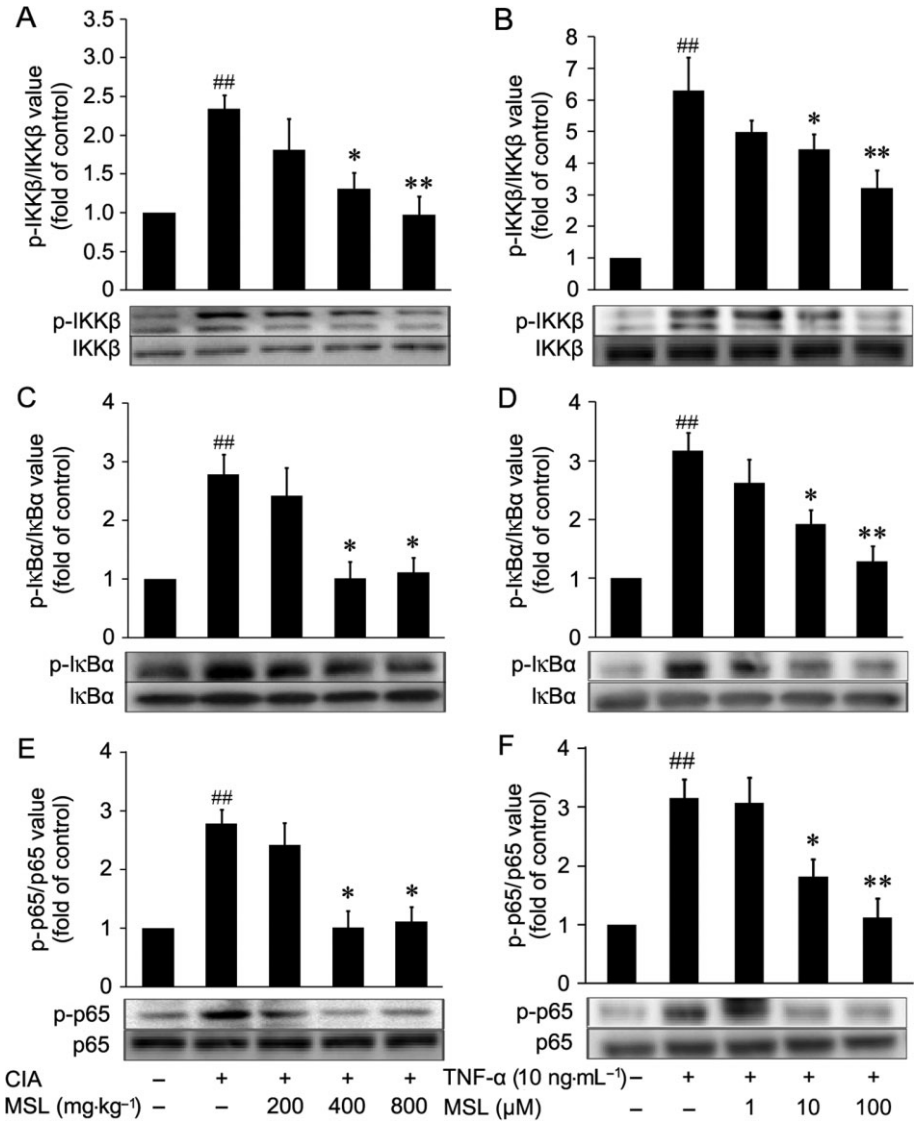

Figure 7

Inhibitory effects of MSL on the activation of the NF-κB signalling pathway in CIA mice joint tissues and human RA FLS. DBA/1 mice (n = 5 mice per group) were treated with vehicle or MSL on day 20 (1 day before second immunization on day 21), and on day 24 after second immunization 3 days later, hind paws were collected and joint tissue homogenates used to measure the phosphorylation levels of IKKβ (A), IκBα (C) and p65 (E) by Western blots. Human RA FLS were pretreated with different concentrations of MSL for 2 h, and then stimulated with TNF-α (10 ng·mL^{-1}) for 30 min, the phosphorylation of IKKβ (B), IκBα (D) and p65 (F) were determined by Western blots. Values are the mean ± SEM of three independent experiments. $^{\#\#}P < 0.01$ versus vehicle-treated DBA/1 mice or untreated cells, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vehicle-treated CIA mice or TNF–α-treated cells.

IκBα and p65 or blocking NF-κBp65 translocation, thus, preventing inflammatory responses in CIA mice.

One of the most important clinical problems in rheumatic disease is the progressive development of joint destruction. Our *in vivo* data demonstrated that MSL markedly suppressed the severity and progression of arthritis in CIA mice after onset of the disease. These therapeutic effects were well supported by arthritis clinical score and paw thickness data. In addition, histopathological and radiological evaluations revealed that MSL significantly reduced the CIA-induced joint destruction. Because of the well-known GI tract toxicity of NSAIDs, MTX (Wessels *et al.*, 2008), the most frequently used disease-modifying anti-rheumatic drug, was selected as a positive control for use in our *in vivo* experiments. Similar to doses of 400 or 800 mg·kg^{-1} MSL, MTX also ameliorated the severity of bone destruction and blocked the progression of arthritis in CIA mice, and MSL did not appear to be superior to MTX in clinical score evaluation at the end of the experiment. It is well known that NSAIDs and MTX exert their therapeutic effects on RA through different pharmacological mechanisms; the major function of MTX is to prevent bone destruction, whereas NSAIDs have an anti-inflammatory effect. In this study, our data indicated that MSL also suppressed the joint destruction, in addition to its

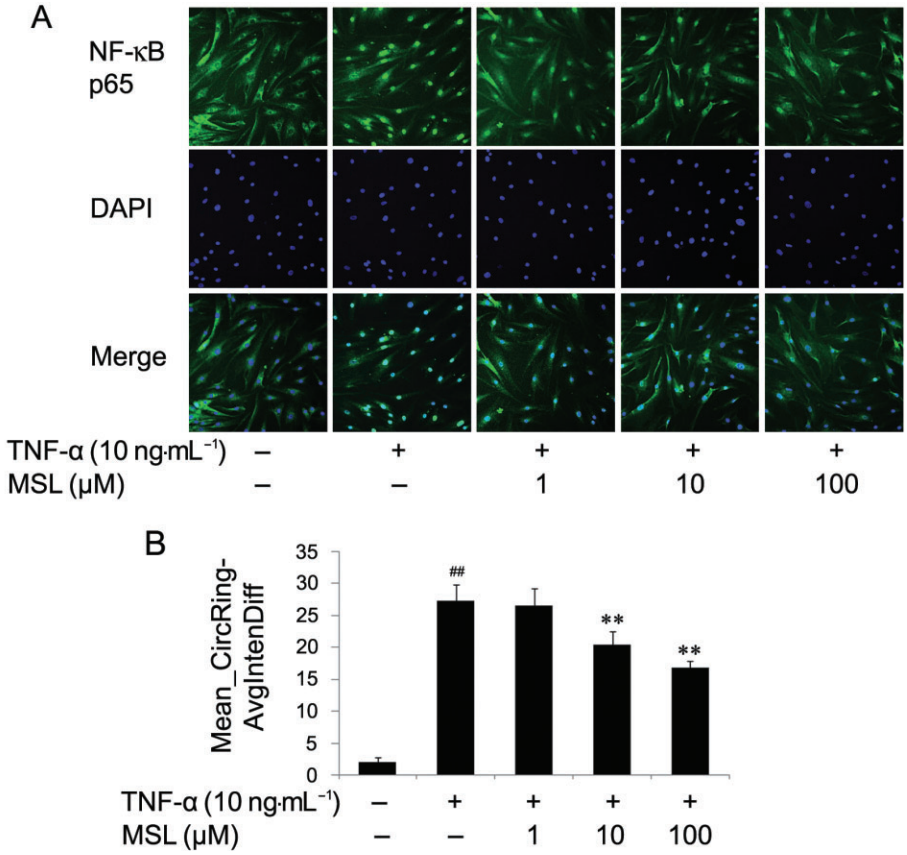

Figure 8

Effects of MSL on the nuclear translocation of p65. For the immunofluorescence assay for NF-κB p65 nuclear translocation, cells were pretreated with MSL for 2 h, and then TNF-α for 30 min. (A) Without TNF-α stimulation, basal NF-κB p65 distributed in cytoplasm with green fluorescence and nucleus in blue fluorescence indicated by DAPI. Incubating 10 ng·mL^{-1} TNF-α with FLS induced a nuclear translocation of NF-κB p65, merged image indicated a cyan fluorescence in the nucleus. (B) The capacity of p65 translocation from the cytosol to the nucleus is presented as values of Mean_CircRingAvgIntenDiff, as described in the Methods section. Values are the means ± SEM of three separate experiments. $^{\#\#}P < 0.01$ versus untreated cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus TNF–α-treated cells.

anti-inflammatory effect. These results suggest MSL has therapeutic effects by blocking the progression of arthritis in CIA mice model.

Although NSAIDs are still the first-line drugs of choice for the treatment of RA due to their anti-inflammatory and analgesic effects, they are not suitable for long-term treatment of RA as they do not improve the progression of RA or reduce bone destruction. In addition, they can induce severe GI damage, especially non-selective NSAIDs, such as aspirin and neproxen (Brooks *et al.*, 2013). It is now thought that the inhibition of COX-1 is an important cause of GI toxicity. COX-1 is a constitutive form and plays a key role in regulating important physiological cytoprotection in the GI mucosa (Russell, 2001). Most conventional non-selective NSAIDs exert their anti-inflammatory effects by inhibiting COX-2, but induce severe GI toxicity because of inhibition of COX-1. Thus, a new class of selective COX-2 inhibitors, known as the coxibs, has been developed to prevent the adverse GI effects while maintaining equivalent anti-inflammatory efficacy. However, the use of selective COX-2 inhibitors to treat RA is likely to increase the risk of cardiovascular events (Baigent and Patrono, 2003). Hence, the selectivity or preference of NSAIDs on COX isozymes is closely associated with the adverse effects they induce. So SI, IC_{50} (COX-1 : COX-2), may be a valuable parameter for estimating the likelihood of an adverse effect (whether it be GI toxicity or risk of cardiovascular events) induced by NSAIDs. From published literature it appears that those drugs that have a low SI, such as the SI of 0.01 for aspirin, 0.5 for ibuprofen and 0.7 for naproxen, are more likely to induce serious GI toxicity (Baigent and Patrono, 2003; White, 2007; Yang *et al.*, 2008). However, the risk of cardiovascular events is enhanced when the SI is increased, such as the SI of 30 for celecoxib and 267 for rofecoxib (Baigent and Patrono, 2003). Hence, we speculated that there might be an optimum SI for NSAIDs with minimum side effects. An optimum SI value is keeping a balance between the inhibitory effects of COX-1 or COX-2 by NSAIDs, and minimizing adverse effects.

Interestingly, our results indicated that MSL hardly had any macroscopic effects on the gastric mucosa after chronic oral administration in CIA mice. Furthermore, it was confirmed that a mega dose and single oral administration of MSL also produced almost no gastric mucosal lesions in the rat. The *in vitro* assay indicated that MSL non-selectively

inhibited the activity of the COX isozyme. The inhibitory effect of MSL on COX-2 was stronger than that on COX-1, and the SI for MSL was about 5. From the perspective of minimal GI toxicity, an SI of 5 might be within the optimum SI value for minimum adverse effects. However, the exact reason for low GI toxicity of MSL needs to be further investigated.

In agreement with the results mentioned earlier, MSL not only exerts anti-inflammatory effects by suppressing the progression of arthritis, but also has little propensity to induce gastric mucosa damage after chronic oral administration in CIA mice. This implies that MSL, in comparison with other NSAIDs, might also exert disease-modifying anti-rheumatic effects as well as an anti-inflammatory effect and may be a potential candidate agent for chronic treatment of RA. Of course, as a candidate for RA, any possible adverse/beneficial effects of MSL on the cardiovascular system have to be further investigated. In the present study, 400 and 800 $mg \cdot kg^{-1}$ of MSL showed similar effects on CIA mice, so further evaluation for a clinically optimum dose along with pharmacodynamics of MSL will be carried out in our next study.

In summary, we demonstrated that a novel natural product isolated from *G. yunnanensis*, MSL, by acting as an anti-inflammatory and suppressing joint destruction, has a significant therapeutic effect on the development and progression of CIA in mice. This therapeutic effect of MSL may be related to the reduced production of pro-inflammatory cytokines and inhibition of the NF-κB signalling pathway, in addition to the non-selective inhibition of COX. Importantly, MSL did not induce any GI mucosa damage in either the long-term or short-term administration studies. MSL has the potential to be developed into a novel therapeutic agent for the treatment of human RA.

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Author contributions

T. Z. and G. D. conceived and designed the study. W. X., C. H., X. Z., X. M., D. Z., Y. L. and S. Z. were involved in data acquisition. W. X., S. X. and Y. Z. have made statistical analyses and interpretation of data. D. M. Z. synthesized the compound of MSL. T. Z. and W. X. wrote the paper. All authors contributed to analysis and interpretation of the data and approved the final paper.

Conflict of interest

The authors declare that they have no competing interests.

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