Triptolide Alleviates Murine Arthritis by Inhibiting Osteoclast Differentiation via TRAF6/MAPKs/NFATc1 Signaling Pathway

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Abstract

**Background:** Bone erosion is an important problem in rheumatoid arthritis (RA). The role of osteoclast in bone resorption is critical in RA, and intervention of osteoclast differentiation can improve bone destruction. *Tripterygium wilfordii Hook F* (TW) can improve the symptoms of RA. The effect of triptolide (TP), which is a purified component of TW, on bone destruction in RA and its mechanism remain unclear.

**Methods:** We investigated the effect of TP on osteoclast differentiation using a co-culture system of synovial fibroblasts and monocytes from adjuvant-induced arthritic rats. We also investigated the effect of TP on murine collagen-induced arthritis (CIA).

**Results:** The results showed that a certain dose of TP inhibited the differentiation of osteoclast, and reduced the levels of interleukin (IL)-1, IL-34, macrophage colony stimulating factor (M-CSF), receptor activator for nuclear factor-κ B Ligand (RANKL), and osteopontin (OPN) in the co-culture supernatant of synovial fibroblasts and monocytes. Furthermore, a certain dose of TP reduced the expression of tumor necrosis factor receptor-associated factor 6 (TRAF6), mitogen-activated protein kinases (MAPKs) and nuclear factor of activated T cells 1 (NFATc1) in osteoclast. Meanwhile, TP reduced the symptoms of arthritis and bone destruction in CIA.

**Conclusion:** Our results suggest that TP can improve the bone destruction of murine arthritis, and the mechanism is related to the inhibition of osteoclast differentiation via TRAF6/MAPKs/NFATc1 signaling pathway.

Introduction

Rheumatoid arthritis (RA) is a clinically common chronic systemic autoimmune disease, with high incidence and disability rate, which is mainly caused by joint bone erosion. Bone erosion occurs at the early stage of RA[1], and delaying or preventing bone destruction has become a hotspot and difficulty in current research.

The excessive proliferation of osteoclast disrupts the balance between osteoblast and osteoclast, leading to the occurrence of bone destruction. Osteoclast is one of the key cells leading to bone destruction in RA. Osteoclast differentiation is closely related to synovial fibroblast in RA [2]. Synovial fibroblast can release interleukin (IL) -1, IL-34, macrophage colony stimulating factor (M-CSF) and other cytokines, and then induce the expression of receptor activator for nuclear factor-κ B Ligand (RANKL) [3-6]. In addition, pro-inflammatory cytokines in synovial of RA stimulate the overexpression of osteopontin (OPN) in monocytes [7]. OPN can regulate osteoclast differentiation and function [8-10]. When OPN is deficient, neither RANKL nor M-CSF can induce bone resorption [11]. RANKL and M-CSF, as key factors to stimulate the fusion and differentiation of osteoclast progenitor cells[12-15], can initiate a variety of intracellular signal transduction pathways[16-20] that cause NFATc1 transcription to mediate osteoclast differentiation and fusion, among which TRAF6/MAPKs signaling pathway has attracted more and more attention.
In our previous study[21], we established a method of inducing osteoclast-like cells by co-culture of synovial fibroblasts and peripheral blood monocytes from adjuvant-induced arthritis (AIA) rats. This co-culture method of osteoclast-like cells induction did not require exogenous stimulation, which could reflect the internal relationship between synovitis and bone destruction in RA, and was suitable for the study of the effect and mechanism of intervening osteoclast differentiation.

*Tripterygium wilfordii Hook F* (TW) can improve RA symptoms. TP is an extract of TW. It had been reported that TP could inhibit cytokine production, influence the osteoclastogenesis in RAW264.7 cells, and improve the symptoms of osteolysis[22-24]. Nevertheless, its effect on bone destruction in RA, and its mechanism on cytokines and signaling pathways during osteoclast differentiation remains unclear.

This study verified the effect of TP on bone destruction in RA by observing the effects of TP on the differentiation of osteoclast induced by co-culture of synovial fibroblast and monocyte and the bone destruction in murine collagen-induced arthritis (CIA). Moreover, the effects of TP on cytokines and TRAF6/MAPKs/NFATc1 signaling pathway in the process of osteoclast differentiation were observed to determine the mechanism of its intervention in osteoclast differentiation.

**Methods And Materials**

**Animals**

Male SD rats weighing 180-220 g were purchased from Zhejiang Experimental Animal Center (Zhejiang, China). Male DBA/1J mice weighing 18-22 g were purchased from the Cavens Laboratory Animal Company (Changzhou, China). All animals were maintained under specific pathogen-free conditions at the Nanjing University of Chinese Medicine Experimental Animal Center. The experimental animals were acclimated for one week before the beginning of the study with free access to rodent diet and water. All experiment processes were in strict accordance with the regulations on the use and management of experimental animals of Nanjing University of Chinese Medicine.

**AIA model preparation**

The AIA rat model was prepared as previously described[25]. Complete Freund's adjuvant (CFA) (including Bacillus Calmette-Guerin (Beijing Institute of Biological Products Co. Ltd., Beijing, China) 7.5 mg/ml) was prepared, and the model of AIA was established by intradermal injection of CFA (0.05 ml) into the right hind foot of SD rats. All rats were continuously fed for 28 days and the changes of joint swelling were observed. The peripheral blood of the rats were collected on the 28th day. Then the rats were sacrificed and the synovial tissues of the joint were separated for the following experiment.

**Establishment of co-culture system of peripheral blood monocytes and synovial fibroblasts from AIA rats to induce osteoclast-like cells**

Osteoclast-like cells were induced according to the method which we have founded in the previous study [25].
The fresh anticoagulant blood of AIA rats was isolated with lymphocyte separation solution, transferred into the cell bottle and cultured for about 12h. Discarded the upper lymphocytes, and the lower adherent cells were mononuclear cells. The monocytes were then collected. The synovial tissues were washed three times with phosphate-buffered saline, and pieces of the superficial layer of synovium of about 1-2 mm³ were cut and placed in Dulbecco's modification of Eagle's medium. The tissue blocks were further divided with scissors in a 100 mm petri dish and were then incubated for 3 or 4h in 2 ml of medium (containing 0.5 ml fetal calf serum and 0.5 ml culture solution) at 37°C. The culture medium was changed every three days. After the synovial fibroblasts gradually grew into sheets, the tissues were removed. The cells were then cultured and transmitted to 3-5 generations.

2 ml of monocytes (1×10⁶ mL⁻¹) were added to the 6-well plates. Synovial fibroblasts in the logarithmic growth phase were prepared into 1×10⁵ mL⁻¹ cell suspension, which were added to the hanging transwell chamber in the 6-well plates. The cells were co-cultured in 5% CO₂ incubator at 37°C to induce osteoclast-like cells. The culture medium was changed every other day. After 21 days, the cells were identified by tartrate-resistant acid phosphatase (TRAP) (Nanjing Jiancheng Bioengineering Institute, China) staining. TRAP-positive multinucleated cells having three or more nuclei were counted as osteoclast-like cells under a microscope. On the other hand, the co-cultured osteoclast-like cells (co-culture for 14 days) (10⁴ /mL) were added to the 96-well plate with preset ivory slices (Immunodiagnostic Systems Limited, Bolton, UK). The bone fragments were taken out and observed by scanning electron microscope after 7 days.

**Detection of the activity of osteoclast-like cells**

After 21 days of co-culture, the cells were treated with different doses of TP (1, 10, 100 μg/L) in 5% CO₂ incubator at 37°C for 48h. The transwell was taken out and the absorbance was detected by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) method.

**Detection of the TRAP level and the bone absorptive capacity of osteoclast-like cells[26]**

The co-culture cells (day 21 of co-culture) were treated with different doses of TP (1, 10, 100 μg/L) for 48h, and the level of TRAP in the supernatants were detected. After co-culture for 14 days, the transwell was taken out, the lower osteoclast-like cells were digested. Then, the osteoclast-like cells (10⁴ /mL) were collected and added to the 96-well plate with preset ivory slices (Immunodiagnostic Systems Limited, Bolton, UK). After 24h of culture, the cells were treated with different doses of TP (1, 10, 100 μg/L) for 48h. Then, after changing the culture medium and continuing to culture for 3 days, the bone slices were taken out and the absorption of osteoclast-like cells in the lacunae was observed by scanning electron microscope, and the bone resorption area in the lacunae was calculated by Leica.

**Detection of IL-1, IL-34, RANKL, M-CSF and OPN levels in culture supernatant of co-culture cells using enzyme-linked immunosorbent assay (ELISA)**
The synovial fibroblasts were co-cultured with monocytes, and treated with different doses of TP (1, 10, 100 μg/L) for 48h on the 7th and 21st days of co-culture, respectively. The culture supernatants were collected to detect the levels of IL-1 (Nanjing Jiancheng Bioengineering Institute, China), IL-34 (Nanjing Jiancheng Bioengineering Institute, China), RANKL (Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China), M-CSF (Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China) and OPN (Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China) by ELISA.

**Detection of the expressions of MAPKs and TRAF6 proteins on osteoclast-like cells using Western blot [27]**

The co-cultured cells (day 21 of co-culture) were treated with different doses of TP (1, 10, 100 μg/L) for 48h. The transwell was removed, and the osteoclast-like cells were collected. The cells were washed twice with ice-cold PBS. Then, the cells were resuspended in cold lysis buffer for 30 min at 4°C and centrifuged at 12000 rpm for 10 min at 4°C. Next, the supernatants were collected and the proteins were quantified by the bicinchoninic acid (BCA) (Beyotime, Shanghai, China) method. Equal amounts of protein (25 ug) were electrophoresed on gradient 10% polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked for 1h at room temperature in 5% nonfat dry milk in TBST and incubated with primary antibodies against p-p38MAPK, p-ERK1/2, p-JNK, p38MAPK, ERK1/2, JNK, TRAF6 and β-actin (each at 1:1000 dilution) (Cell Signaling, USA) with gentle rotation overnight at 4°C. Membranes were next incubated with horseradish peroxidase (HRP) secondary antibody (1:5000 dilution) (Santa Cruz, USA) for 2h at 37°C. The membrane was added with developer, placed at room temperature with 1 min, and exposed the protein on the X-ray film in the darkroom. Develop the image in the processor.

**Detection of the expression of NFATc1 of osteoclast-like cells using immunofluorescence**

Different doses of TP (1, 10, 100 μg/L) were used to treat co-cultured cells (day 21 of co-culture) for 48h. The transwell was taken out, and immunofluorescence detection was performed as previously described[28]. The cells were plated on glass coverslips, fixed with 4% paraformaldehyde for 1-5h and permeabilized with 1% Triton X-100 for 15 min at room temperature. These samples were soaked in a cold solution of 30% H₂O₂ for 15 min after washing with PBS, and covered with 100 μL primary antibody (anti-NFATc1 (1:50, Beijing BoAo Biological Company, China), β-actin (1:1000, Cell Signaling, USA)) for 2h. Then they were added enhancer and incubated for 2h. After soaking in PBS for 5 min and washing 3 times, the osteoclast-like cells were incubated with 50 μL Cy3-conjugated secondary antibody (1:500, Jackson, PA, USA) and counterstained with 4, 6-diamidino-2-phenylindole (DAPI) (1: 500, Abcam, USA) at 37°C. All images were taken with a fluorescence microscopy (Laika, Germany).

**CIA model preparation and TP treatment**

The CIA model was prepared as previously described[28]. In brief, bovine type II collagen (CII) (Redmond, WA, USA) was dissolved in acetic acid (0.1 mol/L) at a concentration of 2 mg/mL. The bovine CII and CFA were mixed at equal volumes (final concentration of 1 mg/mL). The mixed emulsion of 100 μL CII and CFA was intradermally injected into the posterior part of the caudal root of DBA/1J mice for the first
time. On the 21st day after the first immunization, the posterior part of the caudal root of DBA/1J mice were intradermally injected with equal proportional mixture of 100 μL CII and incomplete Freund's adjuvant (IFA) (final concentration of 1 mg/mL) to enhance immunization.

After 7 days of accommodation, CIA mice were randomly divided into two groups (n= 6 per group) as follows: model and TP group. Another 6 DBA/1J mice were used as control group. The mice in TP group were intragastrically administered with 60 μg/kg of TP for 2 consecutive weeks from the 28th day after the first immunization. The mice in the control and model group received the same volume of saline. The effect of TP on joint swelling and arthritis index were observed on the 22th, 26th, 30th, 34th, 38th and 42th days after the first immunization. X-ray observation of the morphological changes of the knee joint were investigated on day 42.

**Arthritis assessments**

During the experiment, the mice were assessed regularly for signs of arthritis. Arthritis index (AI) was used as a measure of CIA. Disease severity was scored 0-4 (0=no edema or swelling, 1=swelling in toe joint, 2=swelling in metatarsophalangeal joint and foot pad, 3=swelling of the feet below the ankle joint, 4=swelling of the entire paw and joint malformation)[29]. The score was less than 16 and AI > 2 was considered as CIA. The investigator was blinded to the experimental group to avoid any bias.

**Statistical analysis**

Quantitative data were presented as the mean ± standard deviation (SD). Unpaired-Student's t-tests, one-way ANOVA with Tukey's Studentized range test and two-way ANOVA for repeated measures with Bonferroni's post hoc test were used to analyze data. Date analysis was performed using GraphPad Prism 8.0. P values <0.05 were considered statistically significant.

**Results**

**The differentiation process and morphological characteristics of osteoclast-like cells**

We have established a mature method for inducing osteoclast-like cells by co-culturing of the synovial fibroblasts and monocytes in AIA rats. The monocytes were co-cultured with synovial fibroblasts, the cells began to differentiate after 5-7 days of co-culturing, and they differentiated into osteoclast-like cells after 12-14 days (Fig.1a-d). After 14 days of co-culture with fibroblasts, monocytes were identified to differentiate into osteoclast-like cells by TRAP staining (Fig.1e). The results of scanning electron microscope showed that the osteoclast-like cells attached and grew on the bone slices, protruded flaky or filamentous pseudopodia, and bone resorption lacunae of different sizes appeared on the bone slices. It was suggested that osteoclast-like cells induced by co-culture of monocytes and synovial fibroblasts had bone resorption function (Fig.1f).

**TP inhibited the cell viability, bone resorption and the TRAP level of co-cultured cells**
The co-cultured cells (day 21 of co-culture) were treated with TP (1, 10, 100 μg/L), respectively. After 48h of culture, the viability of osteoclast-like cells and the TRAP level in culture supernatant were detected. The results showed that the cell viability and the TRAP level decreased after treating with TP (Fig. 1g, h).

In addition, osteoclast-like cells (day 14 of co-culture) were digested and cultured in 96-well plates containing ivory plates for 24h, then treated with different doses of TP (1, 10, 100 μg/L) for 48h. Then after changing the culture medium and culturing for 3 days, the absorption lacunae of osteoclast-like cells on bone plates was observed. The results showed that bone resorption lacunae of different sizes appeared on the bone plates after being cultured with osteoclast-like cells for 6 days, and TP could inhibit the area of bone resorption lacunae caused by osteoclast-like cells (Fig. 1i).

TP reduced the levels of IL-1, IL-34, RANKL, M-CSF and OPN in culture supernatant of co-culture cells

The co-cultured cells were treated with different doses of TP for 48h on the 7th and 21st days of co-culture, respectively. Then, the levels of IL-1, IL-34, RANKL, M-CSF and OPN in the culture supernatant were detected. The results showed that at different stages of osteoclast differentiation, three concentrations of TP reduced the levels of IL-1, IL-34, RANKL, M-CSF and OPN in the culture supernatant (Fig 2).

TP inhibited the expression of TRAF6, MAPKs and NFATc1 in osteoclast-like cells

Osteoclast differentiation is closely related to cytokines and signaling pathways. Our previous study found that osteoclasts were closely related to MAPK signaling pathway[21, 30].

Therefore, we continued to investigate the effect of TP on TRAF6/MAPKs/NFATc1 signaling pathway. Different doses of TP (1, 10, 100 μg/L) were used to treat the co-cultured cells (day 21 of co-culture) for 48h. Osteoclast-like cells were collected, and the expression of TRAF6 and MAPKs were detected by western blot, and the expression of NFATc1 in the nucleus was detected by immunofluorescence. As shown in Fig.3 a-f, 100 μg/L of TP inhibited the expression of TRAF6, 1, 10 and 100 μg/L of TP inhibited the expression of JNK and p-JNK, 10 and 100 μg/L of TP inhibited the expression of p38MAPK and p-p38MAPK, 100 μg/L of TP inhibited the expression of ERK. 100 μg/L of TP also reduced the expression of NFATc1 in the nucleus and inhibited the ratio of NFATc1 positive cells (Fig 4).

These results suggested that TP could play a role in the JNK, ERK and p38MAPK signaling pathways, among which the JNK pathway was the most obvious. Moreover, TP could inhibit the activation of TRAF6/MAPKs/NFATc1 pathway during the osteoclast differentiation.

TP improved arthritic symptoms and bone destruction in CIA mice

Then, we wondered if TP could improve the arthritic symptoms and bone destruction of CIA mice. As shown in Fig.5, Arthritis score and paw thickness significantly decreased in TP group. Meanwhile, on X-ray observation, TP improved bone erosion in CIA mice. The results indicated that TP improved the arthritic symptoms and bone destruction in CIA mice.
Discussion

RA is an autoimmune disease associated with bone destruction. The disruption of the balance between bone resorption and bone formation is the main cause of bone destruction[31]. Osteoclast is one of the key cells that causes bone destruction in RA. Therefore, more and more attention has been paid to the treatment of RA by inhibiting the formation, differentiation and proliferation of osteoclast.

Circulating monocytes and synovial macrophages can be induced to differentiate into functional osteoclasts under certain conditions and become the source of osteoclasts in RA joints[32]. Osteoclast differentiation is closely related to synovitis. Synovial fibroblasts in RA stimulate monocytes to differentiate into osteoclasts by releasing inflammatory cytokines such as IL-1, IL-34 and M-CSF[33-35]. Meanwhile, pro-inflammatory cytokines released by RA synovial fibroblasts stimulate the over expression of OPN in monocytes and participate in the regulation of osteoclast differentiation and maturation[8].

Cytokines and signaling pathways play an important role in the differentiation of osteoclast. IL-1 is involved in regulating the expression of OPN, M-CSF and RANKL. RANKL and M-CSF are important cytokines for osteoclast differentiation. In the presence of M-CSF, RANKL can induce monocyte to differentiate into mature multinucleated osteoclast independent of other cytokines[36]. IL-34, a new cytokine, is a ligand of colony stimulating factor 1 receptor that can be activated by M-CSF[35]. It has been reported that IL-34 can promote RANKL-mediated differentiation of osteoclast by promoting the adhesion and proliferation of osteoclast precursors[14]. OPN is a key factor in the pathogenesis of RA[37-39], and it can regulate osteoclast differentiation by affecting RANKL expression through PI3K and MEK/ERK extracellular matrix pathways[40]. It has been reported that lack of OPN can reduce the level of inflammation and reduce articular cartilage destruction[41]. All the cytokines can further affect the activation of signaling pathway. M-CSF or IL-34 induces the activation of ERK signaling pathways in osteoclast precursor or mature osteoclast[42]. After RANKL binds to RANK on osteoclast progenitor cell, the signal is transmitted downwards to TRAF6[43] and activates downstream signaling pathways, such as MAPKs[44, 45], leading to transcription induction and activation of NFATc1. Cytokines and signaling pathways form complex regulatory networks that jointly participate in the regulation of osteoclast differentiation.

In our previous study, synovial fibroblasts and peripheral blood monocytes from AIA rats were co-cultured[25]. This method successfully induced osteoclast-like cell without adding any stimulating factors, which reflected the key role of factors secreted by synovial fibroblast and monocyte in osteoclast differentiation and better reflected the pathological process of arthritis. In this study, we observed the expression of cytokines and signaling proteins in the co-culture system. The results showed that there were excessive cytokines such as IL-1, IL-34, M-CSF and RANKL in the system. Meanwhile, the expressions of TRAF6, MAPKs, and NFATc1 signaling proteins were higher in the osteoclast-like cells. Therefore, we investigated the effect and the mechanism of TP on osteoclast differentiation using the co-culture system. The results indicated that TP could decrease the levels of differentiation-related cytokines in all stages of osteoclast differentiation. This effect of TP on cytokines could further affect the
activation of TARF6/MAPKs/NFATc1 signaling pathway, which might be one of the mechanisms of its inhibition of osteoclast differentiation. Our results also showed that TP could inhibit the activation of TRAF6/MAPKs signaling pathway in osteoclast and play a role in the JNK, ERK and p38MAPK signaling pathways, among which JNK signaling pathway was the most obvious. TP might further inhibit the expression of NFATc1 in the nucleus through TRAF6/MAPKs signaling pathway to inhibit the proliferation and differentiation of osteoclast.

The effect of TP on the bone destruction of CIA was also investigated. Our data indicated that TP could significantly alleviate arthritic symptoms and bone destruction of CIA. Taken together, TP might improve bone destruction in RA through modulating osteoclast differentiation. The specific role of TP in the inhibitory mechanism of osteoclast differentiation is worthy to be further studied.

**Conclusions**

These findings suggest that TP could improve arthritic symptoms, bone destruction and inhibit osteoclast differentiation in murine arthritis. The mechanism of TP was related to the inhibition of osteoclast differentiation via TRAF6/MAPKs/NFATc1 signaling pathway. This study might provide a new strategy for RA treatment.

**Abbreviations**

AI: arthritis index; AIA: adjuvant-induced arthritis; BCA: bicinchoninic acid; CFA: complete freund's adjuvant; CIA: collagen-induced arthritis; CII: bovine type II collagen; ELISA: enzyme-linked immunosorbent assay; IFA: incomplete Freund's adjuvant; IL: interleukin; MAPK: mitogen-activated protein kinases; M-CSF: macrophage colony stimulating factor; MTT: 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide; NFATc1: nuclear factor of activated T cells 1; OPN: osteopontin; PVDF: polyvinylidene difluoride; RA: rheumatoid arthritis; RANKL: receptor activator for nuclear factor-κ B Ligand; TP: triptolide; TRAF6: tumor necrosis factor receptor-associated factor 6; TRAP: tartrate-resistant acid phosphatase; TW: Tripterygium wilfordii Hook F

**Declarations**

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**Authors' contributions**

All authors contributed to the study conception and design. Baoping Jiang, Lingling Zhou, designed the study, performed experiments, analyzed the data, and drafted the manuscript. Tianyang Liu, Jing Han, Meiyu Shen and Xueping Zhou contributed significantly to perform experiments and analyzed data.
Lingling Zhou designed the study and critically reviewed the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets used in this study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

All procedures performed in studies involving animals were approved by Ethics Committee of Nanjing University of Chinese Medicine.

**Consent to publish**

Yes.

**Competing interests**

The authors declare that they have no conflict of interest.

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**Figures**
Differentiation and morphological characteristics of osteoclast-like cells and the effect of TP on osteoclast-like cells. The monocytes (a) were co-cultured with synovial fibroblasts (b) of AIA rats, and the cells began to differentiate after 5-7 days of co-culturing (multinucleated cells appeared with flaky or filamentous pseudopodia) (c), (200× magnification). The cells differentiated into osteoclast-like cells after 12-14 days of co-culturing (200× magnification). (e) Osteoclast-like cells were identified as osteoclasts by TRAP staining (200× magnification). (f) Scanning electron microscope showed that osteoclast-like cells had bone resorption function (6000× magnification). (g) The effect of TP on viability of osteoclast-like cells was measured by MTT method. (h) The effect of TP on TRAP level of osteoclast-like cells was detected by ELISA. (i) The effect of TP on bone resorption functions of osteoclast-like cells was calculated by Leica. Data were expressed as the mean ± SD of 4 experiments. *p < 0.05, and **p < 0.01 compared with the control group.
Figure 2

Effect of TP on the levels of IL-1, IL-34, RANKL, M-CSF and OPN in culture supernatant of co-culture cells. The levels of IL-1 (a), IL-34 (b), OPN (c), M-CSF (d) and RANKL (e) in culture supernatant of synovial fibroblasts and monocytes co-cultured on the 7th and 14th days were detected by ELISA. Data were expressed as the mean ± SD of 4 experiments. *p < 0.05, and **p < 0.01 compared with the control group.
Figure 3

Effect of TP on the expression of TRAF6 and MAPKs in osteoclast-like cells were analyzed by western blot. (a) Twenty five ug of total protein from the osteoclast-like cells of the different groups were separated using SDS-PAGE, and p38MAPK, p-p38MAPK, JNK, p-JNK, ERK, p-ERK, TRAF6 were detected using western blot. The band intensities of p38MAPK and p-p38MAPK (b), JNK and p-JNK (c), ERK and p-ERK (d), TRAF6 (e) were expressed as a ratio, relative to β-actin. (f) The ratios of p-p38MAPK and p-p38MAPK, p-ERK and ERK, p-JNK and JNK band intensities were expressed as a bar diagram. Data were expressed as the mean ± SD of 3 experiments. *p < 0.05, and **p < 0.01 compared with the control group.
Figure 4

Effect of TP on the expression of NFATc1 in osteoclast-like cells using immunofluorescence. (a) Example of immunofluorescence images of osteoclast-like cells after TP treatment. Intracellular localization of NFATc1 (red) in osteoclast-like cells in each group was detected by immunostaining (200× magnification). (b) The percentage of nuclear NFATc1+ cell was calculated after nuclei (blue) staining. Data were expressed as the mean ± SD of 3 experiments. **p < 0.01 compared with the control group.
Figure 5

Effects of TP on arthritic symptoms and bone destruction in CIA mice. Arthritic scores (a) and paw thickness (b) were measured on the 22th, 26th, 30th, 34th, 38th and 42th days after the first immunization. (c) Photographs of the paws of CIA mice treated with or without TP for 2 weeks. X-ray images of each group were obtained by visualizing joints of normal, CIA and TP-treated mice at 42th day after the first immunization. Data were expressed as the mean ± SD of 6 experiments. *p < 0.05, and **p < 0.01 compared with the model group.