DAP Inhibits the TNF-α-Induced Inflammatory Response by Modulating the MAPK Signaling Pathway in Synovial Cells

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Abstract

Daphnetin(DAP) is extracted from *Daphne odora* var. *marginata* and contains coumarin compounds, which have a good anti-inflammatory analgesic effect. In this study, we investigated whether daphnetin can reduce the TNF-α-induced inflammatory response by inhibiting the MAPK signaling pathway in the synovial cells of CIA rats. A model of synovial cells was constructed using CIA rats induced by TNF-α. The expression of inflammatory cytokines in the synovial cells of CIA rats was observed by real-time PCR and ELISA. The expression and nuclear translocation of MAPK signaling pathway proteins were detected by Western blot and immunofluorescence assays. The results show that the mRNA and protein levels of IL-6, TGF-β, MMP-3 and MMP-13 were significantly lower than those in the culture supernatant of the model control group. The synovial cells of CIA rats induced by TNF-α exhibited decreased expression of p-p38, p-ERK1/2 and p-JNK in the nucleus. In conclusion, daphnetin can affect the activation of the MAPK signaling pathway and reduce the expression of inflammatory factors by inhibiting the MAPK signaling pathway, which plays a role in anti-rheumatic inflammation.

1. Introduction

Rheumatoid arthritis (RA) is a typical systemic autoimmune disease that is characterized by the abnormal hyperplasia of synovial cells, progressive infiltration of inflammatory cells in joint parts, formation of pannus, destruction of bone and cartilage and production of autoantibodies, which lead to joint deformity, loss of function, long disease course, poor prognosis, extensive disability, and serious impacts on the quality of life(1−3). The pathogenesis of RA has not been fully elucidated, but studies have shown that the abnormal activation of a signal transduction pathway is an important factor that causes abnormal proliferation of synovial cells and excessive secretion of inflammatory factors and plays an important role in the pathogenesis of RA(4−7). Mitogen-activated protein kinases (MAPK) are a group that is commonly found in most cells and can be stimulated by a variety of different extracellular stimuli, such as cytokines, hormones, neurotransmitters, cell adhesion and cell activated serine-threonine protein kinase(8). It was found that the mitogen-activated protein kinase (MAPK) pathway was significantly activated in the synovial cells of RA patients compared with those of normal subjects. The main members of p38, ERK1/2 and JNK were all found in the synovial membranes of RA patients, and these signaling molecules all exist in a phosphorylated form. Studies have shown that the MAPK signal transduction pathway has a regulatory role in the pathogenesis of RA, induction of inflammatory cytokine secretion, aggregation of chemotactic inflammatory cells, and promotion of T cell activation, affecting the proliferation, differentiation and apoptosis of a variety of cells (such as synovial cells, osteoblasts, osteoclasts)(9, 10). Therefore, the MAPK signaling pathway plays a very important role in the development and progression of RA. The MAPK pathway is likely to be an important target for drug therapy.

Natural plants contain many anti-inflammatory and antioxidant compounds that can be applied to rheumatoid arthritis and other inflammatory diseases. Daphnetin (7,8-dihydroxycoumarin), extracted from *Daphne odora* var. *marginata* (D. marginata), mainly contains coumarin compounds, which are included

in our laboratory's long-term study of active ingredients in traditional Chinese medicine, with anti-inflammatory, antibacterial, anti-tumor and other pharmacological effects(11–13). Our previous study found that daphnetin has a significant therapeutic effect on CIA rats, significantly reducing the symptoms of arthritis in CIA rats by regulating their immune function, improving Foxp3 + Treg levels in cells, balancing the ratio of Th17/Treg cells, and inducing the apoptosis of synovial cells by a Caspase-dependent pathway(14, 15). This study was based on the results of previous studies; the effect of daphnetin on the synovial membranes of TNF-α-induced CIA rat synovial cells, and the regulation of the MAPK signal transduction pathway was the focus of this study. Tumor necrosis factor-α (TNF-α) is a monocyte or megakaryocyte cell line produced by a variety of efficacies of proinflammatory factors, and there are a variety of ways to increase the inflammatory signal resulting in the over-proliferation and activation of fibroblast synovial cells. This study aimed to analyze the molecular mechanism of daphnetin against rheumatoid inflammation.

2. Materials And Methods

2.1. Chemicals and reagents

Daphnetin (DAP) standard, purity ≥ 98%, was purchased from Shanghai Source Leaf Biological Technology Co., Ltd. Triptolide (TP) standard, purity ≥ 98%, was purchased from Chengdu Pfeiffer Biotechnology Co., Ltd. Recombinant Rat TNF-α was purchased from PeproTech. NT-CROZ, anti-p38, and anti-JNK were purchased from the United States of America Abcam Corporation. Anti-phospho-p38, anti-phospho-p38, anti-phospho-p38, goat anti-mouse IgG-HRP, and goat anti-rabbit IgG-HRP were purchased from the United States KPL company. All primers were designed by the BGI gene software and tested by NCBI BLAST.

2.2. Cell culture and experimental grouping

CIA rat synovial cells were purchased from Shanghai Jinsheng Industrial Co., Ltd. (license number: J2900117872001) and were cultured with 10% FBS (Tianjin Hao Yang Biological Products Company) double anti-DMEM culture medium (Beijing Solarbio Technology Co., Ltd.) at 37°C and 5% CO_2 in an incubator with saturated humidity.

The concentration of TNF-α administered 30 min before grouping was 10 ng/ml according to the literature and preliminary experiments. The concentration of daphnetin used was 20 μg/ml, and the concentration of TP used as the follow-up experimental condition was 10 ng/ml.

2.3. Quantitative real-time polymerase chain reaction (RT-qPCR)

After administration, the cells were collected, and the total RNA was extracted using the RNAsimple Total RNA Kit. The ReverTra Ace qPCR RT Kit (TOYOBO, Japan) was used to reverse transcribe the RNA into cDNA, and the SuperReal The PreMix Plus (SYBR Green) kit (Beijing Tiangen Biochemical Technology Co.,

Ltd.) was quantified on a Real-time PCR instrument (ABI7500) (Applied Biosystems, USA) with thermal cycling as follows: 1 cycle at 95°C for 15 min; 40 cycles of 95°C for 10 seconds and 60°C for 32 seconds; and finally, a melting curve analysis was performed. The primers used are as follows(Table 1):

Table 1
Primer sequences used in real-time PCR

Target gene	Sense sequence (5'-3')	Antisense sequence (5'-3')	Product size (bp)
β-actin	TAGAGCCACCAATCCACACA	TGACAGGATGCAGAAGGAGA	104
IL-6	ACTGGTCTGTTGTGGGTGGT	AGTTGCCTTCTTGGGACTGA	102
TGF-β	AGCCCTGTATTCCGTCTCCT	ATTCCTGGCGTTACCTTGG	120
MMP-3	GGTCATCGACTCAGCCAATGC	GTTGTCAGGGGATTCTGTGG	142
MMP-13	CCCCTTCCCTATGGTGAT	AAGCCAAAGAAAGACTGC	115

2.4. Measurement of cytokines with ELISA

After grouping, the supernatants of each cell culture were collected by centrifugation, and then the ELISA kits were used (Rat IL-6 ELISA Kit was purchased from US ImmunoWay Corporation, Rat MMP-3 ELISA Kit, Rat MMP-13 ELISA Kit, Rat TGF-β ELISA Kit was purchased from Elabscience Biotechnology Co. Ltd). The expression levels of IL-6, TGF-β, MMP-3 and MMP-13 in the cell culture supernatant were measured. Samples were incubated at 37°C for 1 hour. After washing the plates, the biotinylated antibodies were added, and the plates were incubated at 37°C for 1 hour. The plates were washed, the enzyme conjugate was added, and the sample was incubated at 37°C for 30 min. Then, the plate was washed and patted dry, the TBS substrate solution was added, and the samples were incubated at 37°C in light for 10–15 min after the termination solution was added to stop the reaction. The absorbance was measured using a microplate reader at 450 nm, and the results were calculated.

2.5. Immunofluorescence

The nuclear translocation of the MAPK pathway proteins p-p38, p-ERK1/2, and p-JNK was observed. After 48 hours of treatment, the culture medium was discarded, and the climbing tablets were washed with PBS 3 times for 3 min each. The samples were incubated in solution of 4% formaldehyde in PBS at room temperature for 15 min, washed with PBS 3 times for 3 min each, and dried. Next, the samples were incubated in 0.5% Triton X-100 at room temperature for 20 min, washed with PBS 3 times for 3 min each, and dried. Samples were then incubated in 10% normal goat serum DPBS solution at room temperature in a closed container for 30 min, dried without washing, and 200 μl of a good anti-working solution per well was added. The samples were placed into a wet box and incubated at 4°C overnight. The next day, the samples were dipped in PBST 3 times for 5 min each, incubated in FITC-labeled goat anti-rabbit Ig G for 2 hours, and dipped in PBST 3 times for 3 min each. DAPI re-infected the nuclei, and the samples were immersed in PBST 4 times for 5 min each and prepared with a 50% glycerol film. A fluorescence microscope was used to observe and collect pictures.

2.6. Western blot analysis

After 48 hours of treatment, the culture medium was discarded, and the total protein was extracted with a total protein extraction kit (Shanghai Beibo Biotechnology Co., Ltd.). The protein concentration was measured with the BCA method, and the total protein was separated with 5–12% SDS-polyacrylamide gel electrophoresis. Then the expression of proteins from each condition was detected by immunolabeling with enhanced chemiluminescence to determine p-p38/p38, p-ERK1/2/ERK1/2, and p-JNK/JNK.

2.7. Statistical analysis

All results are presented as the mean ± S.E.M. Parametric data were evaluated by one-way ANOVA with SPSS statistical software. P-values less than 0.05 were considered statistically significant in all cases.

3. Results

3.1 Effects of daphnetin on the mRNA expression of IL-6, TGF-β, MMP-3 and MMP-13 in synovial cells of CIA rats induced by TNF-α

To investigate the effect of daphnetin on the mitogen-activated protein kinase (MAPK) signaling pathway in the synovial cells of CIA rats and whether this molecule plays a regulatory role in this signaling pathway, we first observed the effect of 48 hours of treatment on the expression of the MAPK-associated inflammatory cytokines IL-6, TGF- 3, and MMP-13. Our results showed that daphnetin (20 μg/ml) could significantly decrease the expression of IL-6, TGF-β, MMP-3 and MMP-13 mRNA in the synovial cells of CIA rats induced by TNF-α (Fig. 1)

3.2 Effects of daphnetin on the secretion of IL-6, TGF-β, MMP-3 and MMP-13 from the synovial cells of CIA rats induced by TNF-α

We examined the secretion of IL-6, TGF-β, MMP-3 and MMP-13 in the supernatant of cell culture with ELISA. The results showed that daphnetin (20 μg/ml) could significantly decrease the levels of MAPK-related inflammatory factors such as IL-6, TGF-β, MMP-3 and MMP-13 in the TNF-α-induced synovial cells of CIA rats (Fig. 2)

3.3 Effects of daphnetin on the expression of p-p38, p-ERK1/2 and p-JNK in the synovial cells of CIA rats induced by TNF-α

Because of the activation of the MAPK signaling pathway in the synovial cells of rheumatoid arthritis patients, the main proteins in this pathway, p38, ERK, and JNK, can be found in the synoviocytes of rheumatoid arthritis patients, and these proteins all exist in their phosphorylated form. When p38, ERK1/2, and JNK were phosphorylated and activate in the nucleus, they regulate important pathophysiological processes of cells. Therefore, we used cell immunofluorescence to detect the effect of daphnetin on TNF-α-induced CIA rat synoviocytes ($P > 0.05$), and the expression levels of p-ERK1/2 and p-JNK were decreased. The effect of p-JNK was especially notable (Fig. 3a, b, c).

3.4 Effects of daphnetin on the expression of p38, ERK1/2, and JNK and their phosphorylation ratios in the synovial cells of CIA rats induced by TNF-α

p38, ERK1/2, JNK are the three most important families in the study of the MAPK signaling pathway. P38, ERK1/2, and JNK phosphorylation and activation regulate the vital life process of cells; the phosphorylation level of proteins in a pathway and the regulation of that pathway are closely related. To investigate the effect of daphnetin on the MAPK signaling pathway in the synovial cells of CIA rats, we detected the expression of the MAPK signaling pathway factors p-p38/p38 and p-ERK1 in the synovial cells of CIA rats induced by TNF-α with Western blot analysis ($P < 0.05$). The expression of p-p38, p-ERK1/2 and p-JNK were significantly decreased as determined by the Western blot results. The results showed that the expression levels of p-p38, p-ERK1/2 as well as the phosphorylation ratios p-JNK/JNK, p-p38/p38, p-ERK1/2/ERK1/2 were also significantly reduced (Fig. 4).

4. Discussion

Although many experts and scholars have researched the pathogenesis of RA from genetic, pathophysiological, immunological and other aspects for many years, the specific pathogenesis of RA has not yet been fully elucidated(16–18). Studies have shown that fibroblast-like synoviocytes (FLS) is the main cell in synovial hyperplasia. The normal synovial tissue is usually composed of a 1–3 layers of cells; however, through a variety of stimulating activation, proliferation and expansion, this tissue reaches 10–15 layers of cells with the infiltration of a variety of inflammatory cells(19). The proliferation of activated synovial cells continues to produce a variety of inflammatory factors and proteases, such as matrix metalloproteinases (MMP), accelerating the migration and invasion of FLS and the destruction of articular cartilage. At the cellular level, the process of synovial cell proliferation is divided into three steps: first, the extracellular stimulus signal; then intracellular signal transduction; and finally, nuclear translocation and transcriptional activation in the nucleus. IL-1, TNF-α, EGF, TGF-β and other cytokines can stimulate the proliferation of synovial cells, and the gene expression induced by different stimuli is not the same, due to the signal transduction pathway that is initiated. Studies have shown that the abnormal activation of signal transduction pathways is an important factor in the abnormal proliferation of synovial cells and the excessive secretion of inflammatory factors, which plays an important role in the pathogenesis of RA(19).

Mitogen-activated protein kinases (MAPK) are a group of proteins that is commonly found in most cells and can be stimulated by a variety of different extracellular stimuli, such as cytokines, hormones, neurotransmitters, cell adhesion and cell activated serine-threonine protein kinase. These proteins regulate cell proliferation and differentiation, apoptosis, growth, cytokine secretion, cellular recombination and metabolism and other important biological activities. At present, a large number of studies have confirmed that the synovial cells of RA patients exhibit abnormal activation of the MAPK signaling pathway. The main members of the MAPK pathway, p38, ERK1/2 and JNK, can be found in RA patients with joint synovial lesions, and these proteins are phosphorylated. Studies have shown that the MAPK signal transduction pathway regulated the induction of inflammatory cytokine secretion, aggregation of

chemotactic inflammatory cells, and promotion of T cell activation, which in turn have a regulatory role in the pathogenesis of RA and affects the proliferation, differentiation and apoptosis a variety of cells (such as synovial cells, osteoblasts, osteoclasts)(10).

Effects of Daphnetin on MAPK Signaling Pathway in Synoviocytes of CIA Rats Induced by TNF – α On the expression of MAPK related cytokines

IL-6 is a multicellular cytokine that is a key cytokine in RA. The pathogenic effects of IL-6 on RA mainly promote the differentiation of T cells and B cells associated with inflammation. The acute phase of this response promotes protein synthesis induces osteoclast differentiation and the loss of joint tissue, and IL-1 is synergistically induced by the production of MMP, resulting in increased bone and cartilage damage(20). Studies have shown that RA patients with significantly increased serum levels of TGF-β. TGF-β is a growth factor with multiple effects that can induce the secretion of the extracellular matrix and is involved in the expression of inflammatory factors and the differentiation of a variety of immune cells. The activation and proliferation of macrophages and fibroblasts have an important impact promoting angiogenesis, pannus formation, and synovial fibrosis(21). The expression of MMP-3 and MMP-13 in the synovial fluid and synovium of patients with active RA was significantly increased compared to controls. MMP-3, also known as matrix lysin 1, is secreted by synovial cells, chondrocytes, etc., can make collagen types II, III, IV, IX, and XI and multiple proteoglycan matrices, degrades laminin, and is positively correlated with RA. MMP-13, also known as collagenase-3, is mainly secreted by chondrocytes and has the highest degradation efficiency for type II collagen, which is the most abundant collagen in the cartilage matrix. Therefore, MMP-13 is also the limiting enzyme in the speed of matrix collagen degradation. IL-6, TGF-β, MMP-3 and MMP-13 are all cytokines regulated by the MAPK signaling pathway.

Our study found that daphnetin was able to significantly downregulate the expression of IL-6, TGF-β, MMP-3 and MMP-13 mRNA in the synovial cells of CIA rats induced by TNF-α. Additionally, we detected the secretion of IL-6, TGF-β, MMP-3 and MMP-13 in the supernatant of the synovial cells of CIA rats with ELISA and found that the results were consistent with the gene expression analysis. This reduction of the inflammatory response and tissue cell damage by daphnetin is of great significance.

Effects of daphnetin on MAPK pathway protein expression, phosphorylation and nuclear translocation in CIA rat synoviocytes induced by TNF-α

The MAPK signaling pathway undergoes stepwise activation after various stimuli. The phosphorylation of the pathway protein is transferred to the nucleus to be activated, and the activated protein regulates the transcription, translation and synthesis of the downstream proteins and participates in the important life processes in the cell. We investigated the effects of daphnetin on the protein expression and nuclear translocation of the MAPK signaling pathway molecules p-p38, p-ERK1/2, p-JNK in the synovial cells of CIA rats induced by TNF-α with an immunofluorescence assay.

The results showed that the p38, ERK1/2, JNK proteins were significantly activated in the model group and the C group. Additionally, phosphorylated p38, ERK1/2 and JNK were translocated into the nucleus,

and their expression levels in the nucleus were stronger than those in the cytoplasm. The expression of p-p38, p-ERK1/2 and p-JNK was significantly decreased in the nucleus compared with that in the cytoplasm and compared with the expression of p38, ERK1/2 and JNK, respectively. The p-ERK1/2-FITC fluorescence intensity was significantly lower in the nucleus than in the cytoplasm. The expression and phosphorylation of MAPK pathway proteins were detected by Western blot analysis. We found that the p-p38, p-ERK1/2, and p-JNK protein expression levels in the model group were significantly increased, while daphnetin significantly reduced the expression of p38, ERK1/2, and JNK in the synovial cells of CIA rats induced by TNF- α. The phosphorylation levels of ERK1/2 and JNK were lower in the DAP group than in the C and TP groups, and there was no significant difference in p38 phosphorylation level between the C and TP groups.

p-p38, p-ERK1/2, p-JNK immunofluorescence and Western blot results confirmed that daphnetin could inhibit the phosphorylation and activation of the MAPK signal transduction pathway, which affected the regulation of various biological activities of cells by MAPK and was closely related to the pharmacological effects of daphnetin.

5. Conclusions

Daphnetin can significantly inhibit the phosphorylation and activation of the MAPK signaling pathway in the synovial cells of CIA rats induced by TNF-α and decrease the expression level of inflammatory cytokines regulated by the MAPK signaling pathway. It is suggested that daphnetin can regulate the MAPK signaling pathway, inhibiting the activation of this pathway and thereby affecting the important biological processes of cells to inhibit rheumatic inflammation.

Our study was only to explore the effect of daphnetin on the MAPK signaling pathway in the synovial cells of CIA rats induced by TNF-α. We also continue to study the specific sites and mechanisms of daphnetin. In addition, we are further exploring whether rhenium can exert anti-rheumatic inflammatory effects through other RA-related signal transduction pathways.

Declarations

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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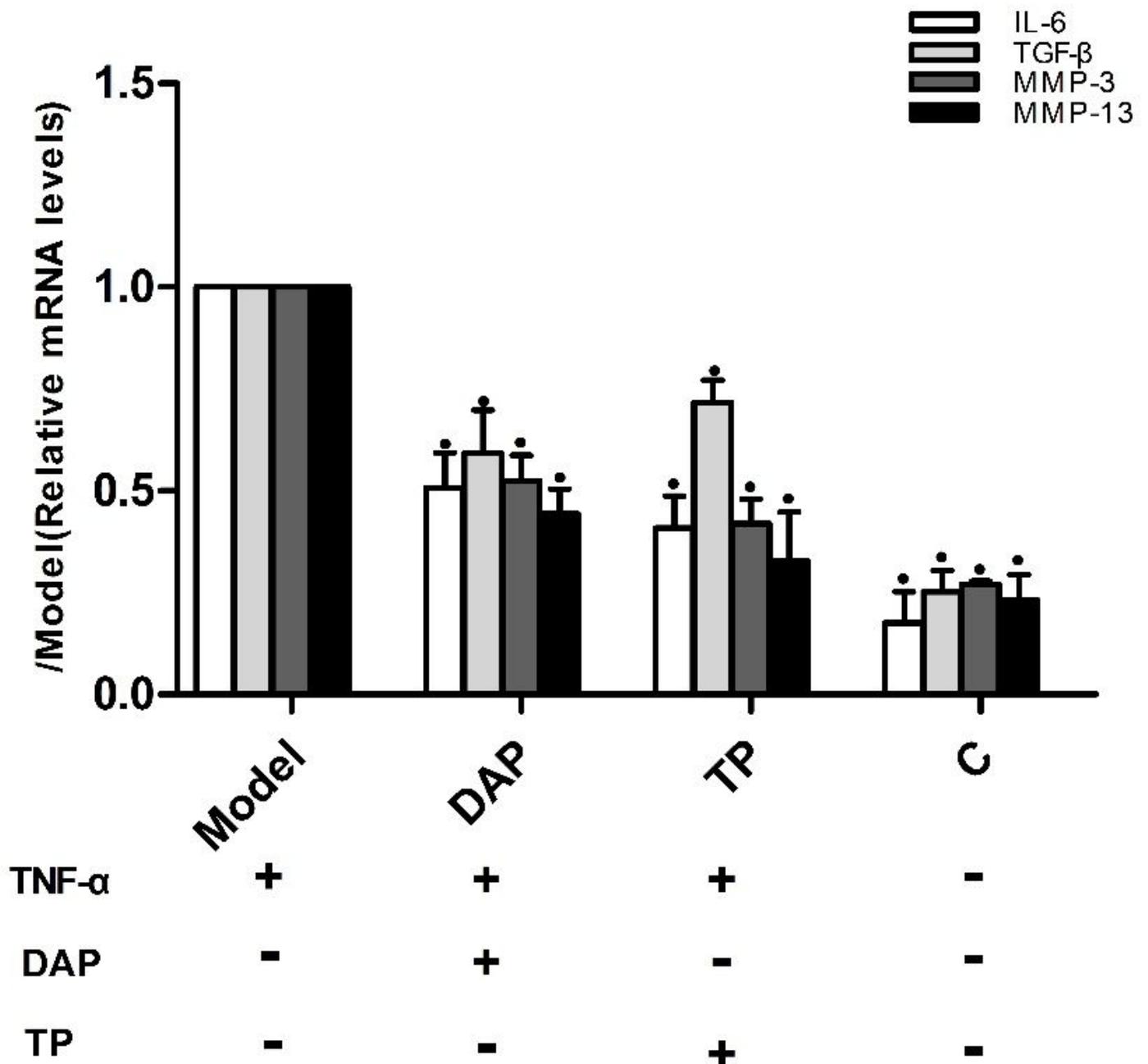
Figures

Figure 1

The relative expression of IL-6, TGF-β, MMP-3 and MMP-13 mRNA in the synovial cells of CIA rats. The expression of IL-6, TGF-β, MMP-3 and MMP-13 mRNA was significantly increased after TNF-α stimulation (P <0.05). The expression of IL-6, TGF-β, MMP-3 and MMP-13 mRNA in the DAP group was significantly lower than that in the model group (P <0.05). There was no significant difference between the two groups (P> 0.05). All experiments were performed in triplicate and repeated three times. The data are presented as the means ± SEM and were analyzed by one-way ANOVA (n=9/group). ●P < 0.05 compared with the model group.
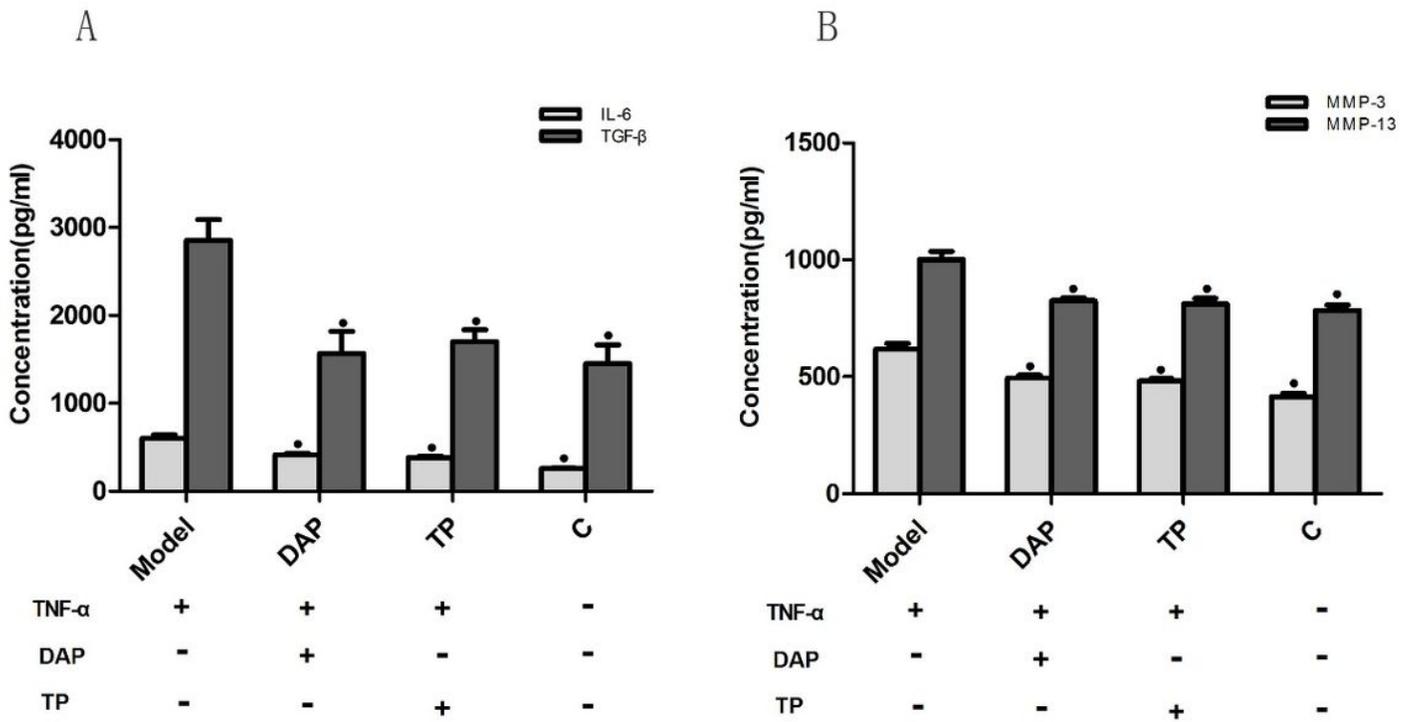
Figure 2

Cytokines in the culture supernatant. (A) IL-6 and TGF-β; (B) MMP-3 and MMP-13. The levels of IL-6, TGF-β, MMP-3 and MMP-13 in the DAP group were significantly lower than those in the model group (P <0.05), but there was no significant difference between the two groups (P> 0.05). The secretion of TGF-β and MMP-13 was close to that of group C (P> 0.05). All experiments were performed in triplicate and repeated three times. The data are presented as the means ± SEM and were analyzed by one-way ANOVA (n=9/group). ●P < 0.05 compared with the model group.
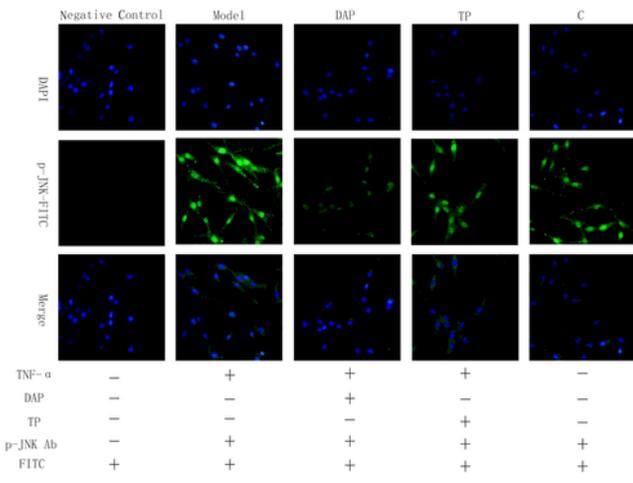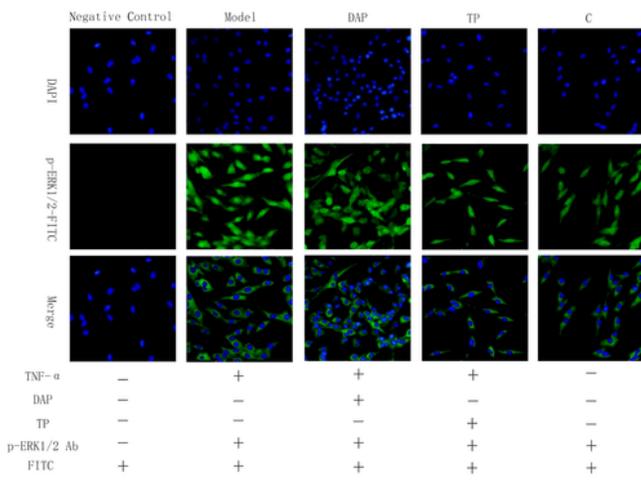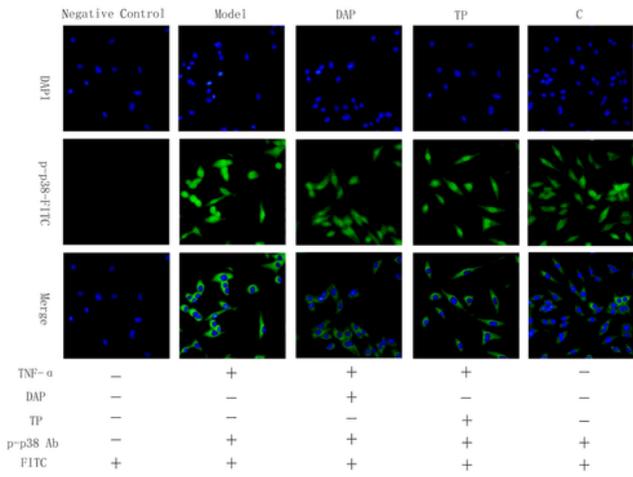
Figure 3

Immunofluorescence of p- p38, p-ERK1/2, and p-JNK in the synovial cells of CIA rats. The p-p38, p-ERK1/2 and p-JNK proteins were labeled with FITC and green fluorescence. The fluorescence intensities of p-p38, p-ERK1/2 and p-JNK in the DAP group was significantly higher than those in the model group. The fluorescence intensity of p- p38, p-ERK1/2 and p-JNK and the expression of it in the nucleus was

significantly reduced. p-ERK1/2 nuclear translocation was significantly inhibited, as shown by the significantly lower p-ERK1/2-FITC fluorescence in the nucleus than in the cytoplasm(n=3/group).
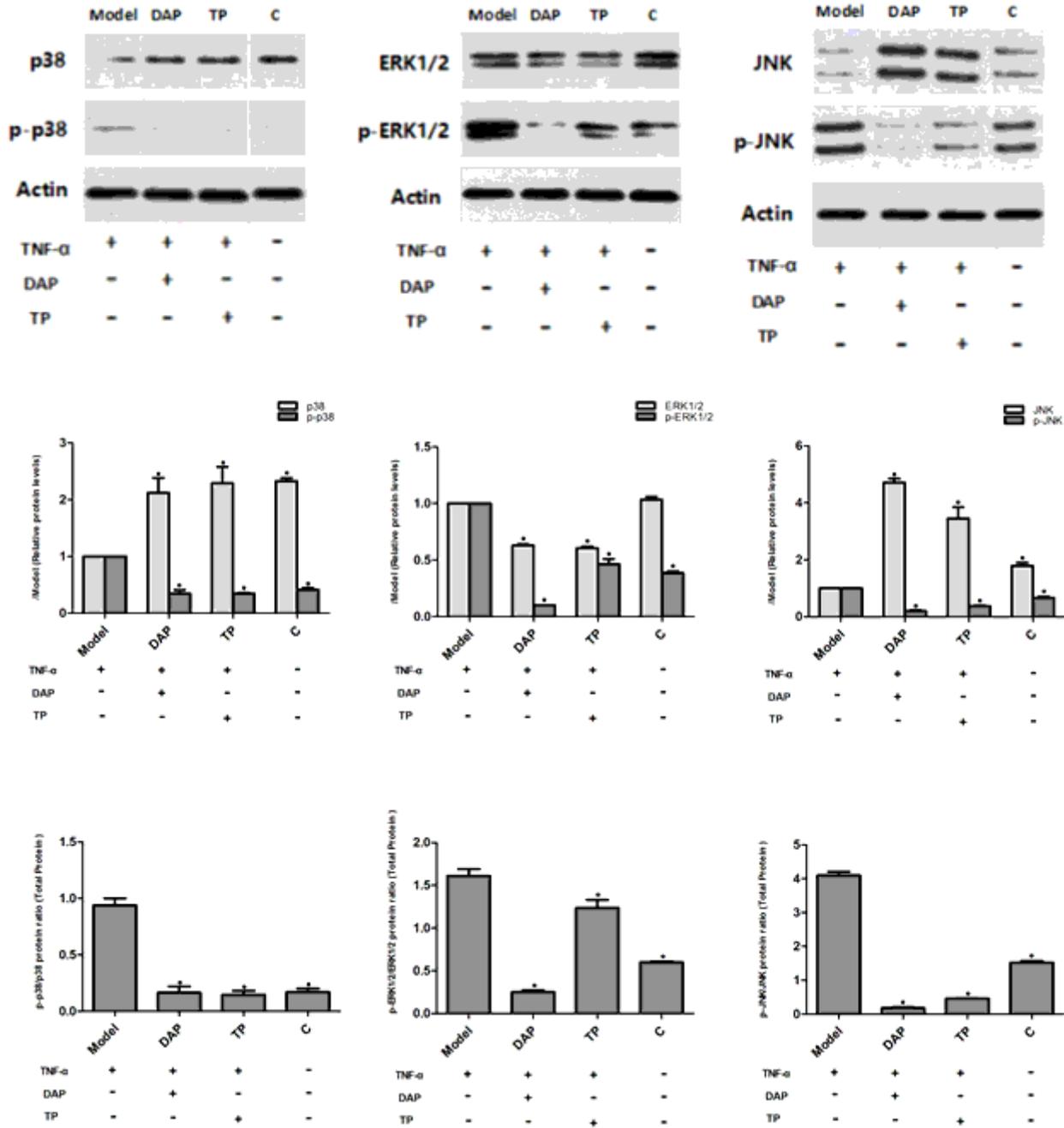

Figure 4

Western blot results and relative protein expression. The expression of the MAPK signaling pathway molecules p38, ERK1/2, JNK and their phosphorylation ratios in the synovial cells of CIA rats induced by TNF-α were detected by Western blotting. The expression of p-p38, p-ERK1/2 and p-JNK was significantly increased after induction with TNF-α, and the expression of p-p38, p-ERK1/2 and p-JNK protein was significantly decreased with daphnetin treatment. The expression of the p-p38/p38, p-ERK1/2/ERK1/2, p-JNK/JNK protein ratios in the DAP group was also significantly lower than that in the model group (P <0.05). The ratio of p-p38/p38 protein expression was significantly lower in the DAP group than in the C

group and TP group (P> 0.05). All experiments were performed in triplicate and repeated three times. The data are presented as the means ± SEM and were analyzed by one-way ANOVA (n=9/group). ●P < 0.05 compared with the model group.