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Imperatorin Inhibits Proliferation, Migration, and Inflammation *via* Blocking the NF-κB and MAPK Pathways in Rheumatoid Fibroblast-like Synoviocytes

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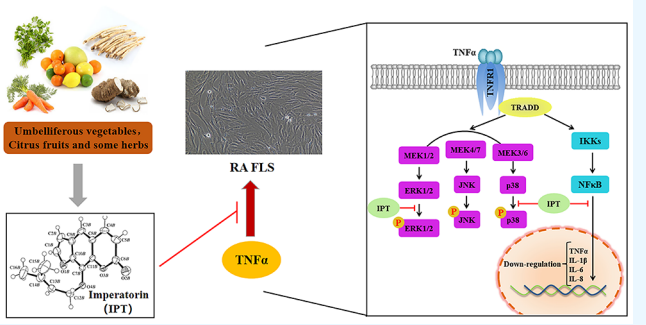
ABSTRACT: Rheumatoid arthritis (RA) is a chronic joint inflammatory disease associated with the aberrant activation of fibroblast-like synoviocytes (FLSs). Searching for natural compounds that may suppress the activation of FLSs has become a complementary approach for RA treatment. Here, we investigated the effects and mechanisms of imperatorin (IPT) on proliferation, migration, and inflammation in primary cultured arthritic FLSs. We found that IPT significantly suppressed TNFα-induced proliferation and migration of arthritic FLSs, but showed little effect on survival and apoptosis. In addition, IPT treatment significantly reduced the TNFα-induced expression of pro-inflammatory cytokines (IL-1β, TNFα, IL-6, and IL-8) in arthritic FLSs. Further mechanism studies suggested that IPT inhibited the activations of p38 and extracellular signal-regulated kinase (ERK). Also, IPT blocked the nuclear factor of κB (NF-κB) activation by suppressing the phosphorylation and degradation of IκBα, thereby preventing the translocation of p65. Collectively, our results demonstrated that IPT could inhibit the over-activated phenotypes of arthritic FLSs *via* the mitogen-activated protein kinase (MAPK) (p38 and ERK) and NF-κB pathways leading to the down-regulation of pro-inflammatory cytokines, which might be beneficial to the anti-proliferative and anti-migratory activities of FLS cells. These findings suggest that IPT has the potential to be developed as a novel agent for RA treatment.



1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease accompanied by long-term synovitis and destruction of cartilage and bone.[1] Although multiple cell types are involved in the pathological process of RA, fibroblast-like synoviocytes (FLSs) have been considered to play crucial roles in both joint damage and the propagation of inflammation.[2,3] FLSs in RA are over-activated and exhibit a uniquely aggressive and transformed phenotype, which is characterized by increased proliferation and migration, evasion of apoptosis, and overproduction of inflammatory cytokines and catabolic enzymes.[3−6] These destructive properties of FLSs have been demonstrated to tightly correlate with histological and radiographic damage in RA and its rodent models.[7−9] Thus, targeting FLSs has been considered to improve clinical outcomes in inflammatory arthritis without suppressing systemic immunity.

It is well known that early diagnosis and treatment are important for the effective management of RA. Current clinical diagnosis of RA often relies on serological tests and imaging techniques such as ultrasonography and magnetic resonance imaging (MRI). However, these conventional methods suffer from low sensitivity and specificity, poor spatiotemporal resolution, and the danger of radiation for early diagnosis. With the gradually wide application of luminescence imaging in biological research and clinical practice, fluorescence optical imaging (FOI) has now become a promising alternative tool for the detection and monitoring of early RA due to its excellent sensitivity, real-time detection, and easy manipulation.[10−13] As RA is varied and vexing, the treatment is still the biggest problem we are facing. Non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying antirheumatic drugs (DMARDs), and biologics are traditionally used in the treatment of RA. NSAIDs and glucocorticoids can effectively reduce inflammation response and control the symptoms; however, these drugs can not block the progression of RA.[14] DMARDs and biologics can prevent the process of bone

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destruction, but long-term use may cause obvious side effects. Furthermore, expensive prices prevent most patients from purchasing biologics. Therefore, the development of new drugs with better curative effects and fewer side effects is becoming an area of active research in RA drug treatment.

Imperatorin (IPT), a naturally occurring furanocoumarin, can be found in citrus fruits, umbelliferous vegetables, and some herbs such as *Angelica dahurica*, *Angelica archangelica*, and *Glehnia littoralis*. This compound has been demonstrated to possess various pharmacological activities such as anticancer,[15] analgesic,[16] antioxidation, anti-inflammation,[17] and diastolic blood vessels.[18] Zhai et al. found that IPT is one of the major active ingredients in the Fengshiding capsule, which is a widely used traditional Chinese medicine for the treatment of RA.[19] In addition, IPT has been reported to protect against collagen-induced arthritis in rats.[20] However, the mechanism of its anti-arthritic action is not yet fully known. Given the therapeutical role of IPT in RA and the importance of FLSs in RA development, we investigated the effect of IPT on pathogenic behaviors of arthritic FLSs and further explored its underlying molecular mechanisms.

2. RESULTS

2.1. Effect of IPT on the Viability of Arthritic FLSs. To explore whether IPT has an inhibitory action on the pathogenic behaviors of FLSs, we first isolated, cultured, and identified arthritic FLSs from the knee synovium of collagen-induced arthritis (CIA) rats. As shown in Figure 1, the isolated cells

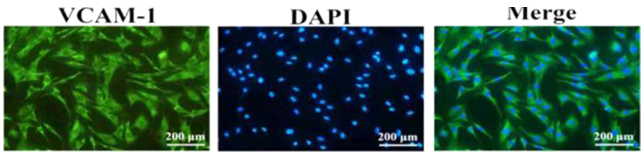

Figure 1. Identification of primary cultured arthritic FLS cells. The cells were isolated from the knee synovium of CIA rats and cultured in the Dulbecco's modified Eagle's medium (DMEM) complete medium. These cells were identified by VCAM-1 and 4′,6-diamidino-2-phenylindole (DAPI) immunofluorescence staining.

displayed a spindle shape which was consistent with FLS morphological feature. Immunofluorescence staining showed that there were more than 98% of cells expressing VCAM-1, suggesting that these cultured cells corresponded most likely to the intimal subpopulation of FLSs (Figure 1). We used these primary cultured arthritic FLS for the subsequent experiments.

To evaluate the possible cytotoxic effect of IPT on arthritic FLSs, an MTX assay was performed. As shown in Figure 2B, IPT had no significant effect on the viability of FLS cells, even at a concentration as high as 160 μM. In the subsequent experiments, concentrations of IPT used were less than 10 μM.

2.2. Effect of IPT on Proliferation and Apoptosis of Arthritic FLSs. The synovial tissues of RA patients are well known to be abnormally hyperplastic due to the enhanced proliferative property of FLSs in an inflammatory environment. Thus, suppression of FLS's proliferation has been proposed as a potential therapeutic strategy for RA. In this study, the 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay showed that TNFα stimulation dramatically increased the proliferative potential of arthritic FLSs; however, this increase was significantly inhibited by 2.5 μM IPT (Figure 2C,D). In addition, we also detected the effect of IPT on synovial cell apoptosis by flow cytometry. As shown in Figure 2E, IPT at the given doses had little effect on apoptosis of arthritic FLSs.

2.3. Effect of IPT on Migration Ability of Arthritic FLSs. The migration of RA FLSs is a critical process in cartilage and bone destruction and functionally differs from proliferation. In view of this, we analyzed the effect of IPT on TNF-induced migration by a wound healing assay. As shown in Figure 2F,G, the migration area of arthritic FLSs was significantly increased upon TNFα induction, while IPT significantly suppressed this increased migration ability at doses greater than 1 μM.

2.4. Effect of IPT on the Expression of Pro-inflammatory Mediators in Arthritic FLSs. In the pathogenesis of RA, synovial cells can produce a large number of cytokines which promote the occurrence and development of RA by acting on a variety of cells and regulating each other to form a complex network. To assess the inhibitory effect of IPT on pro-inflammatory cytokine production, we examined the mRNA expression of *TNFα*, *IL-1β*, *IL-6*, and *IL-8* in TNFα-induced arthritic FLSs. As expected, the transcripts of these pro-inflammatory cytokines were markedly induced after TNFα stimulation. However, the induction was significantly suppressed by IPT treatment (Figure 3A−D).

IL-1β and IL-6 have been demonstrated to play crucial roles in the regulation of the intra-articular microenvironment during RA development.[21,22] Higher levels of IL-1β and IL-6 were found in the serum and synovial fluid of RA patients compared with those of healthy controls.[23] Additionally, the IL-1β inhibitor canakinumab and the IL-6 inhibitor tocilizumab have already been developed in clinics to treat RA patients.[24,25] Therefore, we detected the protein levels of IL-1β and IL-6 in the culture supernatant using ELISA. Consistent with the real-time PCR data, ELISA results showed that IPT treatment significantly suppressed TNFα-induced secretion of IL-1β and IL-6 (Figure 3E,F).

2.5. Effect of IPT on TNFα-Induced Activations of Nuclear Factor of κB (NF-κB) and Mitogen-Activated Protein Kinases (MAPKs). To reveal the mechanisms through which IPT suppressed the pathologic phenotypes of arthritic FLSs, TNFα-induced MAPK signaling pathways were investigated. As shown in Figure 4A,B, the phosphorylations of the extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) were clearly up-regulated after stimulation with TNFα. However, IPT treatment robustly suppressed TNFα-induced activations of ERK and p38. In contrast, IPT had little effect on TNFα-induced JNK phosphorylation (Figure 4A,B). In addition to MAPK signaling pathways, we also tested the activation of NF-κB, which has been demonstrated to play a crucial role in the development and progression of RA.[26,27] As shown in Figure 4C,D, IκBα was effectively phosphorylated and degraded upon TNFα stimulation. However, IPT treatment significantly suppressed the phosphorylation and degradation of IκBα (Figure 4C,D). It is well known that IκB binds to NF-κB in the cytoplasm of normal cells and inhibits NF-κB entry into the nucleus. TNFα-stimulated IκBα degradation can release NF-κB protein (such as p65) into the nucleus and trigger the expression of a subset of NF-κB target genes. In our study, NF-κB p65 was clearly observed to translocate into the nuclei upon TNFα stimulation for 30 min (Figure 4E). And IPT substantially inhibited p65 nuclear translocation, as evidenced by the retention in the cytoplasm of the p65 proteins (Figure 4E).

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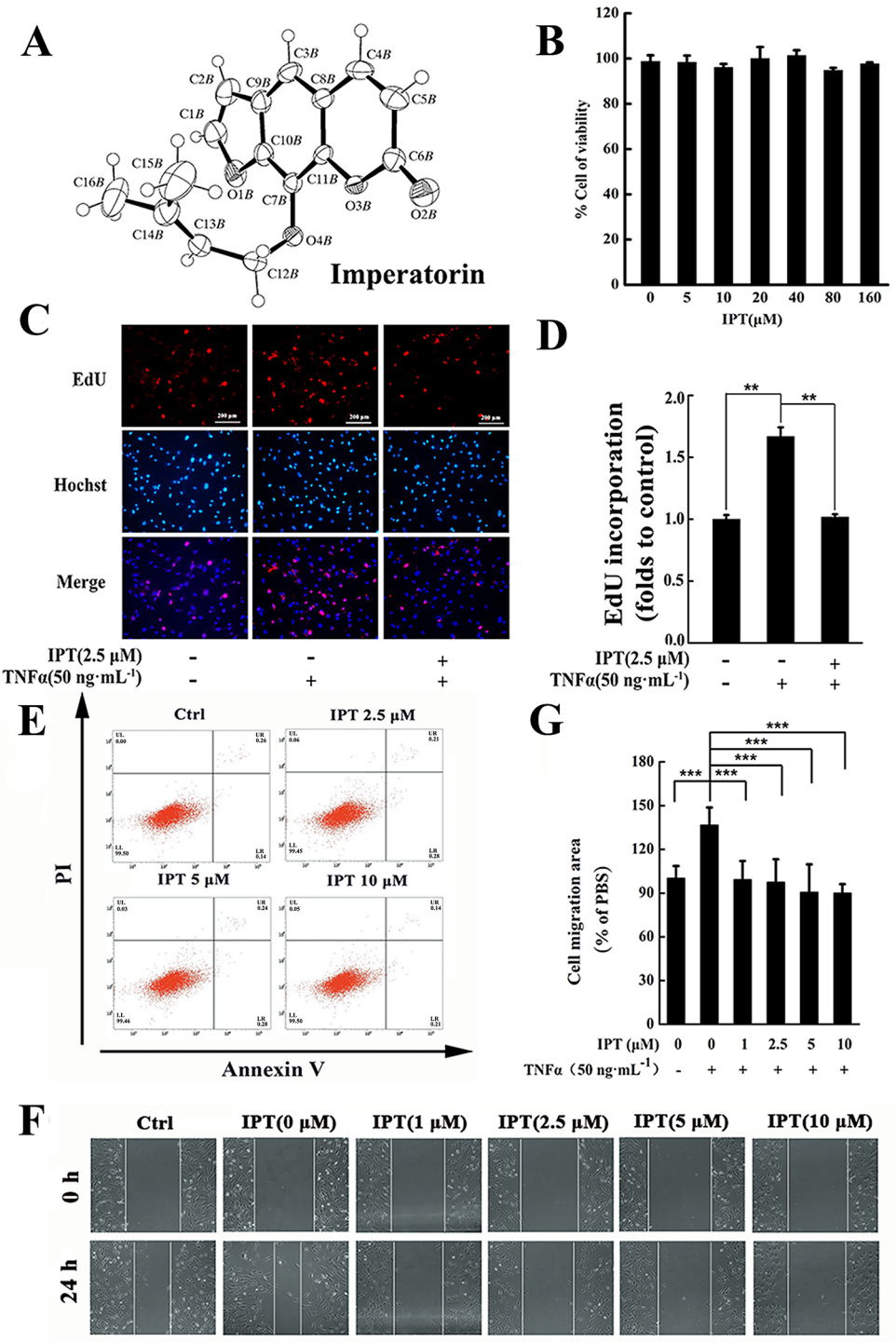

Figure 2. IPT treatment decreases TNFα-stimulated proliferation and migration in cultured arthritic FLS. (A) Chemical structure of imperatorin (IPT). (B) Effect of IPT on FLS viability was assessed by an MTS assay. Data are presented as means ± standard deviation (SD) of three independent experiments. (C, D) 5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay showed that IPT (2.5 μM) significantly suppressed TNFα-induced proliferation of arthritic FLS cells. Data are presented as means ± SD of three independent experiments (**$P < 0.01$). (E) IPT showed little effect on apoptosis of arthritic FLSs. Arthritic cells were incubated with different concentrations of IPT for 24 h, and then an apoptosis assay was performed by flow cytometry. (F, G) IPT reduced the TNFα-induced migration of FLS cells. Arthritic FLSs were pre-treated with varying concentrations of IPT for 1 h and then stimulated with TNFα (50 ng·mL^{-1}) for 24 h. Cell migration was then measured by a wound healing assay. Data are presented as means ± SD of three independent experiments (***$P < 0.001$).

3. DISCUSSION

Synovitis, the predominant pathological change of RA, is characterized by the abnormal proliferation and migration of FLSs, which lead to hyperplasia of synovial tissues and the formation of pannus, with subsequent bone and cartilage destruction.[28] Under normal physiological conditions, FLSs are located in the lining of the synovium, which is involved in regulating the function of leukocytes, nourishing the joint environment, and remodeling the matrix in tissue injury.[29,30] In

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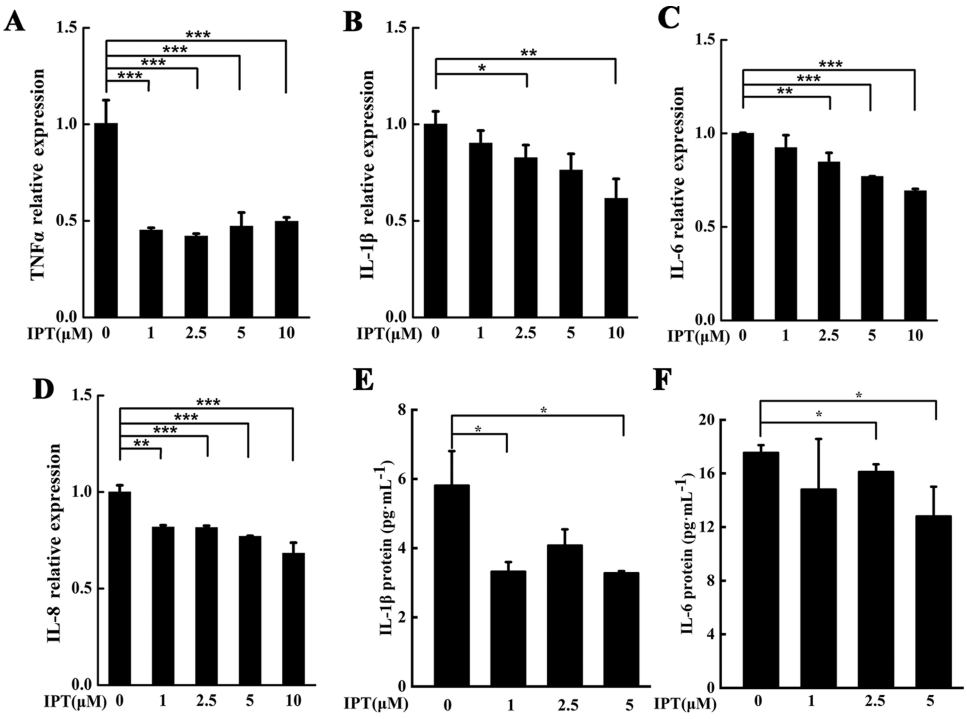

Figure 3. IPT significantly suppresses the expression of pro-inflammatory cytokines in TNFα-induced arthritic FLS cells. Arthritic FLSs were pre-treated with different concentrations of IPT for 1 h and then stimulated with TNFα (50 ng·mL^{-1}) for 24 h. (A–D) Transcriptional levels of pro-inflammatory cytokines were determined by real-time polymerase chain reaction (PCR), including *TNFα*, *IL-1β*, *IL-6*, and *IL-8*. The expression of each target gene was calculated as a relative expression to *β-actin* and represented as a fold change over the TNFα-treated, IPT-untreated cells. Data are presented as means ± SD of three independent experiments (*P < 0.05, **P < 0.01, and ***P < 0.001). (E, F) Protein levels of IL-1β and IL-6 in the culture supernatant were detected by an enzyme-linked immunosorbent assay (ELISA) method. Data are presented as means ± SD of three independent experiments (*P < 0.05).

RA patients with inflamed synovium, FLSs are activated and show a lot of tumor-like biologic behaviors, such as excessive proliferation, apoptosis resistance, escape of growth inhibition, and enhancement of migration rate. Moreover, RA FLSs can also produce and secrete inflammatory mediators that lead to recruitment and activation of immune and non-immune cells along with angiogenesis induction resulting in joint damage.[31] Thus, inhibiting RA FLS proliferation, migration, and over-production of inflammatory cytokines may be a promising strategy for RA treatment.

Primary FLSs from RA patients have commonly been used to study the effects of a variety of drugs and phytochemicals; however, they present certain inconveniences. These RA-derived FLSs produce a broad range of results due to the individual responses of each patient sample,[32] and it is routinely difficult for many labs to acquire synovial tissue samples from RA patients. Thus, in our study, we established a CIA rat model and isolated FLSs from the knee synovium of rats with CIA, as in our previous reports.[33,34] Cell morphology and VCAM-1 immunofluorescence staining both demonstrated that these primary cultured cells belonged to the intimal subpopulation of FLSs. Using these arthritic FLSs, we found that IPT significantly suppressed TNFα-induced proliferation and migration. This suppression of IPT was not due to its cytotoxicity and apoptosis-inducing effect, which could be demonstrated by the MTT assay and flow cytometry. IPT was reported to adequately suppress synovial hyperplasia and pannus formation of collagen-induced arthritis in rats.[20] As we know, the over-proliferation and increased migration of RA FLSs are the main causes of synovial hyperplasia and invasive pannus formation. Therefore, our result could, at least in part, interpret the therapeutic action of IPT in CIA rats reported by the previous study.[20]

Previous studies have demonstrated that IPT has an anti-inflammatory property in multiple cell types and animal models.[35−37] Huang et al. reported that IPT could suppress the protein expression of iNOS and COX2 in LPS-stimulated RAW264.7 cells and thereby inhibited carrageenan-induced paw edema in mice.[36] Zhang et al. demonstrated that oral administration of IPT significantly inhibited inflammatory reactions in different animal models (dimethylbenzene-induced ear edema, acetic acid-induced vascular permeability, and cotton pellet-induced granuloma) and reduced the release of TNFα, IL-1β, and IL-6 through blocking the NF-κB pathway.[37] In consistency with these previous reports, real-time results showed that IPT effectively decreased the expression of *TNFα*, *IL-1β*, *IL-6*, and *IL-8* in TNFα-induced arthritic FLSs. This was further confirmed by the ELISA data. Notably, RA is a multifactorial disease, and multiple other cytokines such as IL-18 and TGFβ are also involved in the pathogenesis of RA.[38,39] Zhang et al. reported that IPT significantly decreased LPS-induced expression of IL-18 and TGFβ1 in FLSs, and thereby effectively inhibited synovitis and synovial fibrosis in the monosodium iodoacetate-induced osteoarthritis rat model.[40] Therefore, we believed that IPT might exert an anti-RA action by inhibiting multiple inflammatory cytokines, including IL-18 and TGFβ1. Compared with the current single mediator therapy such as biological agents, this capacity of IPT to down-regulate a wide spectrum of inflammatory cytokines might have a therapeutic advantage.

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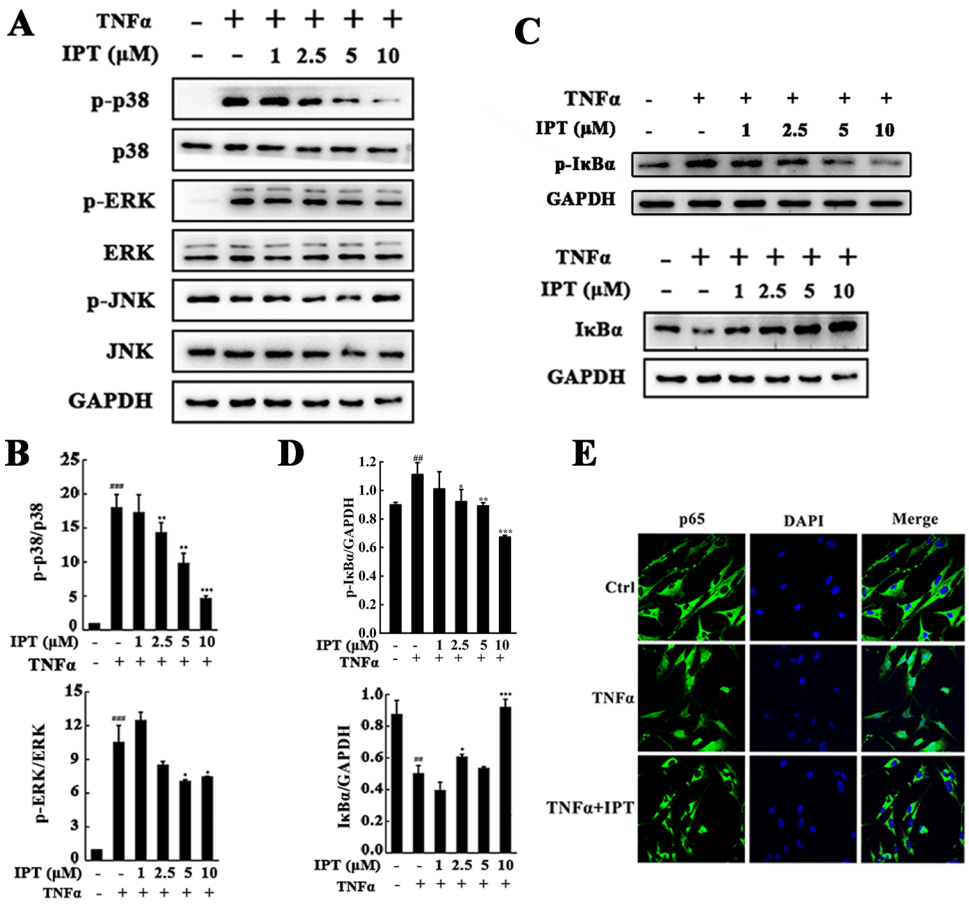

Figure 4. IPT suppresses TNFα-stimulated activations of MAPKs (ERK and p38) and NF-κB in arthritic FLS cells. (A, C) Arthritic FLS cells were pre-treated with different doses of IPT for 1 h and then stimulated with TNFα (50 ng·mL^{-1}) for 15 min. Protein was then extracted for Western blot analysis using antibodies against p-ERK1/2, total ERK1/2, p-p38, total p38, p-JNK, total JNK, p-IκBα, IκBα, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (B, D) Relative expressions of p-p38, p-ERK, p-IκBα, and IκBα were determined by densitometric analysis. Data are presented as means ± SD of three independent experiments ($^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs TNFα-untreated, IPT-untreated controls; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs TNFα-treated, IPT-untreated cells). (E) Arthritic FLS cells were pre-treated with IPT (5 μM) for 4 h and then induced with TNFα (50 ng·mL^{-1}) for 30 min. The localization of p65 was visualized by immunofluorescence analysis.

As we know, IPT has several analogies, such as isoimperatorin, alloimperatorin, and xanthotoxol. Although the effects of these analogies on the pathogenic behaviors of TNFα-induced arthritic FLSs still remain unknown, similar inhibitory functions have been demonstrated in other cell types. Isoimperatorin was found to significantly inhibit the proliferation of nasopharyngeal carcinoma CNE2 cells.[41] Alloimperatorin containing both electron-withdrawing groups (EWG) and electron-donating groups (EDG) could effectively suppress the proliferation and migration of cervical cancer HeLa cells.[42] Xanthotoxol was demonstrated to play an anti-inflammatory role in LPS-induced RAW264.7 cells by suppressing the MAPK and NF-κB signaling pathways.[43] Thus, it is highly possible that these analogies of IPT may also exert an inhibitory effect on the over-activated FLSs, and further studies are needed to explore their anti-RA actions.

Having demonstrated that IPT could suppress the pathogenic behaviors of TNFα-induced arthritic FLSs, we further explored the underlying molecular mechanisms. Increasing evidence demonstrates that MAPKs, including p38, ERK, and JNK, have been abnormally up-regulated in RA synovial tissues and RA-derived FLSs.[44] The reversion of these changes usually is deemed as a therapeutic aim due to their important roles in the pathogenesis of RA. In this study, we found that IPT significantly suppressed phosphorylation of p38 and ERK but without any effect on p-JNK. These results suggested that the inhibitory effect of IPT on FLS's destructive phenotype could occur through suppressing the ERK and p38 pathways. NF-κB is a central regulator of inflammatory signaling in several tissues and cells.[45,46] Moreover, NF-κB plays a crucial role in maintaining the proliferative and aggressive phenotypes of RA FLS.[47] Inhibition of the NF-κB pathway demonstrates a protective effect against RA FLSs. In our study, IPT significantly suppressed the phosphorylation and degradation of IκBα and thereby blocked the nuclear translocation of the p65 subunit of NF-κB. This was further confirmed by the data of real-time PCR and ELISA that IPT could significantly decrease the expression of a subset of NF-κB downstream target genes. Collectively, our data revealed that IPT played an inhibitory effect on over-activated arthritic FLSs *via* multiple targets (Figure 5). The direct binding sites of IPT will be unveiled in future by a quantitative chemical proteomic approach, which can directly detect the binding of small-molecule inhibitors to their targets in cells.[48]

Along with efficacy, safety issues need to be addressed. Furocoumarins, including IPT, have been extensively studied for their phototoxic properties, both *in vivo* and *in vitro*. Stegelmeier et al. demonstrated that the main adverse effect of furanocoumarins was contact photodermatitis rather than

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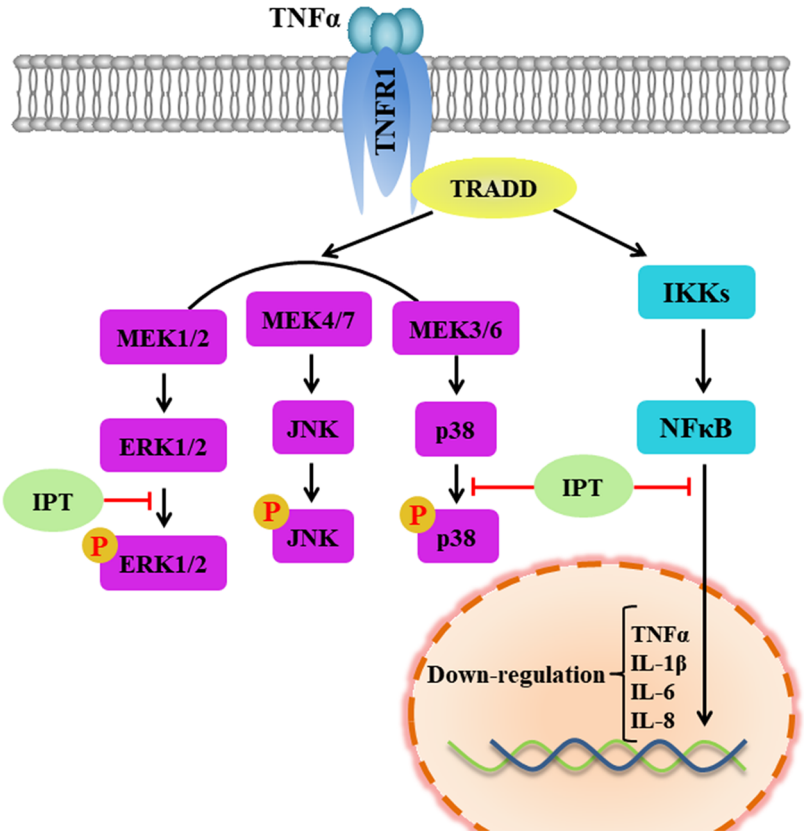

Figure 5. Schematic diagram of the molecular mechanism through which IPT suppressed the pathogenic behaviors of arthritic FLSs. In TNFα-induced FLSs, MAPKs (ERK, p38, and JNK) were all activated. IPT could significantly suppress the phosphorylation levels of ERK and p38. In addition, IPT effectively inhibited the phosphorylation and degradation of IκBα and clearly reduced the nuclear translocation of p65. Consistently, the expression of NF-κB downstream target genes, including TNFα, IL-1β, IL-6, and IL-8, were decreased by IPT treatments.

systemic photosensitization.[49] Systemic toxicity of IPT was found to be primarily directed in the liver and kidneys,[50] but it caused toxicity only at high doses (over 50 mg·kg^{-1}).[51] An *in vitro* study found that IPT had no toxicity to Vero cells between the ranges of 0.125−2048 μg·mL^{-1}.[52] Our study also demonstrated that IPT did not affect FLS viability, even at a concentration as high as 160 μM. Therefore, IPT may be a promising complementary or alternative medicine for RA therapy because of its effectiveness and safety.

In conclusion, we demonstrated, for the first time, that IPT could inhibit proliferation, migration, and inflammation *via* the MAPKs (p38 and ERK) and NF-KB pathways in TNFα-induced arthritic FLSs. These findings suggest that IPT has the potential to be developed as a novel agent for RA treatments.

4. MATERIALS AND METHODS

4.1. Reagents. IPT ($C_{16}H_{14}O_4$, purity ≥ 98%) was obtained from Mansite Bio-Technology Co., Ltd. (Chengdu, Sichuan, China). Recombinant TNFα was purchased from Peprotech (PeproTech, Inc., Rocky Hill, New Jersey). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Gibco BRL, Grand Island, New York). MTS reagents, EdU, lyophilized native chicken type II collagen (CII), Freund's complete adjuvant (CFA), and DAPI solution were purchased from Sigma-Aldrich (St. Louis, Missouri). An Annexin V-FITC apoptosis detection kit was obtained from KeyGen Biotech, Co., Ltd. (Nanjing, Jiangsu, China). TRIzol reagent was from Invitrogen (Carlsbad, California). Antibodies against ERK, phosphorylated ERK (p-ERK), p38, phosphorylated p38 (p-p38), JNK, phosphorylated JNK (p-JNK), phosphorylated IκBα (p-IκBα), and p65 were purchased from Cell Signaling Technology (Beverly, Massachusetts). Antibodies against IκBα and GAPDH were obtained from Santa Cruz Biotechnology (Dallas, California). IL-1β and IL-6 ELISA kits were purchased from SenBeiJia Biological Technology Co., Ltd. (Nanjing, Jiangsu, China). Enhanced chemiluminescence (ECL) solution was purchased from Amersham Pharmacia Biotechnology (Piscataway, New Jersey).

4.2. Isolation, Culture, and Identification of Arthritic FLS Cells. Four female Wistar rats (160−180 g) were purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). A collagen-induced arthritis (CIA) rat model was established as in our previous descriptions.[33,34,53] In brief, the rats were intradermally immunized with 1.5 mg CII emulsified with an equal volume of CFA. Seven days later, a subcutaneous booster was given with half the amount of CII emulsified with Freund's incomplete adjuvant (IFA). The animal experiment was approved by the Experimental Committee of Nanjing Normal University (SYXK 2020-0047). The knee synovium of rats with CIA that had significant signs of disease was dissected and digested by 0.4% type II collagenase and 0.25% trypsin. The primary synovial cells were cultured in H-DMEM supplemented with 10% FBS (v/v), 100 U·mL^{-1} penicillin, and 100 μg·mL^{-1} streptomycin at 37 °C in an atmosphere of 5% CO_2. The FLS cells were identified by staining for vascular cell adhesion molecule-1 (VCAM-1), as in our previous report.[34] Cells obtained from the 3rd to the 10th passages were used in the subsequent experiments.

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4.3. Cell Viability Assay. To determine the effect of IPT on the viability of arthritic FLSs, the cells were seeded into 96-well plates and subsequently treated with different concentrations of IPT (0, 5, 10, 20, 40, 80, and 160 μM) for 48 h. The MTS/PMS complex was then added to each well and incubated for another 4 h. The absorbance of each well was measured at a wavelength of 490 nm using a microplate reader (Model 680, BioRAD, Hercules, California).

4.4. Wound Healing Assay. Arthritic FLSs were cultured into 12-well plates and serum-starved overnight. A linear scratch on the cell monolayer was formed using a sterile 200 μL pipette tip. After washing the suspended cell debris with phosphate-buffered saline (PBS), the cells were pre-treated with different concentrations of IPT (0, 1, 2.5, 5, and 10 μM) for 1 h, followed by stimulation with TNFα (50 ng·mL^{-1}) for 24 h. The effect of IPT on cell migration ability was measured by comparing the remaining cell-free area in the identical fields using ImageJ software.

4.5. Cell Proliferation Assay. Arthritic FLSs were cultured into 24-well plates, pre-treated with 2.5 μM IPT for 6 h, and then stimulated with or without 50 ng·mL^{-1} TNFα for another 24 h. According to the manufacturer's instructions, the cells were incubated with 10 μM EdU for 6 h and then fixed with methanol. The cell nuclei were stained with Hoechst 33342. The numbers of the proliferating cells and total nucleated cells were counted by Image Plus Pro software. The proliferation rate was calculated according to the following formula: proliferation rate = (number of proliferating cells/number of total nucleated cells) × 100%.

4.6. Apoptosis Detection by Flow Cytometry. The apoptosis assay was performed using an annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Briefly, arthritic FLSs were treated with different concentrations of IPT (0, 2.5, 5, and 10 μM) for 24 h and then suspended with a binding buffer. The cells were stained with annexin V and propidium iodide (PI) solution. Flow cytometric analysis was performed with FACScan (Becton Dickinson) with the CellQuest program.

4.7. RNA Extraction and Quantitative Real-Time PCR. Arthritic FLSs were treated with different doses of IPT (0, 1, 2.5, 5, and 10 μM) for 1 h and then stimulated with TNFα (50 ng·mL^{-1}) for 24 h. Total RNA was extracted using TRIzol reagent, and cDNA was synthesized using random primers and oligdT primers. Quantitative real-time PCR amplification was performed using the following primer sets: *IL-1β*, 5′-ATGATGGCTTATTACAGTGGCAA-3′ (forward), 5′-GTCGGAGATTCGTAGCTGGA-3′ (reverse); *IL-6*, 5′-AACCTGAACCTTCCAAAGATGG-3′ (forward), 5′-TCTGGCTTGTTCCTCACTACT-3′ (reverse); *IL-8*, 5′-CATACTCCAAACCTTTCCACCCC-3′ (forward), 5′-TCAGCCCTCTTCAAAAACTTCTCCA-3′ (reverse); *TNFα*, 5′-ATACACTGGCCCGAGGCAAC-3′ (forward), 5′-CCACATCTCGGATCATGCTTTC-3′ (reverse); *β-actin*, 5′-CCACACTGTGCCCATCTACG-3′ (forward), 5′-AGGATCTTCATGAGGTAGTCAGTCAG-3′ (reverse). The PCR reaction conditions were as follows: 95 °C denature for 30 s, followed by 95 °C for 10 s and 60 °C for 30 s for 40 cycles. PCR was performed on Mastercycler ep realplex 2 systems (Eppendorf, Hamburg, Germany). The relative expression of each target gene compared with *β-actin* was calculated using the $2^{-\Delta\Delta Ct}$ method.

4.8. ELISA Assay. Arthritic FLSs were treated with different doses of IPT (0, 1, 2.5, 5 μM) for 1 h and then stimulated with TNFα (50 ng·mL^{-1}) for 24 h. The culture supernatants were centrifuged at 2000 rpm for 20 min to remove the particulate matter. IL-1β and IL-6 were determined using cytokine-specific ELISA kits according to the manufacturer's instructions.

4.9. Western Blot Analysis. Arthritic FLSs were treated with different concentrations of IPT (0, 1, 2.5, 5, and 10 μM) and then stimulated with or without TNFα (50 ng·mL^{-1}) for 15 min. The cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer, and the lysate was collected by centrifugation. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly(vinylidene fluoride) (PVDF) membranes. The membranes were incubated with primary antibodies against p-ERK, p-JNK, p-p38, ERK1/2, JNK, p38, p-IκBα, IκBα, and GAPDH. Immunological responses were detected by ECL solution. Each independent experiment was repeated 3 times, and grayscale was analyzed by ImageJ software.

4.10. Immunofluorescent Staining for p65 Localization. Arthritic FLS cells were cultured into 24-well plates containing sterile coverslips and treated with IPT (5 μM) for 4 h. After stimulation with TNFa (50 ng·mL^{-1}) for 30 min, the cells on coverslips were washed, fixed, and permeabilized with 0.5% Triton-X 100. After blocking with 10% goat serum, the cells were incubated with NF-κB p65 antibody overnight. DAPI solution was used to stain nuclei. The nuclear translocation of p65 was imaged using a Nikon A1R resonance scanning confocal microscope with a spectral detector (Nikon, Tokyo, Japan).

4.11. Statistical Analysis. All data were expressed as the mean ± SD of results obtained from three or more experiments. Statistical comparisons were performed using one-way ANOVA, followed by Tukey's *post hoc* analysis. $P < 0.05$ was considered statistically significant.

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Author Contributions

M.L. and C.W. conceived and designed the experiments. W.L., G.C., Y.M., X.M., J.Z., and X.Y. performed the experiments. M.L. and C.W. analyzed the data. M.L. wrote the manuscript. All authors read and approved the final manuscript.

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Notes

The authors declare no competing financial interest.
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■ ABBREVIATIONS

IPT, imperatorin; FLSs, fibroblast-like synoviocytes; RA, rheumatoid arthritis; NF-κB, nuclear factor of κB; IκBα, inhibitor of nuclear factor κB alpha; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CIA, collagen-induced arthritis; CII, chicken type II collagen; EdU, 5-ethynyl-2′-deoxyuridine

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