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ORIGINAL PAPER

Shikonin inhibits TNF-α production through suppressing PKC-NF-κB-dependent decrease of IL-10 in rheumatoid arthritis-like cell model

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Abstract Shikonin, a major effective component in the Chinese herbal medicine *Lithospermum erythrorhizon* Sieb., exhibits an anti-inflammatory property towards rheumatoid arthritis (RA), but the potential mechanism is unclear. Our aim was to investigate the mechanism of shikonin on the lipopolysaccharide (LPS)-induced fibroblast-like synoviocyte (LiFLS) inflammation model. Fibroblast-like synoviocytes (FLSs) were treated with 200 μg/ml of LPS for 24 h to establish the RA-like model, LiFLS. FLSs were pretreated with shikonin (0.1–1 μM) for 30 min in the treatment groups. Quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assays were used to detect mRNA and protein levels of interleukin (IL)-10 and tumor necrosis factor (TNF)-α. Signal proteins involved in IL-10 production were analyzed by Western blotting. Shikonin significantly reversed the inhibitory effects of LPS on IL-10 expression in FLSs by inactivating the PKC-NF-κB pathway. In addition, shikonin inhibited LPS-induced TNF-α expression in FLSs, and this effect was markedly diminished by IL-10-neutralizing antibody. The IL-10-mediated suppression of TNF-α transcription was demonstrated by no response to the protein synthesis inhibitor cycloheximide and no mRNA decay. Shikonin inhibits LPS-induced TNF-α production in FLSs through suppressing the PKC-NF-κB-dependent decrease in IL-10, and this study also highlights the potential application of shikonin in the treatment of RA.

Keywords Rheumatoid arthritis · Inflammation · *Lithospermum erythrorhizon* · Protein kinase C · Protein synthesis · mRNA stability

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Introduction

Rheumatoid arthritis (RA), one of the most common chronic autoimmune diseases, is characterized by chronic inflammation, articular destruction and abnormal immune response [1]. Fibroblast-like synoviocytes (FLSs) are key cellular participants in RA and are crucial in initiating and driving RA in concert with inflammatory cells [2–4]. The principal treatment for arthritis patients is non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, which has been shown to reduce the expression of inflammatory factors in lipopolysaccharide (LPS)-induced FLSs (LiFLSs) [5]. However, anti-inflammatory agents have the risk of gastrointestinal toxicity, heart failure, etc. [6, 7], and therefore new anti-inflammatory drugs urgently need to be discovered.

Shikonin, the major active substance in *Lithospermum erythrorhizon* Sieb., has been reported to mediate multiple pharmacological activities such as antioxidation, antiviral, cardiovascular protection, antineoplastic and anti-inflammation [8–11]. In RA, researchers found that shikonin could inhibit inflammation, regulate immunity and relieve

pathological joint lesions in a mouse RA model which was induced by collagen type II in the late period [12].

In the collagen type II-induced murine arthritis model, shikonin was found to induce interleukin (IL)-10 production through a GATA-3-dependent mechanism in vivo [13]. IL-10 is an immunosuppressive cytokine in inflammatory responses [14]. Increasing evidence shows that IL-10 is elevated in peripheral blood and synovial joints of RA patients [15–17]. In addition, Shikonin was also found to inhibit tumor necrosis factor (TNF)-α production in human macrophage and murine cells [8, 9]. TNF-α is a proinflammatory cytokine that plays a pivotal role in enhancing the inflammatory response in RA, and is abundantly presented in RA patients' serum and the arthritic synovium [18]. Inhibitors specific for TNF-α have demonstrated efficacy as monotherapy or in combination with NSAIDs in the treatment of RA. Based on these observations, we speculated that shikonin inhibits TNF-α production through induction of IL-10 expression, but no data has fully demonstrated this pathway.

In the present study, we further investigated the effect of shikonin on IL-10 and TNF-α production in a RA-like model, LiFLSs. Our results showed that shikonin could induce the expression of IL-10 and inhibit protein kinase C (PKC) and NF-κBp65 protein in LiFLSs. Moreover, inhibition of either PKC or NF-κB increased shikonin-induced IL-10 production, and the induction of IL-10 further promoted the down-regulation of TNF-α mRNA. This study provides novel insight into the anti-inflammatory mechanisms of shikonin, and suggests the application of shikonin in the treatment of RA.

Materials and methods

Establishment of RA-like model

Fibroblast-like synoviocytes (FLSs) were isolated from knees of rats. Synovial membranes were minced and digested with 1 mg/ml collagen type II (Sigma) in DMEM (Hyclone) at 37 °C for 1 h in 5% CO_2. After centrifugation and washing, the cells were resuspended in DMEM supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 U/ml streptomycin (Beyotime Institute of Biotechnology, Haimen, China). Non-adherent cells were removed after 24 h and adherent cells were trypsinized with 0.25% trypsin (Hyclone) at confluence and then plated in culture flasks. To obtain a homogeneous population of synoviocytes, confluent cultures from passages 3–7 were used. FLSs were verified by immunocytochemistry (BOSTER) as a homogeneous population (phenotype: >99% vimentin and <1% CD68, data not shown). FLSs were treated with 200 μg/ml of LPS for 24 h to establish the RA-like model, LiFLSs.

Western blot analysis

LiFLSs were lysed in RIPA [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS] buffer containing protease inhibitor mixture (0.1% CTAB, Sigma). Cytoplasmic and nuclear extraction was performed using the protocol reported by Jobin [19]. Lysate (50 μg of protein) was separated on 5–12% SDS–polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore) using a Mini Tank Transfer System (Bio-Rad Laboratories, Hercules, CA, USA) at 200 mA for 2 h. The blots were blocked with 5% skim milk, followed by incubation with antibodies anti-p-NF-κBp65 (cat. no. ab86299; 1:2000; Abcam, Cambridge, MA, USA), anti-NF-κBp65 (cat. no. ab16502; 1:2000; Abcam), anti-pIκBα (cat. no. ab92700; 1:1000; Abcam), anti-IκBα (cat. no. ab97783; 1:1000; Abcam), anti-pIKKα (cat. no. ab38515; 1:500; Abcam), anti-IKKα (cat. no. ab32041; 1:10,000; Abcam), anti-PKCβ (cat. no. ab32026; 1:1000; Abcam) and anti-GAPDH (cat. no. 5174; 1:1500, Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4 °C. Blots were then incubated with goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG antibodies (cat. nos. A0208 and A0216; 1:1000; Beyotime) for 1 h at 37 °C. Proteins were detected using Western blotting and visualized by chemiluminescence (Pierce, SuperSignal West Pico Chemiluminescent Substrate).

Quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA expression of TNF-α and IL-10 was analyzed by using the ABI 7300 real-time PCR system (Foster City, CA, USA). Primer pairs were TNF-α [20]: 5′-TTCTCATT CCTGCTCGTGG-3′ and 5′-TTTGGTGGTTCGCCTCCT-3′; IL-10 [21]: 5′-TGCCAAGCCTTGTCAGAAATGATC AAG-3′ and 5′-GTATCCAGAGGGTCTTCAGCTTCT CTC-3′; and GAPDH [22]: 5′-TGGCCTCCAAGGAG-TAAGAAAC-3′ and 5′-GGCCTCTCC CTCTCAGTATC-3′. The reverse transcription reaction was performed with 1 μg total RNA. cDNAs were amplified using SYBR Green Real-time PCR Master Mix (Takara) and 0.4 μmol/L of each primer pair. The reaction was carried out with an initial step at 94 °C for 30 s, followed by 40 cycles of amplification step (94 °C for 30 s, 60 °C for 60 s, and 72 °C for 1 min). Each sample was analyzed in triplicate and the averages of the threshold cycles were used to interpolate curves using 7300 System SDS software.

Results were expressed as the relative expression to the internal control GAPDH.

Enzyme-linked immunosorbent assays

Culture medium was harvested at the indicated time. The concentrations of TNF-α and IL-10 in the supernatant of cultured cells were detected by sandwich enzyme-linked immunosorbent assay (ELISA) kits (Dingguo, Beijing, China) according to the manufacturer's protocol.

Statistical analysis

Results are expressed as mean ± SD. The data were analyzed by one-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. Results were considered statistically significant at $p < 0.05$.

Results

Shikonin induced the up-regulation of IL-10 and down-regulation of TNF-α

IL-10, an important immunosuppressive mediator, is usually down-regulated in RA. In LiFLSs, IL-10 was reduced by LPS, while shikonin reversed the mRNA expression of IL-10 in a dose-dependent manner (Fig. 1a). In particular, 1 μM of shikonin showed similar potency to the positive control indomethacin, an anti-inflammatory agent for RA. We then detected the production of IL-10 protein, as shown in Fig. 2a; shikonin also significantly reversed the IL-10 production which was decreased by LPS. TNF-α is the major macrophage-derived inflammatory cytokine in the rheumatoid joint of RA patients and induces the synthesis and secretion of various proinflammatory cytokines, such as IL-6 and IL-8 in synovial fibroblasts [23]. In our results, shikonin markedly decreased the mRNA and protein level of TNF-α in LiFLSs (Figs. 1b, 2b). As for the inhibitory effect on TNF-α, shikonin showed similar potency to the positive control indomethacin. However, shikonin had no effects on the mRNA expression and production of IL-10 and TNF-α under LPS-free conditions ($p > 0.05$; data not shown).

Shikonin induced the expression of IL-10 through the PKC-NF-κB pathway

In order to determine how shikonin increased the expression of IL-10, we pretreated with various signal protein inhibitors and then carried out shikonin treatment. As shown in Fig. 3a, IL-10 production was promoted by LY333531 (10 nM, protein kinase C inhibitor) and pyrrolidine dithiocarbamate (PDTC; 10 μM, NF-κB inhibitor), but not by JNK inhibitor (40 nM, SP600125), MEK1 inhibitor (2 μM, PD98059), PI3 K inhibitor (1 μM, LY294002), P38 inhibitor (0.5 μM, SB203580) or AP1 inhibitor (100 nM, BMS-536924). However, these inhibitors had no effects on the production of IL-10 under shikonin but LPS-free conditions ($p > 0.05$; data not shown). According to the previous report, PKC was found to be involved in IL-10 production in an LPS-induced endotoxemia model [24], and promoted the downstream activation of NF-κB in mice melanoma cells [25]. Based on these clues, we further examined the protein expression of PKCβ, NF-κBp65 and its upstream components IKKα and IκB [26] in shikonin-treated LiFLSs. The result showed that pretreatment with shikonin could dramatically decrease the expression of pNF-κBp65, pIκB, pIKKα and PKCβ, but increase the expression of IκB (Fig. 3b). However, shikonin had no effect on expressions of NF-κB, pIκB, pIKKα, IKKα and PKCβ under LPS-free conditions ($p > 0.05$; data not shown). These results demonstrated that shikonin induced the expression of IL-10 through inactivating the PKC-NF-κB pathway.

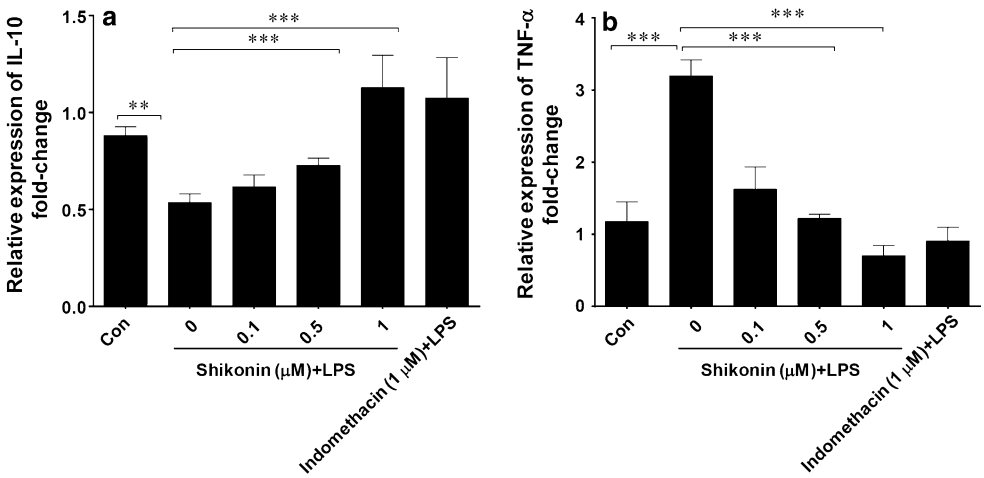
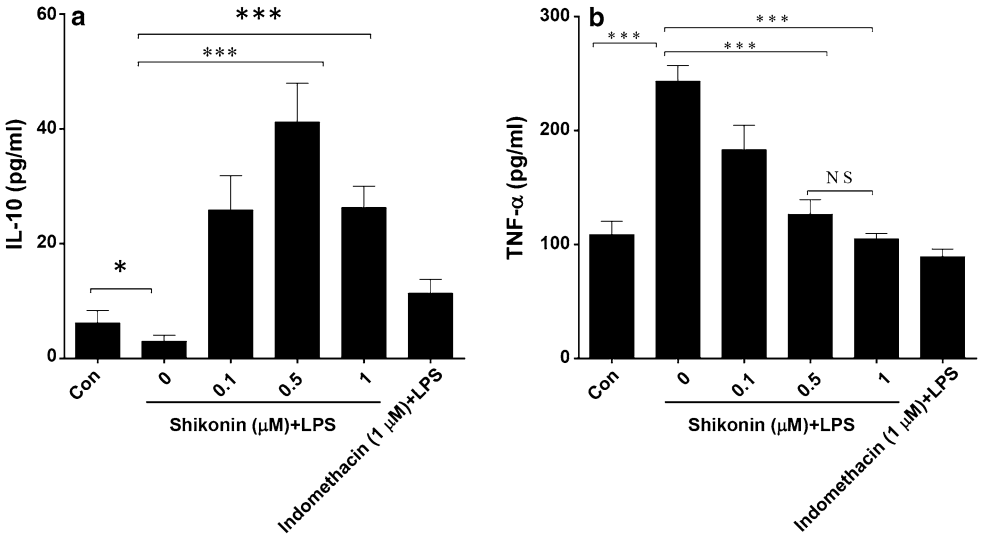


Fig. 1 Effect of shikonin on mRNA expression of TNF-α and IL-10 in LiFLSs. Fibroblast-like synoviocytes were treated with LPS (200 μg/ml) for 24 h to establish the RA-like model, LiFLSs. **a** IL-10 and **b** TNF-α mRNA expression were quantified by qRT-PCR and normalized to GAPDH. Values are mean ± SD ($n = 3$). $^{**}p < 0.01$ and $^{***}p < 0.001$



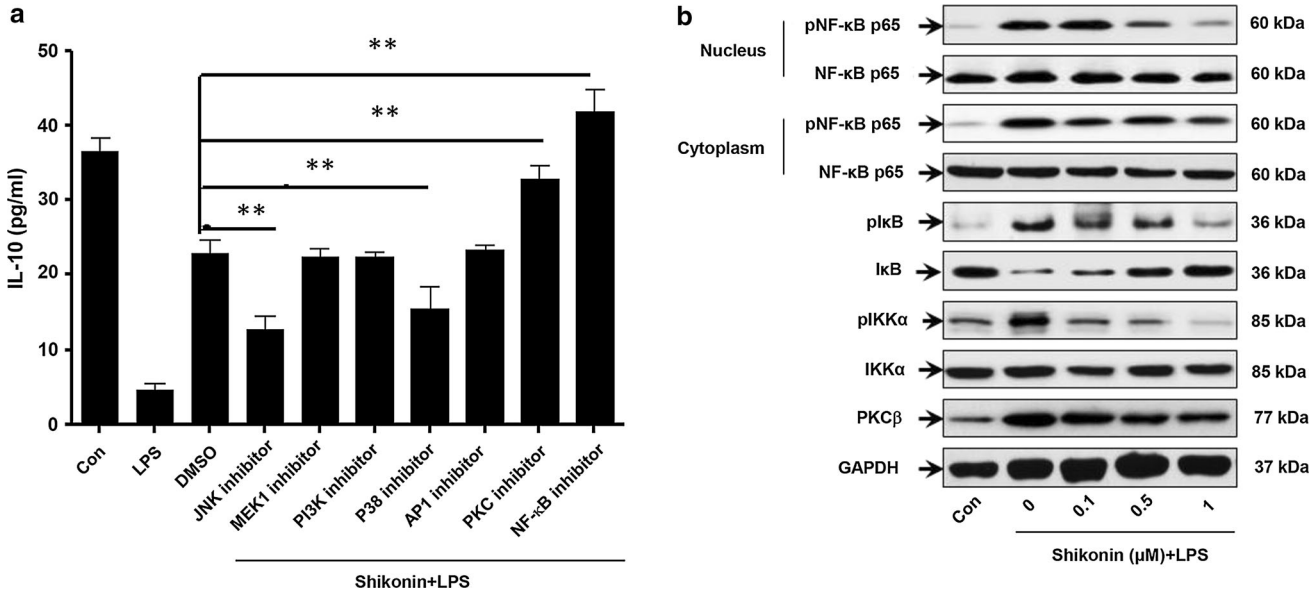


Fig. 2 Effect of shikonin on protein production of TNF-α and IL-10 in LiFLSs. Fibroblast-like synoviocytes were treated with LPS (200 μg/ml) for 24 h to establish the RA-like model, LiFLSs. **a** IL-10 and **b** TNF-α protein production in supernatant were quantified by ELISA. Values are mean ± SD ($n = 3$). $^{*}p < 0.05$ and $^{***}p < 0.0001$, *NS* no significant difference

Fig. 3 Shikonin induces IL-10 production through PKC-NF-κB pathway in LiFLSs. **a** All signal protein inhibitors (10 nM LY333531, 10 μM PDTC, 40 nM SP600125, 2 μM PD98059, 1 μM LY294002, 0.5 μM SB203580 and 100 nM BMS-536924) treated LiFLSs 1 h before shikonin treatment; 0.1% DMSO was solvent control to inhibitors. IL-10 protein in supernatant was quantified by ELISA. Values are mean ± SD ($n = 3$). **b** Cells were pretreated with shikonin for 30 min at different indicated concentrations. Proteins were quantified by Western blotting and normalized to GAPDH. $^{***}p < 0.0001$

Shikonin decreased TNF-α expression through IL-10

It was reported that IL-10 inhibits the transcription of TNF-α [27, 28], so we investigated whether there is a connection between IL-10 and TNF-α that is induced by shikonin. LiFLSs were pretreated with IL-10 antibody, as shown in Fig. 4; the decrease in TNF-α protein secretion and mRNA expression induced by shikonin was reversed by the addition of IL-10 neutralizing antibody (Fig. 4a, b).

To determine whether protein synthesis was involved in the IL-10-mediated suppression of TNF-α, the protein synthesis inhibitor cycloheximide was used. However, considering that cycloheximide inhibits all protein synthesis, as shown in Fig. 4b, cycloheximide totally blocked the inhibitory effect of shikonin on TNF-α transcription. This may be due to cycloheximide inhibiting IL-10 production induced by shikonin, so we added recombinant IL-10 (50 U/ml) or replaced the medium with the supernatant from shikonin-treated LiFLSs to exclude the inhibitory effect of cycloheximide on IL-10 production. In the presence of cycloheximide (5 μg/ml), which at this dose has been shown to have an inhibitory effect on translation [29],

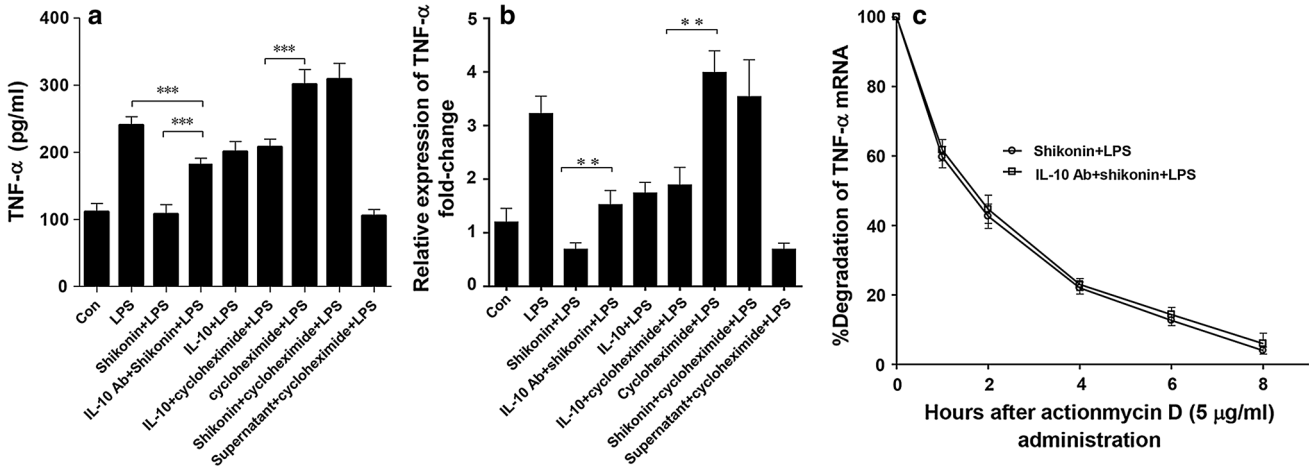


Fig. 4 IL-10 induced by shikonin inhibited TNF-α production in LiFLSs at least in part by down-regulated expression of TNF-α mRNA. **a** Cells were treated with 1 μM shikonin 30 min before LPS treatment with or without 4 μg/ml IL-10 antibody. TNF-α protein production in supernatant was quantified by ELISA. Values are mean ± SD ($n = 3$). **b** In addition to some groups the same as **a**, cells were treated with recombinant IL-10 (50 U/ml) with or without cycloheximide (5 μg/ml) 1 h before LPS treatment; *supernatant* means medium used to culture FLSs was replaced by supernatant from shikonin-treated LiFLSs before the addition of cycloheximide. TNF-α mRNA expression was quantified by qRT-PCR and normalized to GAPDH. **c** Shikonin with or without IL-10-antibody-treated cells 30 min before LPS treatment. Following incubation with LPS for 1 h, 5 μg/ml actinomycin D was added and cells were lysed at 1, 2, 4, 6 and 8 h following the addition of actinomycin D. TNF-α mRNA expression was quantified by qRT-PCR and normalized to GAPDH. Values are mean ± SD ($n = 3$). $^{**}p < 0.01$ and $^{***}p < 0.0001$

IL-10 decreased TNF-α mRNA levels (Fig. 4b). In other words, in the presence of cycloheximide, the supernatant from shikonin-treated LiFLSs also down-regulated TNF-α expression, which might be caused by IL-10 or other inhibitory molecules in the supernatant of shikonin-treated LiFLSs. Overall, these data demonstrated that IL-10 induced by shikonin suppresses the transcription of TNF-α independent of protein synthesis, and other substances induced by shikonin may also be involved in the inhibition of TNF-α mRNA expression.

In order to exclude the possibility that IL-10 could shorten the half-life of TNF-α mRNA and thus decrease TNF-α production, we also assessed the half-life of TNF-α mRNA in the presence of 5 μg/ml actinomycin D, which at this dose has been shown to inhibit translation [30, 31]. Following actinomycin D treatment, mRNA was extracted at different time points. No significant difference was seen in the rate of TNF-α mRNA decay in the shikonin-treated groups with or without IL-10 antibody (Fig. 4c). This result indicated that TNF-α was not reduced by IL-10 through increasing its mRNA decay.

Discussion

RA is characterized by the proliferation of the synovial membrane into a pannus, which includes resident fibroblast-like synoviocytes (FLSs) and infiltrating mononuclear cells capable of producing inflammatory cytokines [32]. Prompt treatment is the key to preventing joint destruction and organ damage; traditionally RA has been treated with disease-modifying anti-rheumatic drugs, NSAIDs, but NSAID tablets can increase the risk of serious stomach problems, and thus new drugs urgently need to be found.

Shikonin, a major active chemical component isolated from the dried root of *Lithospermum erythrorhizon* (LE), possesses numerous pharmacological properties, including anti-inflammatory and antitumor properties and promotion of wound healing activity [33]. It was reported that shikonin significantly inhibits the concentrations of TNF-α, IL-6 and IL-1β in bronchoalveolar lavage fluid and primary macrophage cultures induced by LPS [10, 11, 34]. In RA, shikonin also exerts beneficial effects on collagen-induced arthritis (CIA), a mouse RA model, and markedly reduces joint swelling and cartilage destruction [12, 13].

In this study, we investigated the potential beneficial effects of shikonin in an RA-like model: LPS (200 μg/ml)-induced fibroblast-like synoviocyte (LiFLS) inflammation. However, others have reported that LPS at a lower concentration of 1 μg/ml could also induce IL-6 secretion, but activated FLSs did not release any detectable amount of mature IL-18 [35]. Similarly, increased IL-6 and CXCL-10 production and p38 phosphorylation were found in LPS (1 μg/ml)-activated RA LiFLSs [36]. Our result showed that shikonin significantly increased the level of the anti-inflammatory cytokine IL-10. IL-10 can effectively block the production of the proinflammatory cytokines TNF-α, IL-1 and IL-8 in synovial macrophages and synoviocytes

[37–39]. IL-10 was also found to inhibit TNF-α production in interferon-γ-activated macrophages [40], and has been used in the treatment of RA in clinical trials [41, 42]. In this study, we found that shikonin inhibited TNF-α production through IL-10 in LiFLSs, and exerted a similar potency to indomethacin. These results suggested that shikonin may serve as an effective candidate for the treatment of RA.

We investigated which pathway is involved in the shikonin-induced up-regulation of IL-10. In bone marrow-derived dendritic cells, glycogen synthase kinase-3 inhibition has been shown to induce PKC and ERK1/2 activation, and thus provoke the production of IL-10 [24]. NF-κB activation is normally correlated with the production of proinflammatory cytokines, such as IL-6 and IL-8 [43], and mediates inflammation [44]. To date, only one report on HIV transactivating Tat protein found that Tat protein [45] induces NF-κB activation and thus promotes production of IL-10 through at least three signaling pathways concurrently, including the classical and alternative IKKa pathways. However, we demonstrated IL-10 production in shikonin-treated LiFLSs in the presence of seven signal protein inhibitors; this result showed that PKC inhibitor and NF-κB inhibitor both significantly enhanced this effect. Decreased NF-κB phosphorylation could increase the expression of IL-10 and is associated with PKC signaling [46, 47]. In our study, shikonin also markedly decreased PKC expression, IKKα phosphorylation and NF-κBp65 translocation.

It was found that IL-10 could inhibit TNF-α production in synoviocytes [37], and that shikonin suppresses the basal transcription and activator-regulated transcription of TNF-α by inhibiting the binding of transcription factor IID protein complex (TATA box-binding protein) to the TATA box [8]. In our results, we found that IL-10 antibody markedly reduced the inhibitory function of shikonin on TNF-α, though still less than the concentration of TNF-α in LiFLSs. Before adding cycloheximide into the medium of FLSs, we replaced the medium with the supernatant from shikonin-treated LiFLSs. Compared with the exogenous IL-10-treated group, the supernatant inhibited TNF-α transcription more effectively than exogenous IL-10, implying that shikonin may have other ways to down-regulate TNF-α production. mRNA half-life analysis showed that IL-10 has no influence on TNF-α mRNA degradation. Taken together, these results demonstrated that IL-10 inhibits TNF-α through its transcription level.

In summary, this study demonstrated the mechanism of shikonin in the treatment of RA. By using a RA-like cell model, LiFLSs, we found that shikonin has anti-inflammatory effects on LiFLSs by targeting IL-10 and TNF-α, two key cytokines in the development of RA pathogenesis. Shikonin inhibited TNF-α transcription by suppressing the PKC-NF-κB-dependent decrease in IL-10 production. Our work highlights the potential application of shikonin in the treatment of RA in future.

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Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interests exists.

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