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Inhibitory effect of triptolide on interleukin-18 and its receptor in rheumatoid arthritis synovial fibroblasts

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Abstract. *Objective:* To determine the effects of triptolide (TP) on the expression of interleukin-18 (IL-18) and its receptor in phorbol 12-myristate 13-acetate (PMA)-stimulated rheumatoid arthritis synovial fibroblasts (RASF).
Materials and Methods: RASF were obtained from the synovial tissue of patients with RA. RASF were pretreated with TP (0~100 ng/ml) for 2 h before stimulation with PMA (50 ng/ml). The bioactivity of IL-18 in the supernatant was detected based on IFN-γ secretion from IL-18-responding human myelomonocytic KG-1 cells. IL-18 level was analyzed by ELISA. In situ expression of IL-18Rα was determined by immunofluorescence assay. To estimate the protein and mRNA expression of IL-18 and IL-18Rα in RASF, western blot and quantitative RT-PCR were performed. Nuclear factor-κB (NF-κB) activity in the whole-cell extract of treated RASF was also measured using an ELISA-based method.
Results: TP effectively inhibited the bioactivity of IL-18 in PMA-stimulated RASF. The expression of IL-18 and IL-18R at protein and gene levels was reduced by TP. NF-κB activity in PMA-stimulated RASF was profoundly suppressed by TP. These effects showed a high correlation with TP concentration (0~100 ng/ml).
Conclusion: TP effectively inhibited the expression of IL-18 and its receptor in PMA-stimulated RASF. These results suggest a mechanism of TP in RA therapy.

Key words: Triptolide – Rheumatoid arthritis – Synovial fibroblasts – Interleukin-18 – Interleukin-18 receptor

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with chronic inflammation of multiple arthrosis synovial tissue, which is characterized by synovial fibroblast hyperplasia and large amount of invasive lymphocytes, macrophages, and plasmacytes [1–2]. One of the critical pathogenesis of RA is the overexpression of inflammatory cytokines, including interleukin-18 (IL-18), tumor necrosis factor (TNF), and IL-1 [2–3]. These inflammatory cytokines activate nuclear factor-κB (NF-κB), promote production of cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), and induce synthesis of prostaglandin E2 (PGE2) and nitric oxide (NO), which lead to synovial inflammation associated with arthrosis, swelling, hyperplasia, angiogenesis, and finally arthritic degeneration [2–4]. These findings indicate that the inhibition of overexpression and activity of inflammatory cytokines and the subsequent reduction in the production of inflammatory mediators would improve the state of RA and prevent further progression.

Tripterygium wilfordii Hook f. (TWHF), a Chinese traditional medicinal herb, shows anti-inflammatory and immunosuppressive effects in RA [5]. Triptolide (TP) is an active component of TWHF [6]. The main antirheumatoid mechanism of this component includes inhibition of systemic immune response and downregulation of the activity of T cell helper type 1 (Th1) cells and their cytokines [5], which subsequently reduces the expression and production of inflammatory factors and mediators.

IL-18, also known as interferon (IFN)-gamma-inducing factor (IGIF), is a cytokine belonging to the IL-1 family [7]. IL-18 mRNA is mainly produced in the pancreas, kidney, skeletal muscle, lung, and liver tissue and is highly expressed in macrophages [8]. IL-18 was reported to play an important role in RA process and therapy [9–11]. In RA, IL-18 is mainly produced by macrophages [12]. IL-18 activates T cells and macrophages to produce proinflammatory cytokines, chemokines, and adhesion molecules, which in turn maintain chronic inflammation and induce bone and cartilage destruction [12]. Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis in rheu-

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matoid synoviocytes [13]. IL-18 increases the production of VEGF via AP-1-dependent pathways in a dose-dependent manner [13]. It was suggested that the downregulation of IL-18 activity could be a potential therapeutic strategy for RA. Based on the effects of TP and IL-18 in the immune response, we investigated the effect of TP on IL-18 and its receptor in rheumatoid arthritis synovial fibroblasts (RASF), discuss the possible mechanism, and finally provide a new theory and experimental data for RA therapy.

Material and Methods

Isolation and culture of RASF [14]

Synovial tissue samples were obtained from 5 patients with RA that met the criteria of the American College of Rheumatology; these patients had undergone total knee replacement surgery or synovectomy. Fresh synovial tissues were minced aseptically and then dissociated with 4 mg/ml collagenase (Sigma, LA, USA) for 4 h in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) at 37 °C. The dissociated cells were plated on culture dishes and allowed to adhere in RPMI 1640 supplemented with 10 % fetal bovine serum (Hyclone, Utah, USA) at 37 °C in a humidified atmosphere containing 5 % CO_2. After removal of nonadherent cells, the plated cells were further incubated for 18 h and then thoroughly washed with D-Hank's solution. The adherent synovial cells were harvested using trypsin (0.25 % w/v)-EDTA (0.02 % w/v) solution, followed by washing with D-Hank's solution containing 2 % FBS. The collected synovial cells with homogeneity of more than 98 % (as determined by flow cytometry (FCM)) were used at the third or fourth passage for subsequent experiments, and less than 1 % of all cells were positive for CD3, CD20, CD68, or von Willebrand factor.

Grouping and treatment for RASF

RASF (1×10^6) were cultured in a 6-well plate (Falcon), treated with different concentrations (0, 10, 50, and 100 ng/ml) of TP (Calbiochem, purity: ≥98 %) for 2 h, and finally stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Calbiochem) for 18 h. Cells and culture supernatant were collected for the assay.

Assay for bioactivity of IL-18

The bioactivity of IL-18 in culture supernatant was detected by enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN), based on IFN-γ secretion from IL-18-responding human myelomonocytic KG-1 cells. The protocol was in accordance with the method reported by Yamamura [15]. The activity of IL-18 in the samples was determined by the difference in the levels of IFN-γ between cultures treated with and without anti-IL-18 monoclonal antibody (mAb) (R&D Systems, 2 μg/ml).

ELISA for IL-18

IL-18 level in the supernatant was detected by a specific hIL-18 ELISA kit (MBL, Nagoya, Japan) according to the manufacturer's instructions. The sensitivity of the assay was 12.5 pg/ml.

Immunofluorescence assay for IL-18Rα

Cells were cultured in slide (Lab-Tek Chamber Slide System, Nalge Nunc International), treated with different concentrations of TP (0~100 ng/ml) for 2 h, and finally stimulated with PMA (50 ng/ml) for 18 h. RASF was fixed with 4 % paraformaldehyde at 4 °C for 20 min and incubated with anti-hIL-18Rα mAb (R&D Systems) at room temperature for 1 h. The cells were then further incubated with fluorescein isothiocyanate (FITC)-coupled secondary antibody (Santa Cruz, CA, USA) for 30 min. Fluorescence was detected by FAScan flow cytometry (Becton Dickinson, San Jose, CA, USA) and photographed by fluorescence microscopy (Olympus IX81, Japan).

Western blotting for IL-18 and IL-18Rα

Cells were collected and lysed by a lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % Triton, 1 % NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM leupeptin, 1 mM phenyl methylsulfonyl fluoride (PMSF)) for 30 min at 4 °C. The insoluble material was then removed by centrifugation at 8000×*g* for 10 min at 4 °C. The concentration of protein in each cell lysate was determined using a BCA protein assay kit (Pierce, IL, USA) with bovine serum albumin (BSA) as the standard. An identical amount of protein (40 μg) from each sample was loaded onto a 10 % SDS-PAGE gel and electrophoresed at 200 volts. Proteins were then transferred to nitrocellulose membranes (0.45 μm, Millipore, MA, USA) that were blocked with 5 % BSA (Sigma, LA, USA) in TBS (25 mM Tris-HCl, 150 mM sodium chloride, pH 7.2) for 1 h at room temperature. Blots were incubated with anti-IL-18 or anti-IL-18Rα (R&D Systems) or anti-β-actin-specific primary antibody (Santa Cruz) at 1:800 dilution at room temperature for 2 h. Blots were washed 3 times and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution) for 1 h at room temperature. All blots were developed using enhanced chemiluminescence (ECL) reagents (Supersignal Dura kit, Pierce) according to the manufacturer's instructions. The signals were captured on an X-ray film.

Quantitative real-time polymerase chain reaction assay

Total RNA was isolated from RASF using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. All procedures were carried out using RNase-free reagents. RNA (1 μg) was reverse transcribed into cDNA by using SuperScript II reverse transcriptase (Invitrogen) in 20 μl reaction volume. Quantitative RT-PCR (QRT-PCR) was performed by LightCycler technology (Roche Molecular Biochemicals) with SYBR Green I detection. In all assays, cDNA was amplified using a standardized program (a 10-min denaturing step; 50 cycles of 5 min at 95 °C, 15 min at 65 °C, and 15 min at 72 °C; melting point analysis in 0.1 °C steps; and a final cooling step). Each LightCycler capillary was loaded with 1.5 μl DNA Master Mix, 1.8 μl $MgCl_2$ (25 mM), 10.1 μl H_2O, and 0.4 μl of each primer (10 μM). The final amount of cDNA per reaction corresponded to 5.0 ng of RNA used for reverse transcription. Relative quantification of target gene expression was performed using a mathematical model, as recommended by Roche Molecular Biochemicals. The results are expressed as the ratios of IL-18 to β-actin and IL-18R to β-actin. The following primers were used for the experiments: IL-18 sense: 5′-TTC GGG AAG AGG AAA GGA AC-3′, antisense: 5′-AAG GAT ACA AAA AGT GAC AT-3′ (480 bp); IL-18R sense: 5′-CCC AAC GAT AAA GAA GAA CGC C-3′, antisense: 5′-TGT CTG TGC CTC CCG TGC TGG C-3′ (419 bp); β-actin sense: 5′-CGC TGC GCT GGT CGT CGA CA-3′, antisense: 5′-GTC ACG CAC GAT TTC CCG CT-3′ (619 bp). The reaction specificity was confirmed by 1.5 % agarose gel electrophoresis of products after real-time PCR and melting curve analysis.

Activity assay for NF-κB p65 in treated RASF

NF-κB p65 transcription factor assay kits (Active Motif, CA, USA), which combine all the benefits of fast, sensitive, and specific assays, were used to monitor NF-κB activation. Cell extracts (5 μg/well) were added to a 96-well plate coated with an oligonucleotide probe containing the site for NF-κB binding. Each well was then incubated with a primary antibody specific for the active form of the bound transcription

factor. The wells were then incubated with an HRP-conjugated secondary antibody. The plate was washed 3 times at each step, and 100 µl of a standard developing solution (TMB) was added to each well. The absorbance results were read using a spectrophotometer (Biotek ELX800) at 450 nm with a reference wavelength of 650 nm. Both positive control wells and blank wells were set for this assay. The specificity of binding was determined by prior addition of a 20-fold excess of unlabeled competitor consensus oligonucleotide.

Data and statistical analysis

The data presented are means ± SD. Significance of the differences between the experimental conditions was determined by a paired two-sample Student's *t* test.

Results

Bioactivity and expression level of IL-18

PMA (50 ng/ml) induced the expression of IL-18 in RASF. TP (≥50 ng/ml) obviously inhibited the expression and bioactivity of IL-18 in PMA-stimulated RASF (Table 1).

In situ expression of IL-18Rα

Immunofluorescence assay (Fig. 1) revealed that high concentration (50 and 100 ng/ml) of TP obviously inhibited the expression of IL-18R in RASF, whereas low concentration (10 ng/ml) did not. In the FAScan flow cytometry assay, the expression of IL-18Rα in the control group was 9.42 % ± 2.41 %; in the PMA group, 46.17 % ± 4.08 %; and in the TP groups (10, 50, and 100 ng/ml), 43.23 % ± 3.11 %, 31.34 % ± 4.62 %, and 15.12 % ± 3.14 %, respectively. The inhibition ratios of TP to IL-18R expression were 6 % (10 ng/ml), 32 % (50 ng/ml), and 67 % (100 ng/ml).

Table 1. The level and bioactivity of IL-18 in culture supernatants of treated RASF

Groups	IL-18 (pg/ml)	IFN-γ (pg/ml, bioactive of IL-18)
Normal	32.67 ± 7.02	26.00 ± 5.29
PMA + TP0ng/ml	254.33 ± 33.65	271.66 ± 35.83
PMA + TP10ng/ml	222.60 ± 24.44	152.00 ± 12.49*
PMA + TP50ng/ml	102.00 ± 17.78*	63.00 ± 4.58*
PMA + TP100ng/ml	41.33 ± 19.21*	26.33 ± 2.08*

RASF (1 × 106) were cultured in a 6-well plate, treated with TP for 2 h, and finally stimulated with 50 ng/ml PMA for 18 h. The culture supernatant was collected for the assay. The bioactivity of IL-18 in the supernatant was detected based on IFN-γ secretion from IL-18-responding human myelomonocytic KG-1 cells. IL-18 level in the supernatant was analyzed by ELISA. Data are expressed as means ± SD.
* Compared with other groups, $p'<0.05$, by Student's *t* test.

Inhibitory effect of TP on protein expression of IL-18 and IL-18Rα

As shown in the western blotting assay (Fig. 2), TP differentially inhibited the expression of IL-18 and its receptor IL-18Rα induced by PMA in RASF. The inhibitory effect was

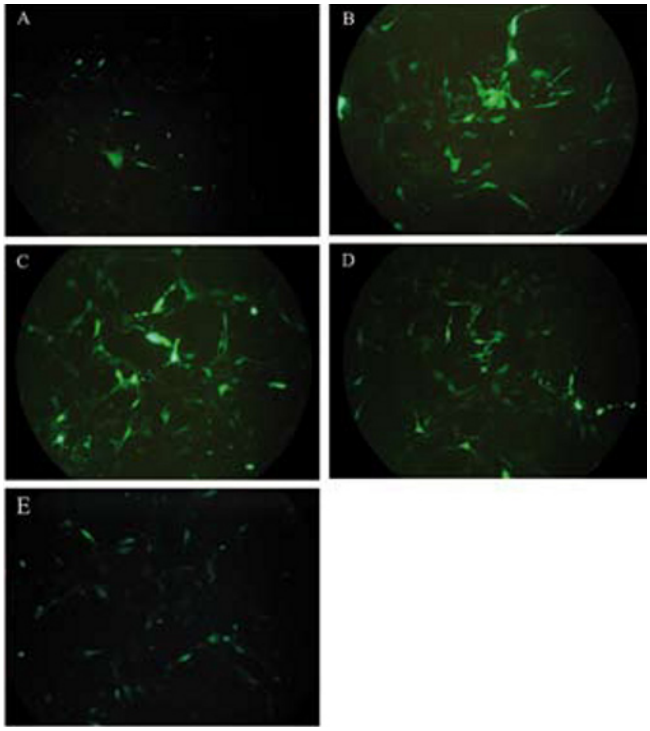

Fig. 1. Immunofluorescence assay for in situ expression of IL-18R. RASF were cultured in a glass slide, treated with TP (0~100 ng/ml) for 2 h, and finally stimulated with PMA (50 ng/ml) for 18 h. Immunofluorescent analysis was performed using primary antibody specific to IL-18Rα and FITC-coupled secondary antibody. The fluorescence was detected and photographed by fluorescence microscopy. A: Normal; B: PMA + TP (0 ng/ml); C: PMA + TP (10 ng/ml); D: PMA + TP (50 ng/ml); E: PMA + TP (100 ng/ml).

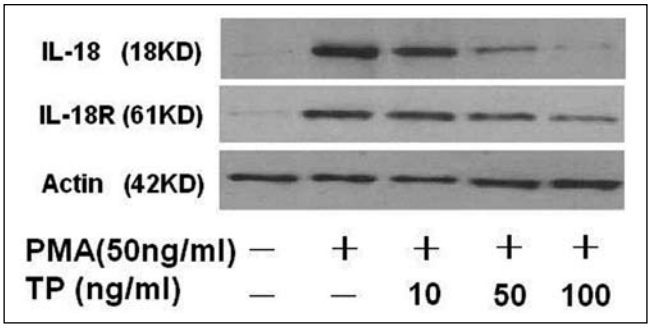

Fig. 2. Western blot analysis of protein expression of IL-18 and IL-18R in treated RASF.
RASF (1×10^6) were cultured in a 6-well plate, treated with TP (0~100 ng/ml) for 2 h, and finally stimulated with 50 ng/ml PMA for 18 h. The cells were collected and lysed in a lysis buffer. Cellular proteins (40 µg/sample) were subjected to western blot analysis using primary antibody specific to IL-18 or IL-18Rα, followed by incubation with HRP-conjugated secondary antibody. The amount of β-actin was used as the loading control. The reaction was visualized by ECL, and signals were captured on an X-ray film.

related to TP concentration. Moreover, the inhibitory effect on IL-18 was more obvious than that on IL-18Rα.

Inhibitory effect of TP on mRNA expression of IL-18 and IL-18Rα

QRT-PCR (Fig. 3) revealed that 50 ng/ml TP markedly inhibited the mRNA expression of IL-18, whereas 100 ng/ml TP was required to inhibit IL-18R. This result indicated that TP could more efficiently inhibit the expression of IL-18 than IL-18R in RASF. The inhibition ratios of TP to IL-18R expression were 1.5 % (10 ng/ml), 51.3 % (50 ng/ml), and 78.5 % (100 ng/ml).

Inhibitory effect of TP on NF-κB activity in RASF

The NF-κB activity assay (Fig. 4) revealed that TP effectively inhibited the NF-κB activity induced by PMA in RASF. The inhibitory effect was related to TP concentration. The inhibition ratios of TP to IL-18R expression were 23.7 % (10 ng/ml), 61.9 % (50 ng/ml), and 77.7 % (100 ng/ml).

Discussion

Triptolide (TP), the active component of the Chinese medicinal herb *Tripterygium wilfordii* Hook f., was reported as a novel immunosuppressive and anti-inflammatory agent [6]. It suppresses the production of PGE2 by blocking the upregulation of COX2 (16) and inhibits the production of NO by decreasing the transcription level of the iNOS gene [6]. The anti-RA mechanisms of TP was shown to include immunosuppression, anti-inflammation, induction of cell apoptosis, inhibition of vascular proliferation, protection of articular cartilage, and gene regulation [5, 17]. RA, an autoimmune disease, is associated with Th1 immune response and IL-18. IL-18 plays a critical role in the genesis and progression of RA [18]. IL-18R is expressed on the surface of Th1 cells and not on Th2 cells [19]. IL-18 is involved in Th1-induced immune response and induces macrophage to produce monokines [20]. The activation of macrophages induces local inflammation, resulting in the production of IL-1, TNF-α, and MMPs (matrix metalloproteinases) [20]. Additionally, IL-18 triggers the production of NO, mediates the secretion of IFN-γ [21], and stimulates angiogenesis in RA [22]. Kinoshita K [23] reported that blockade of IL-18R signaling delayed the onset of autoimmune disease in MRL-Faslpr mice. We assume that a relationship exists between TP and IL-18/IL-18R in RA.

The activity of mature IL-18 is closely related to that of IL-1 [7]. IL-18 induces Th1 lymphocytes to secrete IFN-γ, increases the expression of Fas/FasL, and promotes the proliferation of T cells and natural killer (NK) cells. The bioactivity of IL-18 was evaluated by detecting IFN-γ secretion. Our studies found that TP effectively inhibited the expression of IL-18 and IFN-γ. IFN-γ is a prime proinflammatory cytokine in inflammation and autoimmune disease [24]. IFN-γ plays its role by inducing the production of first iNOS and then NO. TP decreases the expression level of IL-18,

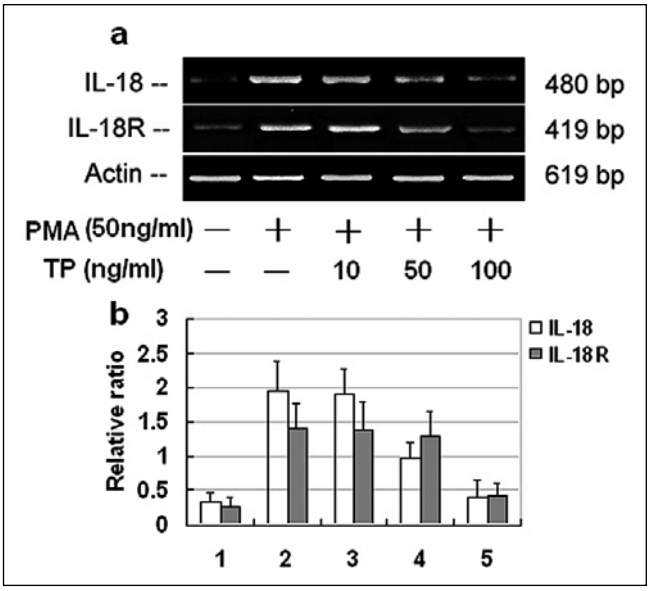

Fig. 3. Detection of mRNA expression of IL-18 and IL-18R in treated RASF by real-time quantitative RT-PCR.
RASF (1×10^6) were cultured in a 6-well plate, treated with TP for 2 h, and finally stimulated with PMA for 18 h. Total RNA was isolated from RASF using TRIzol reagent. Quantitative RT-PCR was performed by LightCycler technology with SYBR Green I detection. The reaction specificity was confirmed by 1.5 % agarose gel electrophoresis of products after real-time PCR and melting curve analysis.
a. PCR products were separated on 1.5 % agarose gel with ethidium bromide in TBE buffer.
b. The expression of IL-18 and IL-18R mRNA in treated RASF was detected by LightCycler technology with SYBR Green I detection. The results are expressed as the ratios of IL-18 to β-actin and IL-18R to β-actin.

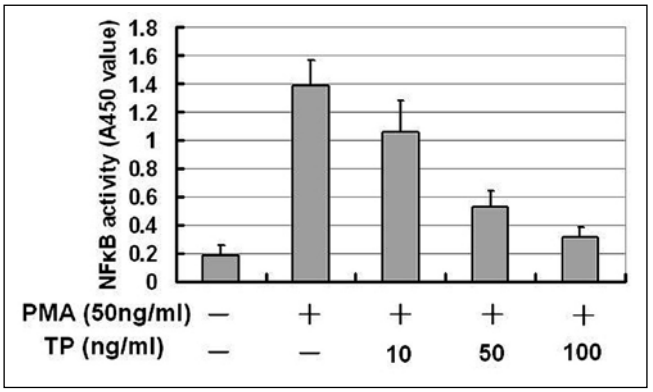

Fig. 4. NF-κB activity in cell extracts of treated RASF was measured using an ELISA-based method.
RASF were cultured and treated with TP for 2 h and finally stimulated with PMA for 18 h. The cells were collected, and whole-cell extracts were prepared. Cell extracts (5 μg/well) were added to a 96-well plate coated with an oligonucleotide probe containing the site for NF-κB binding. NF-κB activity was measured using an ELISA-based method of Active Motif. The results were expressed as A450 values.

which indirectly induces the decrease in IFN-γ secretion and NO production.

Th1 secretes IL-2 and IFN-γ to maintain the inflammatory environment. T-cell helper type 2 (Th2) secretes cytokines such as IL-4 and IL-10 to provide protective effect. The overexpression of IL-18 induces Th1 to secrete more IFN-γ and stimulates differentiation of Th1; this impairs the balance of Th1/Th2 and aggravates the state of RA. TP inhibits IL-18 expression to maintain the balance of Th1/Th2 and alleviates the inflammatory state in RA.

Our immunofluorescence and FAScan studies showed that IL-18 and IL-18R were differentially inhibited by TP. Only high concentration (100 ng/ml) of TP could effectively inhibit the expression of IL-18R. Western blot analysis and LightCycler RT-PCR revealed that TP could suppress the expression of IL-18 and IL-18R at both protein and gene levels. Compared to IL-18R, the inhibitory effect of TP on IL-18 was more obvious. These results indicate that IL-18 is the main target of TP. As inhibition of IL-18 expression, IL-18R feeds back to decreases subsequently.

NF-κB is an important transcription factor that regulates the expression of inflammatory mediators. Han [25] reported that NF-κB expression was increased and its activity was enhanced in RA. Activated NF-κB improved the transcription level of multiple genes, including IL-1β, TNF-α, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and COX2. Moreover, the overexpression of inflammatory factors such as TNF-α and IL-1β induces the activation of NF-κB [26]. This results in the formation of a positive regulatory cycle, which may amplify and maintain the progression of RA [26]. Additionally, NF-κB suppressed the apoptosis of synovial cells in RA. Our results showed that TP obviously suppresses the activity of NF-κB in a concentration-dependent manner. The NF-κB pathway may serve as another therapeutic target in RA. IL-18 transmits signals mainly via the NF-κB signaling pathway [27]. IL-18 significantly enhanced the production of angiogenic factors in RASF, depending on JNK, p38 MAPK, phosphatidylinositol 3-kinase (PI3K), and NF-κB [28]. The activity of IL-18 depends on an IL-18 receptor (IL-18R) complex [7]. This IL-18R complex is composed of a binding chain known as IL-18Rα and a signaling chain, which is a member of the IL-1 receptor family. IL-18Rα is a member of the IL-1R family, which was previously identified as IL-1 receptor-related protein (IL-1Rrp) [7]. The IL-18R complex recruits IL-1R-activating kinase (IRAK) and TNF receptor-associated factor-6 (TRAF-6), which phosphorylates NF-κB-inducing kinase (NIK) with subsequent activation of NF-κB. Sylvester J [29] reported that TP suppressed the expression of MMP genes in articular chondrocytes by inhibiting NF-κB activities. TP effectively inhibited the expression of IL-18, IL-18R, and NF-κB in RASF treated with PMA. It is presumed that the inhibitory effect of TP on IL-18 and its receptor is related to NF-κB activity.

In conclusion, TP exhibits therapeutic effect on RA by effectively inhibiting the expression of IL-18 and IL-18R at protein and gene levels and subsequently suppressing the activity of NF-κB, which decreases IFN-γ secretion, NO production, and angiogenesis. TP also maintains the balance of Th1/Th2 in RA. It has been reported that IL-18 monoclonal antibody, dissoluble IL-18 receptor (IL-18R), and IL-18 binding protein (IL-18BP) were applied for RA therapy [30–33]. In this case, TP cooperates with these IL-18 inhibitors to suppress the expression of IL-18 and IL-18R; this strategy could be applied for clinical therapy of RA.

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