Abstract

Gouty arthritis (GA) is an inflammatory disease caused by the deposition of monosodium urate (MSU) crystals into joints. Tetrandrine (TET) is a bisbenzylisoquinoline alkaloid extracted from the root of Stephania tetrandra and can exert an anti-inflammatory function in different diseases. Nevertheless, the specific function of TET in GA remains unclear. We established the GA mouse model by MSU injection into joints of mice. Paw volume and gait score were detected for measuring the degree of joint swelling and the situation of joint dysfunction. Western blot were utilized to test the alterations of M1-related factors (IL-6, IL-1β, TNF-α, IL-12, and iNOS) and M2-related factors (Mgl1, Mgl2, Pgc1-β, Arg-1, and IL-10). The activity of NF-κB p65 in tissues was determined. The interaction of NF-κB p65 and Lcp1 was measured by ChIP and luciferase reporter assay. Lcp1 KO mice were utilized to detect the effect of Lcp1 depletion on GA process. TET treatment markedly suppressed MSU-induced joint swelling, joint dysfunction, and joint injury in GA mice. TET can also reduce inflammatory reactions in MUS-induced mice. Furthermore, we proved that TET facilitated M2 macrophage polarization and inhibited M1 macrophage polarization in GA mice. In addition, TET was found to inhibit NF-κB activity and NF-κB-mediated Lcp1 expression. Lcp1 knockdown can improve joint injury and promote M2 macrophage polarization in GA mice, while this effect was further enhanced by TET. TET alleviates inflammation and facilitates macrophage M2 polarization in GA by NF-κB-mediated Lcp1.

Keywords: Gout arthritis, Lcp1, Macrophage polarization, NF-κB, Tetrandrine

1. Introduction

Gouty arthritis (GA) is an inflammatory disease caused by disorders of purine metabolism and decreased uric acid excretion [1]. GA is characterized by an innate immune disorder and causes a systemic inflammatory reaction which could result in the deposition of monosodium urate (MSU) crystals in the joints and surrounding tissues [2]. The incidence rate of GA is about 1-2% and is increasing every year, especially in developing countries [3]. It can cause assorted complications, such as hypertension, type 2 diabetes, coronary artery disease, and kidney disease [3]. GA is paroxysmal and commonly occurs in the human extremities, usually presenting as redness, swelling, warmth and pain in individual joints and limiting movement [4]. Current research on the subject suggests that episodes of GA are associated with people’s diets and lifestyles, such as high protein, high purine compounds and high-stress lifestyles [4]. First-line medications commonly used in GA include NSAIDs, colchicine and glucocorticoids, which can quickly suppress the inflammatory response and relieve pain [5]. These western drugs have shown good efficacy in treating single episodes of GA, but repeated use tends to develop resistance and cause adverse effects such as gastrointestinal, liver and kidney damage, which leads to diminished utility and effectiveness of the drugs. Active extracts of natural herbs are increasingly receiving more and more attention because of their promising efficacy, less adverse effects and wide availability [6]. Therefore, the study of traditional herbal medicines is necessary to develop new GA treatments.

Macrophages are the crucial components of the immune system. Macrophage polarization has been confirmed by many studies to exert a crucial function in assorted pathophysiological processes, including inflammatory reaction, tissue repair and metabolism [7, 8]. Macrophages have two polarized phenotypes, the classically activated M1 and the alternatively activated M2. M1 macrophages play mainly pro-inflammatory, pathogenic microbial clearance and anti-tumor roles, whereas M2 macrophages play the opposite role, suppressing inflammatory responses, promoting tissue remodeling, and participating in immune regulation [9]. Studies have shown that macrophages acquire a pro-
inflammatory M1 phenotype after MSU stimulation [10]. Then, endothelial cells are damaged by the activation of inflammatory mediators [11]. Studies have confirmed that Kinsenoside alleviates osteoarthritis via macrophage polarization through the inactivation of NF-κB/MAPK pathway [12]. Simiao Wan attenuates MSU-stimulated arthritis by regulating macrophage M2 polarization [13]. Tetrandrine (TET) is a bisbenzylisoquinoline alkaloid extracted from the root of *Stephania tetrandra* and possesses pharmacological actions such as anti-inflammation, analgesia, anti-fibrosis, and anti-tumor [14], which is widely utilized in human diseases such as tuberculosis, hyperglycemia, malaria, and cardiovascular diseases [14, 15]. Studies have demonstrated that TET can suppress the NF-κB pathway via the inhibition of IkBα and NF-κB p65 phosphorylation, thereby decreasing the release of proinflammatory factors [16]. TET suppresses rheumatoid arthritis via inhibiting neutrophil activity [17]. TET inhibits migration and invasion of rheumatoid arthritis fibroblast-like synoviocytes via activating PI3K signaling [18]. However, TET function in GA process remains unclear.

In this study, the MSU-induced GA mouse model was established, and we explored whether TET can regulate macrophage M2 polarization to alleviate GA process.

2. Materials and methods

2.1. Animals

All animal studies were approved by the Ethics Committee of YHANGZHOU HIBIO TECHNOLOGY CO. LTD (HB2208016). Lcp1 knock-out (KO) mice were purchased from Cyagen Biosciences Inc. (Guangzhou, China) and C57BL/6J mice were purchased from HANGZHOU HIBIO TECHNOLOGY CO. LTD (HB2208016). Mice were housed in the standard environment (23–25°C, 40–60% humidity, and a 12 h light/dark cycle) and adaptively raised for a week.

2.2. The establishment of MIA mice

Sixty C57BL/6J mice were randomly divided into 6 groups (n=10 per group): the control group, the MIA group, the colchicine (COL; 0.3 mg/kg) group, the 2 mg/kg TET group, the 4 mg/kg TET group, and the 8 mg/kg TET group. Mice in the control group and MIA group were intragastrically administered an equal volume of normal saline for 7 days. Mice in the COL group and TET groups received COL or TET (Sigma Aldrich, St. Louis, MO, USA) intragastrically once a day for 7 days. On day 6, mice of the MIA group, the COL group, and the TET group were subjected to intra-articular injection of MSU crystals (0.5 mg in 20 µl of sterile PBS; Sigma–Aldrich) into the left paw under 1% isoflurane anesthesia. Mice in the control group and MIA group were injected with an equal volume of PBS. After 24 h MSU, the paw volume of the left hind limb was assessed through an electronic caliper and the gait score was measured. Next, the mice were anesthetized with isoflurane and the blood was gathered. Then, mice were euthanized and the ankle joint tissues of mice were collected for further assays.

2.3. Measurement of gait score

Gait score was utilized for measuring the behavior disorder. It is divided into four grades from 0 to 3: 0 represents normal gait; 1 represents slight limp; 2 represents moderate limp; and 3 represents severe limp. The gait score was used by two observers who did not know the experimental protocol.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Serum was gathered by centrifugation at 6000 × g for 10 min. The contents of IL-1β and IL-10 in serum were determined by their corresponding ELISA kit (Solarbio, Inc., China) in line with user guides.

2.5. H&E staining assay

The ankle joint tissues were fixed with 4% paraformaldehyde, decalcified in 10% EDTA, and then embedded in paraffin. Next, they were sliced at 4 µm intervals. Sections were stained with Hematoxylin for 15 min and re-stained with Eosin for 5 min. Later, they were dehydrated with alcohol, hyalinized, and sealed with neutral resins. The microscope (Olympus) was applied for observation.

2.6. Detection of NF-κB activity

Based on the user guides, NE-PER nuclear extraction kit (Thermo Fisher, USA) was applied to obtain the nuclear extracts. Tissues were homogenized in the CER I buffer and cultured on ice. After 10 min, CER II buffer was supplemented into the tubes and subjected to centrifugation. The supernatants were removed and the pellets were resuspended in ice-cold nuclear extraction reagent. After centrifugation, the supernatant containing the nuclear extract was performed with NF-κB p65 Transcription Factor Assay Kit (Abcam) in accordance with user guides. The activity of NF-κB p65 in the sample was quantified by reading the absorbance value at 450 nm.

2.7. Cell culture and transfection

RAW264.7 macrophage cells and HEK-293T cells were obtained from Procell (Wuhan, China) and incubated in DMEM (Gibco, USA) added with 10% FBS (Hycolon, USA) at 37°C with 5% CO₂. To silence p65 expression, cells were transfected with 25 nM of p65 siRNA (si-p65; Genechem, Shanghai, China) and the negative control siRNA (si-NC; Genechem) by Lipofectamine 3000 (Invitrogen, USA) for 48 h.

2.8. RT-qPCR

TRIzol (Invitrogen) was used to extract RNA from cells or tissues. Reverse transcription was performed with ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) in line with user guides. Next, qPCR was performed utilizing SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Gene expression was calculated with the 2 −ΔΔCt methods normalized to U6 or GAPDH.

2.9. Western blot

The total proteins from collected tissues were extracted using RIPA lysis buffer. Proteins were separated by 10% SDS-PAGE and blotted on PVDF membranes (Millipore, Billerica, MA, USA). After blocked with 5% skim milk, membranes were cultured with primary antibodies (Abcam, USA) at 4°C for one night. Then, membranes were cultured with the secondary antibody for 2 h. The membranes were visualized by ECL reagent (Beckman Colter, Brea, CA, USA). The relative densities of protein bands were analyzed by ImageJ.
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2.10. ChIP assay
Cells were cross-linked with 1% formaldehyde. Next, lysis buffer was placed into the treated cells and chromatin was sheared to DNA fragments of 150–900 bp by sonication. After that, anti-p65 (Abcam) or anti-IgG was added into the sonicated mixtures for incubation. The precipitated complexes were rinsed and reverse cross-linked. After purification, the extracted DNA was subjected to amplification by RT-qPCR analysis.

2.11. Luciferase reporter assay
The p65 binding sites to Lcp1 promoter were inserted into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA). Cells were subjected to co-transfection with pGL3-Lcp1 and si-p65 or si-NC for 48 h. Dual-Luciferase Reporter System (Promega) was applied to test the luciferase activity.

2.12. Statistical analysis
GraphPad Prism software (version 7.0, USA) was utilized to analyze the data. The measurement data were expressed as means ± SD from three individual repeats. Student’s t-test was applied for comparison between two groups. The comparison among multiple groups was analyzed by the one-way ANOVA. *p<0.05 indicated a statistically significant difference.

3. Results
3.1. Effects of TET on paw volume, gait score, and histological score of MIA mice
Through the utilizing of pubchem database (https://pubchem.ncbi.nlm.nih.gov/), we obtained the 2D and 3D structural formulas of TET and they were shown in Figures 1A, B. In order to explore the function of TET on GA process, we established the MIA mouse model and treated mice with different doses of TET (2 mg/kg, 4 mg/kg, and 8 mg/kg). Paw volume and gait score were commonly utilized for measuring the degree of joint swelling and the situation of joint dysfunction (Figures 1C, D). The results displayed that paw volume and gait score were significantly elevated in MIA mice in comparison to control mice. The treatment of TET notably reduced the two test indicators in a dose-dependent manner in comparison to MIA mice. COL is clinically used to treat gout, and here is used as a positive control. Obviously, COL can also reduce paw volume and gait score in MIA mice. Then, it was illustrated by HE staining that, compared with the control mice, there was obvious inflammatory cell infiltration, synovial hyperplasia and tissue necrosis in the ankle joint tissues of MIA mice. TET (4 mg/kg and 8 mg/kg) treatment significantly reduced this phenomenon, while 2 mg/kg of TET has little effect (Figure 1E). Thus, we confirmed that TET could alleviate GA in mice.

3.2. Effects of TET on macrophage repolarization markers in MIA mice
The effect of TET on inflammatory reaction and macrophage repolarization was further investigated. ELISA results indicated that IL-1β content in serum of MIA mice was significantly increased compared with control mice, while TET treatment (4 mg/kg, and 8 mg/kg) markedly reduced its content (Figure 2A). By contraries, IL-10 content reduced in serum of MIA mice was gradually recovered by TET treatment (4 mg/kg, and 8 mg/kg) (Figure 2B). Then, western blot was utilized to detect the levels of iNOS (M1 macrophage marker) and Arg-1 (M2 macrophage marker) in tissues. As a result, iNOS levels were increased in tissues of MIA group, while TET treatment reduced its levels. Arg-1 levels decreased in MIA mice were promoted by TET treatment (Figure 2C). Next, the expression of M1/M2-related genes was further detected. We found that TET declined IL-6, IL-1β, TNF-α, IL-12, and iNOS (M1-related genes) expression in tissues of MIA mice, but it elevated Mgl1, Mgl2, Pgc1-β, Arg-1, and IL-10 (M2-related genes) expression (Figures 2D, E). These outcomes suggested that TET can inhibit M1 macrophage repolarization and promote M2 macrophage repolarization in GA.

3.3. TET inhibits NF-κB-mediated Lcp1 expression in MIA mice
The NF-κB pathway is well-known as an inflammation-related pathway and has been repeatedly shown to be activated in GA [19, 20]. We found that NF-κB p65 activity was enhanced in tissues of MIA mice by using
that compared with the significant inflammatory infiltration (Figures 4A, B). Furthermore, HE staining showed the effect of Lcp1 silencing on joint swelling and joint function in MIA mice. TET treatment further enhanced the alleviation on joint swelling and joint dysfunction in MIA mice. We discovered that, compared with the control MIA mice, Lcp1 KO MIA mice showed less inflammation and tissue necrosis at the joint injury site, and its expression was decreased after TET treatment (Figure 3C). ChIP assay was used to detect the interaction between p65 and Lcp1. As a result, p65 can bind to the promoter region of Lcp1 in tissues and RAW264.7 cells (Figure 3D). Through the JASPAR website (https://jaspar.genereg.net/), we obtained the binding sites of p65 on the promoter region of Lcp1, and the schematic diagram is shown in Figure 3E. Then, the luciferase activity of the Lcp1 promoter was observed to decline when knocking down p65 in HEK293T and RAW264.7 cells, further confirming the combination of Lcp1 promoter and p65 (Figure 3F). We also observed that the expression level of Lcp1 was markedly suppressed upon knockdown of p65 (Figure 3G). These results confirm that p65 can transcriptionally activate the expression of Lcp1. We therefore concluded that TET inhibited NF-κB-mediated Lcp1 expression in MIA mice.

3.4. Effects of Lcp1 silencing on joint swelling and joint dysfunction in MIA mice

We purchased the Lcp1 KO mice to further detect the effect of Lcp1 silencing on joint swelling and joint dysfunction of MIA mice. We discovered that, compared with the control MIA mice, Lcp1 KO MIA mice showed less paw volume and lower gait score, suggesting Lcp1 silencing notably alleviated joint swelling and joint dysfunction in MIA mice. TET treatment further enhanced the effect of Lcp1 silencing on joint swelling and joint function (Figures 4A, B). Furthermore, HE staining showed that compared with the significant inflammatory infiltration, synovial hyperplasia and necrosis in the tissues of MIA group, we observed that these pathological changes were significantly alleviated in MIA+Lcp1 KO group, and the treatment effect was more obvious after TET administration (Figure 4C). The same trend appeared in the histological score (Figure 4D). Thus, we confirmed that TET improved GA by regulating Lcp1 expression.

3.5. Effects of Lcp1 silencing on macrophage repolarization markers in MIA mice

ELISA was implemented to test the impact of Lcp1 depletion on inflammatory factors. The results illustrated that Lcp1 silencing reduced the IL-1β content and increased IL-10 content and TET treatment further enhanced the effect of Lcp1 silencing (Figures 5A, B). Then we observed that in comparison of control MIA mice, iNOS, IL-6, IL-1β, TNF-α, and IL-12 levels in Lcp1 KO MIA mice were notably decreased, while Arg-1, Mgl1, Mgl2, Pgc1-β, and IL-10 levels were increased. Furthermore, TET administration further strengthened the function of Lcp1 knockdown (Figures 5C-E). In short, these results proved that TET promoted M2 macrophage repolarization by regulating Lcp1 in GA.

4. Discussion

GA is an inflammatory disease caused by the deposition of MSU crystals into joints [1]. In recent years, with the in-depth study of the pathogenesis of GA, a variety of traditional Chinese medicines have been proven to have a significant improvement effect on the development of GA, such as Simiao Decoction [21], Resveratrol [22], and Si-Miao-San [10]. As a bisbenzylisoquinoline alkaloid, TET is widely used in different diseases due to its anti-inflammatory, analgesic and other effects [14]. For instance, TET regulates Rheb-mTOR pathway-mediated autophagy to protect against pulmonary fibrosis [23]. TET relieves silicosis via suppressing NLRP3 inflammasome in lung macrophages [24]. TET alleviates ischemia/reperfusion-induced neuronal damage in the subacute phase [25]. Furthermore, studies have confirmed that TET also exerts protective effects on arthritis by alleviating neutrophil activities [17], inactivating NF-κB [16], and inhibiting osteoclastogenesis [26]. In this study, the GA mouse model was established by MSU injection into ankle joint. We found that 4 mg/kg and 8 mg/kg of TET significantly alleviated joint swelling and dysfunction. Histopathological analysis showed that TET alleviated inflammatory cell infiltration and tissue necrosis at the joint injury site, and its effect was equivalent to that of colchicine. Therefore,
we believe that TET can alleviate the process of GA.

After MSU is recognized and activated in the joint tissues, a large number of pro-inflammatory mediators, including IL-1β, IL-6, TNF-α and chemokines, are transcribed and released, resulting in an acute inflammatory response [27, 28]. Subsequently, neutrophils enter the joint or peri-articular tissues and engulf the deposited MSU crystals, releasing inflammatory cytokines and mononuclear macrophage to amplify and maintain joint inflammation, resulting in further joint swelling [27, 28]. M1 macrophages can secrete lots of pro-inflammatory cytokines. In contrast, M2 macrophages mainly produce anti-inflammatory factors, such as IL-10, TGF-β, and Arg1 [29]. The core of pro-inflammatory switch is the expression of iNOS, which produces a large amount of nitric oxide [30]. Similarly, a key biomarker of M2 activation is the increase of Arg-1 level, which can decompose arginine and inhibit the production of nitric oxide [31, 32]. This study found that M1-related factors (IL-6, IL-1β, TNF-α, IL-12, and iNOS) and M2-related factors (Mgl1, Mgl2, Pgc1-β, Arg-1, and IL-10) in tissues.

\[ *p<0.05, **p<0.01, ***p<0.001. \]

**Fig. 5.** Effects of Lcp1 silencing on macrophage repolarization markers in MIA mice. (A, B) ELISA was utilized to detect the contents of IL-1β and IL-10 in serum of mice in the MIA group, the MIA+Lcp1 KO group, and the MIA+Lcp1 KO+TET group. (C) Western blot of iNOS and Arg-1 levels in different groups. (D, E) Western blot of M1-related factors (IL-6, IL-1β, TNF-α, IL-12, and iNOS) and M2-related factors (Mgl1, Mgl2, Pgc1-β, Arg-1, and IL-10) in tissues.

**Informal Consent**
The authors report no conflict of interest.

**Availability of data and material**
We declared that we embedded all data in the manuscript.

**Authors' contributions**
FL and SR conducted the experiments and wrote the paper; LY analyzed and organized the data; XX and HF conceived, designed the study and revised the manuscript. We thanked Zhoushan Hospital of Zhejiang Province, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine and Zhoushan Medical and Health Science and Technology Project of Zhejiang Province (No. 2023ZD01), and Zhoushan Medical and Health Research Special Funds.

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**References**

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**Fig. 5.** Effects of Lcp1 silencing on macrophage repolarization markers in MIA mice. (A, B) ELISA was utilized to detect the contents of IL-1β and IL-10 in serum of mice in the MIA group, the MIA+Lcp1 KO group, and the MIA+Lcp1 KO+TET group. (C) Western blot of iNOS and Arg-1 levels in different groups. (D, E) Western blot of M1-related factors (IL-6, IL-1β, TNF-α, IL-12, and iNOS) and M2-related factors (Mgl1, Mgl2, Pgc1-β, Arg-1, and IL-10) in tissues.

\[ *p<0.05, **p<0.01, ***p<0.001. \]

**Overall, this study confirmed that TET can alleviate inflammation and promote macrophage M2 polarization to suppress GA process by NF-κB-mediated Lcp1. These discoveries suggest TET may become a new and effective agent for GA.**
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