Abstract. Total saponins extracted from *Dioscorea collettii* (TSD), extracts of the Chinese herb *Dioscorea*, are thought to exhibit therapeutic benefit in gouty arthritis. However, its exact mechanism remains unclear. The current study aimed to elucidate the underlying mechanisms by investigating the effects of TSD on the inflammation induced by monosodium urate (MSU) crystals in THP-1 macrophages. The viability of THP-1 macrophages was examined using the MTT assay and the levels of inflammatory cytokines, including interleukin (IL)-1β, IL-18 and tumor necrosis factor (TNF)-α, released by the cells were quantitatively measured using ELISA kits. The results revealed that the protein level of cluster of differentiation 11b increased in THP-1 cells treated with 100 ng/ml phorbol ester, suggesting that monocytic THP-1 cells were successfully differentiated into macrophages. TSD decreased the levels of inflammatory cytokines, including TNF-α, IL-18 and IL-1β, secreted by THP-1 macrophages. As the release of IL-1β and IL-18 is dependent on the NLR family pyrin domain containing 3 (NALP3) inflammasome and caspase-1, the current study investigated the effect of TSD on the aforementioned proteins. The results revealed that TSD decreased the protein levels of NALP3 and apoptosis-associated speck-like, which serve important roles in the assembly of the NALP3 inflammasome. Furthermore, NALP3 inflammasome-related proteins were also decreased by TSD in rotenone induced THP-1 macrophages, TSD inhibited the activation of caspase-1 and rotenone-induced NALP3 inflammasome activation in THP-1 macrophages. The results obtained in the current study revealed that TSD attenuated MSU crystal-induced inflammation by inhibiting rotenone-induced activation of the NALP3 inflammasome and caspase-1, suggesting that these two proteins may be novel targets for the treatment of gouty arthritis.

Introduction

Gout, a type of inflammation caused by the disturbance of purine metabolism and deposition of monosodium urate (MSU) crystals in synovial fluid and other tissues, is one of the most common forms of inflammatory arthritis, usually in the presence of prolonged hyperuricemia. A longitudinal study reported that the prevalence of gout and hyperuricemia in China were 2.8 and 18.1%, respectively (1). The incidence of gout and hyperuricemia are increasing due to obesity, insulin resistance, metabolic syndrome, renal impairment, cardiovascular disease and hypertension (2).

*Dioscorea collettii* is a traditional Chinese medicine that been used for the treatment of inflammatory conditions, such as gouty arthritis and hyperuricemia, in China for several years (3). Total saponin from *Dioscorea collettii* (TSD) extracted from *Dioscorea* has been reported to have significant anti-inflammatory, analgesic and anti-hyperuricemia effects. A previous study in rats with chronic hyperuricemia revealed that TSD exhibited its effects through the down-regulation of solute carrier family 22 member 12 and solute carrier family 2 member 6 and the upregulation of solute carrier family 22 members 6 and 8 (4). A previous study (5) suggested that the monosodium urate (MSU)-induced inflammatory response is dependent on the inflammatory cytokine interleukin (IL)-1β. The IL-1-dependent innate inflammatory phenotype relies on the formation of the macromolecular NLR family pyrin domain containing 3 (NALP3) inflammasome complex in response to the MSU ‘danger signal’ (6). Therefore,
the NALP3 inflammasome may be a potential target of TSD in gouty arthritis. The present demonstrated that TSD inhibited the secretion of inflammatory cytokines, including IL-1β, IL-18 and tumor necrosis factor (TNF)-α, in THP-1 macrophages treated with MSU crystals. Furthermore, the present study revealed that TSD inhibited the assembly of the NALP3 inflammasome and the activation of caspase-1.

Materials and methods

Drug and reagents. Dioscorea rhizomes were purchased from The First Affiliated Hospital of Anhui University of Chinese Medicine. According to the literature, the saponins were extracted from Dioscorea (4). The total content of TSD in the extract of Dioscorea was 53.1% (4). Urate sodium was purchased from Sigma-Aldrich (Merck KGaA). Colchicine and rotenone were purchased from Shanghai Aladdin Biochem Technology Co., Ltd. ELISA kits for IL-1β (cat. no. F0179A), IL-18 (cat. no. F0138A) and TNF-α (cat. no. F0121A) were purchased from Shanghai Fankewe Technology Industry Co., Ltd (www.shfksc.com).

Preparation of MSU crystals. MSU was prepared according to the method of Huang et al's study (7). Briefly, 1 g uric acid was dissolved in 200 ml boiling water and the solution pH was adjusted to 7.2 and sterilized by heating at 180˚C for 2 h and stored in a sterile environment until use. The crystals were suspended in PBS at a concentration of 50 mg/ml and sonicated 10 min in 40 kHz at room temperature. 10 min to obtain rod-shaped crystals with uniform sizes (5-25 µm in length). A Limulus amebocyte cell lysate assay (cat. no. L00350; GenScript) was used to verify the absence of endotoxin in the preparation. The assay was performed according to the manufacturer's protocol.

Cell culture and drug treatments. The human THP-1 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences. THP-1 cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare), containing 10% FBS (Zhejiang Tianhang Biotechnology Co., Ltd.). The air in the cell incubator was humidified and contained 5% CO₂ and 95% air at 37°C. The medium was changed every 2 days. In order to certify the effect of macrophages on MSU crystals, THP-1 cells were induced into macrophage-like cells. THP-1 cells (2x10⁶ cells/well) were seeded in six-well culture plates and incubated with phorbol 12-myristate acetate (PMA) from 25-200 ng/ml for 24 h, then cells were washed by PBS and observed the morphology under an inverted light microscope at x200 magnification. Images were captured of each well in at least 5 random fields, the result was calculated by the ratio of adhered or pseudopodia-formed THP-1 cells to the total cells. The cells were identified by morphology and cluster of differentiation (CD)11b protein level was characteristic of macrophages.

Viability assays. To evaluate the effects of MSU crystals or TSD on the viability of THP-1 macrophages, THP-1 macrophages were treated with MSU (0, 25, 50, 100, 200, 300 and 400 µg/ml) or TSD (0, 0.1, 0.3, 1, 3, 10 and 30 µg/ml) for 24 h. The viability of THP-1 macrophages was examined by MTT assay and the formazan was dissolved by DMSO (≥99.7%; Sigma-Aldrich; Merck KGaA). Every well was measured at a wavelength of 490 nm (optical density at 490) with the Thermo Varioskan Flash (Thermo Fisher Scientific, Inc.). Cell viability was expressed as a percentage of control cells, which were defined as 100% viable. All the assays were performed in triplicate.

Inflammatory cytokine ELISAs. In order to investigate the most appropriate MSU crystals concentration in THP-1 macrophages, cells were treated with MSU crystals at different concentrations (0, 50, 100, 200, 300 and 400 µg/ml). In follow-up experiments, THP-1 macrophages were treated with MSU (0.3, 1.0 and 3.0 µg/ml) or colchicine (0.5 and 5 µM) for 24 h prior to the stimulation with MSU crystals (400 µg/ml) or rotenone (80 µM). The level of the inflammatory cytokines, such as IL-1β, IL-18 and TNF-α in the supernatants of media were quantitatively measured with ELISA kits as listed above. The ELISA plates were measured using a Thermo Varioskan Flash (Thermo Fisher Scientific, Inc.).

Western blot analysis. THP-1 macrophages grouping was the same as above. Cells were lysed in RIPA (Beyotime Institute of Biotechnology) buffer containing 1 mM PMSF (Beyotime Institute of Biotechnology). Protein samples (30 µg/lane) extracted from cell lysate were separated by SDS-PAGE. The separated proteins in the gel were transferred onto a polyvinylidene difluoride membrane (EMD Millipore), blocked with 2.5% TBST milk [10 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.5% Tween-20, 2.5% skim milk] for 2 h at room temperature and probed with anti-CD11b antibody (cat. no. ab133357; Abcam), anti-NALP3 antibody (D2P5E; cat. no. 13158; Cell Signaling Technology, Inc.), anti-ASC antibody (ab133357; Abcam), anti-β-actin antibody (cat. no. 4872; Cell Signaling Technology, Inc.); respectively. All antibodies were used at 1:1,000 dilution at 4˚C overnight. All antibodies were diluted with TBST milk. The secondary antibodies, 1:10,000, (Alexa Fluor® 680 goat anti-mouse IgG; cat. no. A11022; Jackson ImmunoResearch, Inc.) and the grey value were analyzed with Odyssey (LI-COR Biosciences), respectively. All antibodies were used at 1:1,000 dilution at 4˚C overnight. All antibodies were diluted with TBST milk. The secondary antibodies, 1:10,000, (Alexa Fluor® 790 goat anti-rabbit IgG, cat. no. A11-655-144 and Alex Fluor® 680 goat anti-mouse IgG; cat. no. A11-625-146) were incubated with the membranes for 2 h at room temperature and detected with an Odyssey® CLX Infrared Imaging System (LI-COR Biosciences), and the grey value were analyzed with ImageJ 1.52a (National Institutes of Health). The assay was performed in triplicate.

Assay of mRNA expression level of NALP3 with reverse transcription (RT)-PCR. The THP-1 macrophage grouping was the same as above. The mRNA expression levels of NALP3 gene were determined by RT-PCR. Total RNA was extracted from THP-1 macrophages with different treatments using the total RNA extraction kit. RT-PCR was performed using SuperScript™ III One-Step RT-PCR system (Thermo Fisher Scientific, Inc.). The β-actin gene was used as an internal control for normalization of transcription levels. The following
primer sequences were used: NALP3 (NALP3 forward, 5'-TTCTCTCTGTACGAGTTCTCAAG-3' and NALP3 reverse, 5'-GGATCTTCTGAGGTAGTCG-3'); and β-actin (β-actin forward, 5'-GAGACCTTCAACACCCAGCC-3' and β-actin reverse, 5'-GGATCTTCTGAGGTAGTCG-3'). RT-PCR amplification (Thermo Fisher Scientific, Inc.) was performed using a protocol of 50˚C for 2 min and 95˚C for 10 min, then 95˚C for 15 sec followed by 53˚C for 1 min for 40 cycles. The analysis method of RT-PCR was carried out using the 2-ΔΔCq method (8).

Statistical analysis. Data were expressed as the mean ± standard deviation. The results were analyzed for statistical significance using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. All data analysis was performed with SPSS 17.0 software (IBM Corp.).

Results

THP-1 cells are successfully differentiated into macrophages by PMA. THP-1 cells were exposed to 25-200 ng/ml PMA for 24 h and examined under an inverted microscope (9,10). As shown in Fig. 1A, THP-1 cells treated with PMA at concentrations >25 ng/ml exhibited cell adhesion, spreading and formed pseudopodia, suggesting that the cells successfully differentiated into macrophages (11,12). Furthermore, the level of CD11b, a macrophage surface marker (13), was analyzed in the THP-1 macrophages. There was a significant increase in the CD11b protein level in cells treated with PMA at concentrations exceeding 100 ng/ml (P<0.05; Fig. 1B). Therefore, 100 ng/ml PMA was used in subsequent experiments.

Cytotoxic effect of MSU and TSD on THP-1 macrophages. The cytotoxic effect of MSU and TSD on differentiated THP-1 cells was measured using the MTT assay. As shown in Fig. 2, no significant changes in cell viability were observed in THP-1 macrophages treated with MSU at concentrations up to 400 µg/ml or TSD at concentrations up to 3 µg/ml for 24 h (48 or 72 h-treatment showed the similar results with 24 h, Fig. S1). Based on these results, MSU at a concentration of 400 µg/ml and TSD at a concentration range of 0.1-3 µg/ml were used for further experimentation.

MSU induces THP-1 macrophages to secrete IL-1β. Previous studies reported that macrophages respond to MSU crystals during the progression of the gout inflammatory response (14-16). MSU crystals subsequently lead to the production of inflammatory cytokines such as IL-1β, which is a key regulatory cytokine in gout (6). The present study investigated whether MSU induces the secretion of IL-1β in THP-1 macrophages. As shown in Fig. 3, the level of IL-1β in the
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In cell culture medium increased ~1.79- and 1.92-fold following treatment with MSU at concentrations of 300 and 400 µg/ml, respectively. Based on these results, MSU at a concentration of 400 µg/ml was selected for subsequent experimentation.

**TS D** significantly inhibits MSU-induced secretion of inflammatory cytokines in THP-1 macrophages. In order to investigate whether TSD inhibits the production of inflammatory cytokines in THP-1 macrophages treated with MSU crystals, cells were treated with or without TSD for 24 h prior to stimulation with 400 µg/ml MSU. Cells treated with colchicine (0.5 or 5 µM) served as the positive controls. As shown in Fig. 4, treatment with MSU crystals significantly increased the protein levels of IL-1β, IL-18 and TNF-α in THP-1 macrophages (P<0.05). However, the levels of IL-1β decreased to ~72.2 and 71.2% in cells treated with 1 and 3 µg/ml TSD, respectively (P<0.01; Fig. 4A). The levels of IL-18 significantly decreased to ~57.0 and 66.6% in cells treated with 1 and 3 µg/ml TSD, respectively (P<0.01; Fig. 4B). The levels of TNF-α decreased to ~50.1% in cells treated with 3 µg/ml TSD (P<0.01; Fig. 4C). Cells treated with colchicine exhibited similar results. These data indicated that TSD decreased the secretion of IL-1β, IL-18 and TNF-α from THP-1 macrophages.

**T S D** inhibits the activation of the NALP3 inflammasome.

The release of IL-1β and IL-18 is dependent on the NALP3 inflammasome and caspase-1 (17-19). The present study therefore investigated whether TSD inhibited the secretion of IL-1β via the NALP3 inflammasome. As NALP3 and apoptosis-associated speck-like (ASC) serve an important role in NALP3 inflammasome assembly (20,21), the protein levels of NALP3 and ASC in TSD-treated THP-1 macrophages were measured in the current study. As shown in Fig. 5A, the protein level of NALP3 decreased to ~64.5 and 38.8% in cells treated with 1 and 3 µg/ml TSD compared with the MSU + PBS (control) group, respectively (P<0.05). The protein level of ASC decreased to ~66.3, 63.2 and 42.9% in cells treated with 0.3, 1 and 3 µg/ml TSD, respectively (P<0.05; Fig. 5A). Additionally, the protein levels of caspase-1 p45 and p20, which are activated by the NALP3 inflammasome, were investigated. As presented in Fig. 5A, the protein levels of caspase-1 p45 and p20 significantly decreased to ~64.4 and 88.1% in cells treated with 3 µg/ml TSD compared with the MSU + PBS (control) group, respectively (P<0.05). The same trend was observed in cells treated with colchicine (Fig. 5A). However, there were no significant differences in the protein levels of NALP3, ASC, caspase-1 p45 and p20.
in cells treated with MSU + PBS compared with the MSU group (Fig. 5A).

NALP3 is a critical inflammasome component which recognize numerous exogenous and host ligands (14). Once activated, NALP3 recruits the adapter ASC, which in turn recruits pro-caspase-1 (5,17). It was reported that MSU crystals directly increased intercellular NALP3 mRNA expression in human FLS cells (22). Additionally a previous study showed that NALP3 transcript levels are increased after treatment of macrophages for 6 h with MSU (23). Therefore it was investigated whether TSD could inhibit the transcriptional level of NALP3. As shown in Fig. 5B, the level of NALP3 decreased ~45.0 and 61% in cells treated with 3 µg/ml TSD, respectively, compared with rotenone + PBS-treated cells (P<0.05). The protein level of ASC decreased ~45.0 and 61% in cells treated with 1 and 3 µg/ml TSD, respectively.

Mitochondrial reactive oxygen species (ROS) may be a major trigger for the activation of the NLRP3 inflammasome and production of inflammatory cytokines (6). The current study investigated the effects of TSD in rotenone-induced THP-1 macrophages. Rotenone is a respiration chain complex I inhibitor and induces mitochondrial ROS production. As shown in Fig. 6, THP-1 macrophages NALP3 inflammasome-associated protein levels were decreased in THP-1 macrophages which were incubated with TSD before and after incubation with rotenone for 6 h. The protein levels of NALP3, caspase-1 p45 and caspase-1 p20 significantly decreased ~78.7, 38.7 and 47.4% in cells treated with 3 µg/ml TSD, respectively, compared with rotenone + PBS-treated cells (P<0.05). The protein level of ASC decreased ~45.0 and 61% in cells treated with 1 and 3 µg/ml TSD, respectively.

Discussion

MSU deposition is the major source of inflammation in gouty arthritis (24-26). Current treatment strategies for gout remain suboptimal. Clinical studies have revealed that a number of novel drugs, including IL-1R antagonists, may reduce the pain and inflammation associated with gout (27-30). Furthermore, several trials demonstrated that IL-1 inhibitors prevent flares during the initial stages of allopurinol therapy (31).

Dioscorea colletii has been widely used for the treatment of gouty arthritis in traditional Chinese medicine. TSD, the extract prepared from Dioscorea colletii, is primarily used for the treatment of gout and hyperuricemia in rats (32). However, mechanisms by which TSD exerts its effects in gout have not been fully elucidated. The present study therefore investigated the mechanism of TSD in gouty arthritis.

Previous studies demonstrated that MSU crystals recruit monocytes and promote their differentiation into proinflammatory M1-like macrophages (33-35). The resulting inflammation is likely to serve a role in the deposition of MSU crystals in gout (36). A growing body of evidence suggests that the MSU crystal-induced differentiation of monocytes into macrophages is associated with the initiation, progression and resolution of acute gouty inflammation (5,37). In addition, it was reported that MSU crystals increase the secretion of cytokines and chemokines, including TNF-α, IL-1β, IL-18, IL-6, and IL-8, by macrophages and monocytes (38-40). IL-1β and TNF-α have been implicated in the development of gout flare-ups (41).

A previous study demonstrated increased levels of NALP3 mRNA and protein in MSU-treated human fibroblast-like synoviocytes 6 to 48 h after treatment (42). Additionally, MSU crystals induced a significant increase in IL-1β in the culture medium with a peak concentration at 6 h following MSU treatment (21). The authors’ previous study revealed that 400 µg/ml MSU significantly increased the level of IL-1β and NALP3 inflammasome activation in THP-1 macrophages 6 h following treatment (43). Similar results were obtained in the current study. Additionally, the present study demonstrated that TSD inhibited the secretion of inflammatory cytokines, including IL-1β, IL-18 and TNF-α, from THP-1 macrophages treated with MSU crystals, suggesting that TSD may exert a therapeutic effect in gout by decreasing the inflammation induced by MSU.

A previous study reported that innate immunity is involved in the pathogenesis of gout (44), suggesting that MSU crystals may be recognized by pattern-recognition receptors (PRRs), such as...
nucleotide-binding oligomerization domain-like receptors (NLRs) and toll-like receptors (TLRs). NALP3 is a member of the NLR family, intracellular PRRs that recognize pathogen-associated molecular patterns and danger-associated molecular patterns (14).

MSU crystal-induced inflammation relies on the activation of the NALP3 inflammasome, which consists of NALP3, ASC and caspase-1 (17,45,46). Following activation by MSU crystals, NALP3 assembles with the adaptor protein ASC to form a protein-complex termed the NALP3 inflammasome. The NALP3 inflammasome subsequently cleaves caspase-1 to the active enzyme form. The activated caspase-1 cleaves pro-IL-1β to IL-1β (47-49). Several studies have indicated that the aberrant activation of the NALP3 inflammasome is associated with the pathogenesis of autoimmune and chronic inflammatory and metabolic diseases, including...
Atherosclerosis, type 2 diabetes and gout (50-53). A previous study revealed that macrophages deficient of the NALP3 inflammasome components caspase-1 and ASC exhibited significantly reduced MSU crystals-induced inflammatory responses, and did not produce IL-1β. This suggests that the NALP3 inflammasome and activated caspase-1 play an important role in MSU crystal-induced inflammation (14). The current study revealed that TSD decreased the protein levels of NALP3 and ASC in MSU-treated THP-1 macrophages. Additionally, the present study demonstrated that TSD inhibited the activation of caspase-1 in THP-1 macrophages treated with MSU crystals. The aforementioned results suggested that TSD may attenuate MSU-induced inflammation by inhibiting the activation of the NALP3 inflammasome and caspase-1. Zhou et al (54) reported that the Chinese herbal medicine Dioscorea nipponica...
decreased the activities of β-galactosidase, β-N acetyl glucosamine, β-glucuronidase, acid phosphatase and malonaldehyde and decreased the levels of TNF-α, IL-1β and IL-8 in rats with gouty arthritis, suggesting that Dioscorea nipponica decreased the extent of the self-limiting responses by the NALP3 inflammasome.

ROS are major mediators of the NALP3/IL-1β signaling pathway. Production of pro-inflammatory cytokines is associated with the generation of ROS (55). Mitochondria-and NADPH oxidase-derived ROS have been implicated in pro-inflammatory microglia activation (56). As the abnormal activation of the NALP3 inflammasome is linked to the pathogenesis of autoimmune and chronic inflammatory and metabolic diseases, including atherosclerosis, and type 2 diabetes and gout (50-52), regulating the NALP3 inflammasome may prevent unwanted host damage and excessive inflammation. Dysfunction of the mitochondrial respiratory chain (MRC) is associated with the activation of the NALP3 inflammasome partially due to the release of mitochondrial ROS and DNA. Impairment of the MRC by rotenone confers a selective priming signal for the activation of the NALP3 inflammasome (57).

Ma et al (24) demonstrated that intracerebral hemorrhage-induced inflammatory activation was associated with the activation of the NALP3 inflammasome and revealed that rotenone induced ROS production as well as NALP3 inflammasome activation. Similarly, the present study demonstrated that rotenone induced the activation of the NALP3 inflammasome, which was subsequently suppressed by TSD. The aforementioned results suggested that ROS may trigger the activation of the NALP3 inflammasome and that the NALP3 inflammasome is a potential therapeutic target of TSD in gouty arthritis.

While the present study suggested that TSD may exert a therapeutic effect in gouty arthritis, future in vivo experimental models are required to substantiate the results obtained. Additionally, the effects of TSD on the expression of TLRs and the pro-IL-1β or NALP3 signaling pathway warrant further investigation, and these are the main areas of interest for the next study.

In summary, the current study demonstrated that TSD attenuated MSU-induced production of inflammatory cytokines, such as TNF-α, IL-1β and IL-18, via inhibition of the NALP3 inflammasome and caspase-1 in THP-1 macrophages. The NALP3 inflammasome and caspase-1 may therefore serve as therapeutic targets for TSD in gouty arthritis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GC and LL conceived and designed the study, LW, LZ and LL performed the experiments and data analysis. LW, LZ and LL wrote the manuscript. All authors reviewed, edited and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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