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NLRP3 inflammasome inhibitor cucurbitacin B suppresses gout arthritis in mice

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Abstract

Gouty arthritis is a common inflammatory disease characterized by monosodium urate (MSU) crystal-induced nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome activation with upregulated caspase 1 protease and IL-1β in macrophages. Cucurbitacin B (CuB) is a tetracyclic triterpene that possesses a potential anti-inflammatory activity. However, the immunomodulatory and anti-inflammatory effects of CuB on gout have not been well characterized. Therefore, the purpose of the present study was to determine whether CuB exhibits anti-inflammatory effects on gout and to analyze the underlying molecular mechanism. We examined the effects of CuB on various stimuli-activated bone marrow-derived macrophages (BMDMs) and in a mouse model with MSU-induced acute gouty arthritis. Our results demonstrated that CuB effectively suppressed multiple stimuli-activated IL-1β secretion by interrupting NLRP3 inflammasome complex formation, inhibiting NLRP3 inflammasome activation and suppressing key enzymes of glycolysis in macrophages. Consistent with this, CuB pretreatment also ameliorated MSU-induced arthritis in vivo models of gout arthritis, manifested by reduced foot swelling and inflammatory cell infiltration. Taken together, our data provide the evidence that CuB is an NLRP3 inflammasome inhibitor with therapeutic potential for treating NLRP3 inflammasome-mediated diseases, especially gouty arthritis.

Introduction

Cucurbitacin B (CuB) is a tetracyclic triterpene isolated from Cucurbitaceae (Zhang et al. 2011, Kim et al. 2015). It has a variety of biological activities, including anticancer (Haritunians et al. 2008, Liu et al. 2008, Yin et al. 2008, Thoennissen et al. 2009, Lee et al. 2011a, Dakeng et al. 2012) and anti-inflammatory activities (Kim et al. 2015). So far, most studies on CuB have focused on its potential anti-tumor effects, that is, CuB is considered to be an inhibitor of the growth, migration, and invasion of a wide variety of cancer cells and is an inducer of apoptosis of various cancer cells, including myeloid leukemia, lymphomas, breast cancer, pancreatic cancer, melanomas, and osteosarcoma (Oh et al. 2002, Haritunians et al. 2008, Liu et al. 2008, Yin et al. 2008, Thoennissen et al. 2009, Lee et al. 2011a, Dakeng et al. 2012). However, recent studies have found the anti-cancer mechanisms of CuB differ among different cancer cells (Thoennissen et al. 2009, Jin et al. 2011, Gao et al. 2014, Zheng et al. 2014). Furthermore, CuB could potentially be used as an anti-inflammatory agent (Kim et al. 2015). The recent study also has demonstrated the immunosuppressive characteristics of CuB on LPS-activated peritoneal macrophages (Kim et al. 2015).
CuB suppressed the LPS-activated expression of CD80, CD54, CD40, and MHC II molecules and attenuated the release of LPS-activated pro-inflammatory mediators, such as interleukin (IL)-1β, TNF-α, IL-12, etc. (Kim et al. 2015). However, the immunomodulatory and anti-inflammatory effects of CuB on inflammatory disease remain to be elucidated.

Gout is a multifactorial inflammatory disease characterized by monosodium urate (MSU) crystal-induced gouty arthritis and hyperuricemia (Liu-Bryan 2010, Neogi 2016). Acute gout flares can be initiated by MSU-induced activation of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome and subsequent secretion of IL-1β (Martinson et al. 2006, Liu-Bryan 2010). NLRP3 inflammasome is strongly associated with the initiation and development of various disorders, including chronic metabolic diseases (VandanaMagsar et al. 2011, Lee et al. 2013), cardiovascular diseases (Sandanger et al. 2013, Wang et al. 2014b) and neurological diseases (Jto et al. 2015, Yan et al. 2015). In addition, NLRP3 inflammasome plays a crucial role in the onset and progression of auto-immune and auto-inflammatory diseases (Lissner et al. 2015, Jenko et al. 2016, Pellegrini et al. 2017). NLRP3 inflammasome has been recognized as a receptor and an innate immune system sensor. It manages the activation of caspase 1, and its inappropriate activation can contribute to the onset and progression of the above-mentioned diseases (Fusco et al. 2020). Accumulating evidence has indicated that the NLRP3 inflammasome responds to a diverse set of stimuli and requires two signals for activation: the priming signal activated by a range of infectious and sterile stimuli, including lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA), and the second signal activated by MSU, ATP or Nigericin (Martinson et al. 2006, Cruz et al. 2007, Bauerfeind et al. 2009, Schroder et al. 2010, Freigang et al. 2011, Heid et al. 2013, Rock et al. 2013, Mian et al. 2019). Previous studies have demonstrated the key role of NLRP3 inflammasome in the beginning and progression of gout (Martinson et al. 2006, Liu-Bryan 2010). Upon the stimulation of MSU crystals, the NLRP3 inflammasome forms a macromolecular complex via recruitment of the adaptor protein, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and precursor of caspase 1 (pro-caspase 1) (Schroder et al. 2010, Latz et al. 2013), leading to the cleavage of inactive pro-caspase 1 to the active form of caspase 1 (Davis et al. 2011, Ghaemi-Oskouie & Shi 2011). Subsequently, the inflammasome assembly activates downstream immune responses, including the maturation and secretion of pro-inflammatory cytokines, such as IL-1β and IL-18 (Sutterwala et al. 2006, Franchi et al. 2009, Broderick et al. 2015). Since the activation of the NLRP3 inflammasome and subsequent IL-1β secretion are crucial steps in the pathogenesis of gouty arthritis, inhibition of the NLRP3 inflammasome activation and blocking IL-1β production could serve as potential therapeutic targets for acute gout flares (Schroder et al. 2010, Burns & Wortmann 2011).

Glycolysis has been shown to be a major metabolic pathway involved in immune responses (Krawczyk et al. 2010, Masters et al. 2010, Zhou et al. 2010, Tannahill et al. 2013), especially in NLRP3 inflammasome activation in macrophages (Moon et al. 2015). Recent studies have revealed that the NLRP3 inflammasome is regulated by mitochondrial reactive oxygen species (ROS) and important components of glycolysis, including the transcription factor hypoxia-inducible factor-1α (HIF-1α) (Gupta et al. 2017), hexokinase-1 (HK1) (Moon et al. 2015, Hughes & O’Neill 2018). In addition, HIF-1α serves as a key mediator for succinate-induced IL-1β production (Kim et al. 2006, Tannahill et al. 2013). Previous studies indicated that HIF-1α translocated to the nucleus and interacted with the hypoxia response element to transcribe pro-IL-1β (Kim et al. 2006, Tannahill et al. 2013). It has also been confirmed that chaetocin (a fungal metabolite with anti-inflammatory effects) attenuated MSU-mediated IL-1β release via the HIF-1α pathway in macrophages (Mian et al. 2019), indicating HIF-1α might be a potential target in treating MSU crystal-induced gout flares.

In the current study, LPS was used as a stimulus of priming signal to induce the formation of the IL-1β precursor, and ATP or MSU crystal was used as a second stimulus to trigger the complete activation of the NLRP3 inflammasome, thereby mimicking gouty inflammation in vitro according to the previous studies (Mian et al. 2019). Although CuB has been reported to be a good anti-inflammatory reagent, the effects of CuB on MSU-induced gouty arthritis remain incompletely understood. The aims of this study are to investigate the immunomodulatory activity of CuB on reducing NLRP3 inflammasome activation and IL-1β production via the HIF-1α pathway in bone marrow-derived macrophages (BMDMs) isolated from mice, and the role of CuB in ameliorating inflammation in a mouse model of gout induced by intra-articular MSU crystals.

Materials and methods

Animals

Male C57BL/6 mice, 8-12 weeks old, were purchased from Model Animal Research Center of Nanjing University and
housed in a specific pathogen-free environment at the animal facility of Tongji University for at least 7 days before experiments. An acute gouty arthritis mouse model was performed as previously described (Mian et al. 2019). First, mice in the CuB group were injected intraperitoneally with CuB (2.5 mg/kg) in PBS once a day 72 h prior to MSU crystal administration. The control group was administrated intraperitoneally with the same amount of DMSO in PBS. Secondly, after 72 h, mice were intra-articular injected with MSU crystals suspended in PBS (1 mg/50 μL) into the right hind footpad to establish the mouse model of gouty arthritis. At the same time, PBS was injected into the left foot hind pad as self-control. Eight hours after MSU crystal injection, joint swelling was examined by digimatic caliper. Joint inflammation was measured as the ratio of the footpad thickness of right hind pad over the left hind pad, according to the previous study (Mian et al. 2019). Paw tissues were fixed in formalin for H&E staining. Mouse experimental protocols were carried out in accordance with relevant Chinese institutional laws and guidelines, and were approved by the local ethics committee of Tongji University (Shanghai, China).

Reagents
CuB was purchased from Selleck (Shanghai, China). Anti-TXNIP antibody and anti-pro IL-1β antibody were purchased from Invivogen (Hongkong, China). Anti-NLRP3 antibody was purchased from Novas (Centennial, CO, USA), and anti-ASC antibody was obtained from Santa Cruz Biotechnology. Ultra-pure LPS and ATP were purchased from Invivogen (Hongkong, China). Nigericin was obtained from Sigma-Aldrich. Anti-IL-1β antibody (AF-401-NA) was from R&D Systems. Antibodies against β-tubulin, HIF-1α, caspase 1, HK1, HK2, pyruvate dehydrogenase (PDH), pyruvate kinase M2 (PKM2), and lactate dehydrogenase A (LDHA) were obtained from Cell Signaling Technology. Dulbecco’s modified Eagle’s medium (DMEM) and penicillin-streptomycin (PS) were obtained from Invitrogen. Fetal bovine serum (FBS) was purchased from HyClone Laboratories.

Preparation of MSU crystals
MSU crystals were prepared as described in the previous study (Denko & Whitehouse 1976, Mian et al. 2019). Briefly, 1.68 g uric acid was dissolved in 500 mL of deionized water, heated to 70°C, adjusted to pH 7.2–7.4 with 0.1 M NaOH, and crystallized overnight at room temperature. MSU crystals were recovered by centrifugation, washed with ethyl alcohol and dried at 55°C for 24 h. MSU crystals were sterilized and then suspended to 50 μg/μL by PBS before each experiment. Crystal shape and birefringence were assessed by compensated polarized light microscopy. We measured < 0.015 EU/mL endotoxin in MSU crystal preparations using a Limulus amebocyte lysate assay (Sigma-Aldrich).

Cells culture and treatments
BMDMs were differentiated from precursors which were isolated from femurs and fibula aseptically dislocated from Male C57BL/6 mice as described previously (Mian et al. 2019). Isolated cells were cultured for 1 week in DMEM containing 10% (v/v) FBS, antibiotics (penicillin 100 IU/mL and streptomycin 100 μg/mL), and 50 ng/mL recombinant mouse M-CSF (R&D Systems) as previously described (Mian et al. 2019). After differentiation, BMDMs were pretreated with or without 0.01/0.1/1 μmol/L CuB for 12 h. After that, to stimulate NLRP3 inflammasome, pretreated BMDMs were stimulated with or without 100 ng/mL LPS for 4 h for priming and then cultured with or without 500 μg/mL MSU for 4 h, 5mM ATP for 1 h separately. Then, treated BMDMs were collected and frozen at −80 °C for qPCR and Western blot.

Cell viability assay
The effects of CuB on cell viability in BMDMs were assessed using Cell Counting Kit-8 (YEASEN, China). BMDMs were seeded in a 96-well plate at a density of 1000 cells/well, and subsequently incubated with different concentrations of CuB (0.01/0.1/1 μmol/L) in the presence of LPS plus ATP (or MSU) for 12 h. At the end of CuB treatment, the supernatants were replaced with 100 μL of CCK-8 reagent. After 2 h incubation, the absorbance of each well was then read at 450 nm using the SpectraMaxi3 microplate spectrophotometer (Molecular Devices, San Francisco, CA, USA).

RT-qPCR
Total RNA was extracted from BMDMs using TRIZOL reagent (Invitrogen). cDNA was obtained using a PrimeScript RT reagent Kit (TAKARA) and subsequently quantified by ABI real-time system (Applied Biosystems) using a SYBR Premix Ex Taq kit (TAKARA) following the manufacturer's
instructions. The relative expression of each mRNA after normalization to β-actin was calculated using the ΔΔCt method. The information of all primers was listed in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

Western blot

Following treatment with the various concentrations of CuB, BMDMs were lysed and the protein concentration was determined using a Bio-Rad protein assay following the manufacturer’s instructions. Then, proteins were subjected to standard Western blot analysis. In general, equal amounts of protein were separated electrophoretically using SDS-PAGE, followed by transferring to a nitrocellulose membrane. The blots were incubated with primary antibodies (1:1000), except for anti-IL-1β (1:2000) and ASC (1:500), followed by secondary antibodies (1:5000). Specific proteins were then detected using ECL chemiluminescent substrate (Millipore,) according to the instructions of the manufacturer.

ELISA assays

Culture supernatants were collected, and measurements of IL-1β concentrations were performed using ELISA kits according to manufacturer’s instructions (MultiSciences, China). Serum IL-1β levels of mice were also determined by ELISA assay (MultiSciences).

Statistical analysis

Data were analyzed with the statistical package for social sciences for windows version 18.0 (SPSS), and were presented as the mean ± S.D. Representative data were presented from at least three independent experiments. Significant differences between the groups were analyzed by one-way ANOVA followed by the least significant differences (LSD) or Dunnett’s T3 post hoc multiple comparison test. Differences were considered significant if P < 0.05.

Results

Increase of IL-1β in ATP-induced Inflammation in BMDMs

The cytotoxicity of CuB in BMDMs was evaluated by treatments of BMDMs with different concentrations of CuB (0.01/0.1/1 µmol/L) in the presence of LPS plus ATP (or MSU) for 12 h. The results of CCK-8 assay demonstrated that there was no obvious influence of CuB on the cell viability of BMDMs (Supplementary Fig. 1A and B).

ATP is a common IL-1β stimulus in vitro. In order to investigate whether ATP could induce IL-1β maturation, the effect of ATP on IL-1β levels was examined in LPS primed BMDMs. Compared with the control group, the levels of inflammatory IL-1β in BMDMs and the release of IL-1β in supernatants were notably enhanced upon stimulation of LPS plus ATP (Fig. 1A, B and E).

CuB attenuates IL-1β production in ATP-activated BMDMs

In order to investigate whether CuB could decrease IL-1β maturation, the effect of CuB on ATP-induced IL-1β release was examined in BMDMs. BMDMs were pretreated with different concentrations of CuB (0.01, 0.1, 1 µmol/L) for 12 h. As indicated in Fig. 1C and D, the levels of inflammatory IL-1β in the BMDMs were decreased in a CuB-concentration dependent manner. The results of qPCR showed that IL-1β levels induced by ATP were significantly decreased in BMDMs pretreated with 1 µmol/L CuB (Fig. 1C). Those results were confirmed by Western blot (Fig. 1D). The results of the ELISA test indicated that IL-1β release in the culture medium of LPS primed BMDMs upon stimulation of ATP was significantly increased, and it was reversed in BMDMs pretreated with 0.1 µmol/L CuB (Fig. 1E). Our results suggested incubation of CuB in the activated macrophage cultures showed a significant inhibition of IL-1β levels.

In addition, the mRNA expressions of Tnf-α and Il-18 cytokines were also significantly attenuated by CuB of 0.01, 0.1 or 1 µmol/L upon ATP-activated BMDMs (Supplementary Fig. 2A and B). Taken together, our data demonstrated that CuB could suppress the production of proinflammatory cytokines IL-1β, TNF-α and IL-18 in the ATP-activated macrophages.

CuB inhibits Toll-like receptors 4 (TLR4) signaling in ATP-activated BMDMs

Recent study has revealed that ATP-induced IL-1β release needs a ‘priming’ process to produce a precursor of inflammatory cytokine, which is triggered by TLR ligands (Mian et al. 2019). TLR4, a classic mediator of ‘priming’ signal, can be induced by LPS (a TLR4 agonist) (De Nardo 2015), and stimulates the upregulation of NLRP3 expression in macrophages (Qiao et al. 2012, Liao et al. 2013).

We analyzed mRNA levels for Tnf-α, an NF-κB target gene, which is classically activated by TLR4 agonists (Kishore et al. 2004, Bauernfeind et al. 2009). We found
that the mRNA levels for \textit{Tnf-\alpha} were significantly higher in the LPS plus ATP group and were reversed by CuB (Supplementary Fig. 2A). Then, we assessed the influence of CuB on the expression of the NLRP3 inflammasome complex upon the stimulation of LPS plus ATP. Incubation of CuB of 0.01, 0.1 or 1 \textmu mol/L in the cultures significantly reduced the NLRP3 mRNA and protein expression (Fig. 2A and B). We also found that CuB inhibited LPS plus ATP-induced pro-IL-1\textbeta production (Fig. 3A and B). Our data indicated that CuB might reduce the expression of NLRP3 inflammasome and inhibit the production of pro-IL-1\textbeta by regulating TLR4 signaling.

\textbf{CuB inhibits NLRP3 inflammasome activation in ATP-activated BMDMs}

It has been widely reported the maturation and secretion of IL-1\textbeta can be mediated by the NLRP3 inflammasome. To further investigate the anti-inflammatory properties of CuB, we continued to measure the effects of CuB on the level of key elements of the NLRP3 inflammasome activation upon the stimulation of ATP, such as the adapter ASC, and the effector protein caspase 1 in BMDMs. As shown in Fig. 4A and B, ASC expression was significantly attenuated by the addition of CuB (concentrations of 0.01, 0.1, or 1 \textmu mol/L) into the cultures of LPS plus ATP-treated BMDMs. ASC oligomerization is an essential step for NLRP3 activation. Our data suggested that CuB inhibited ACS expression to suppress NLRP3 activation.

Furthermore, the expression of \textit{Caspase 1} mRNA in LPS plus ATP-activated BMDMs was significantly inhibited by CuB of 0.01, 0.1 or 1 \textmu mol/L (Fig. 4C). Cleaved caspase 1 level was also significantly suppressed in LPS plus ATP-activated BMDMs by CuB of 1 \textmu mol/L (Fig. 4D).

Previous studies have indicated thioredoxin (TRX)-interacting protein (TXNIP) participates in the reactive oxygen species (ROS)-mediated inflammasome activation (Schroder & Tschopp 2010, Zhou \textit{et al.} 2010), and the TXNIP-dependent activation of NLRP3 inflammasomes may be a means for sensing intracellular oxidative stress (Mian \textit{et al.} 2019).
We found that CuB could significantly decrease ATP plus LPS-induced TXNIP and NLRP3 expression in BMDMs (Figs 4E, 2A and B). Taken together, our data demonstrated that CuB can inhibit IL-1β production and cleaved Caspase 1 production via suppressing NLRP3 inflammasome activation and the formation of NLRP3 inflammasome complex in LPS plus ATP-activated macrophages.

**CuB inhibits aerobic glycolysis in macrophages**

It has been reported that HIF-1α signaling is critically involved in glycolysis and is important for IL-1β production (Kim et al. 2006, Lee et al. 2011b, Tannahill et al. 2013). As shown in Fig. 5A, the expression of HIF-1α was significant inhibited by CuB in LPS plus ATP activated BDMs. To investigate the impact of CuB on glycolysis, we examined the effect of CuB on the expression of key enzymes in glycolysis.

As seen in Fig. 5B, the expression of HK1, HK2, PKM2, LDHA and PDH were influenced following CuB treatments. All those key enzymes in glycolysis were significantly downregulated by CuB of 1 μmol/L. According to these data, we suggested that HIF-1α signaling and key enzymes in glycolysis pathway were downregulated by CuB, which would be responsible for the inhibitory effect of CuB on IL-1β production.

**CuB relieves MSU-induced Inflammation in BMDMs**

To further confirm anti-inflammatory effect of CuB in BMDMs, MSU, another typical IL-1β stimulus was used. In addition to ATP, IL-1β production can be activated by MSU (Fig. 6A). CuB treatment resulted in significant decreases in the production of IL-1β, which was tested by Western blot (Fig. 6B). Consistent with the above results, the levels of IL-1β in the culture medium of activated BMDMs were significantly increased, and the addition of CuB remarkably reduced IL-1β levels (Fig. 6F).

To determine whether CuB was a common inhibitor for NLPR3 inflammasome activation, we examined the impact of CuB on MSU-induced NLPR3 inflammasome activation. Treatment with CuB significantly inhibited cleaved Caspase 1, ASC and TXNIP levels triggered by MSU in BMDMs (Fig. 6C and D). Taken together, these results demonstrate that CuB can specifically inhibit NLPR3 inflammasome activation and the subsequent IL-1β production. Consistent with the inhibitory effects of CuB on NLPR3 inflammasome activation, CuB remarkably suppressed pro-IL-1β production (Fig. 6C) and key enzymes in glycolysis such as HK2 and PDH (Fig. 6E) in MSU plus LPS-activated macrophages.

Taken together, our data indicated that CuB suppressed NLPR3 inflammasome activation, glycolysis, and finally IL-1β production in MSU-activated macrophages, suggesting that CuB is a common anti-inflammatory agent, which can inhibit inflammation induced by various stimuli in vitro.
Cucurbitacin B is a NLRP3 inhibitor

MSU is a common IL-1β stimulus in vitro, as well as a major cause of gout attacks. To assess whether the observed inhibitory effect of CuB on inflammasome activation in BMDMs could be extended to in vivo models of gout arthritis, we assessed the impact of CuB on an MSU crystal-induced mouse model of gout arthritis. As shown in Fig. 7A and B, amelioration of foot swelling was observed in CuB treatment group compared with the control group in a MSU-induced mouse model of gout arthritis. We determined the serum levels of IL-1β at 8 h after MSU injection in the control group and CuB group by ELISA assays. CuB treatment could significantly reduce the rise of serum IL-1β after MSU injection (Fig. 7C). As shown in Fig. 7D, H & E staining indicated that inflammatory cell infiltration was significantly increased in the soft tissue and joint space of the right paw after MSU injection compared with the left paw after PBS injection in the control group. In contrast, CuB remarkably reduced the inflammatory cell infiltration in the right paw of the CuB group. Our data revealed inflammatory responses elicited by MSU crystals in the sterile environment of joint space in vivo, which can be significantly reduced by CuB. Thus, CuB could exert a therapeutic effect on a gout flare.

CuB relieves MSU-induced arthritis in a mouse model of gout
Discussion

In the current study, we describe the anti-inflammatory effects of CuB in ATP or MSU plus LPS-activated BDMDs and mouse models of experimental arthritis representative of gouty flare. Our results suggested that CuB suppressed ATP or MSU plus LPS-induced NLRP3 inflammasome activation and glycolysis that finally culminated in the decrease of IL-1β production in vitro. In addition, our data showed that CuB relieved MSU-induced arthritis in the mouse model of gout, indicating that CuB could exert a therapeutic effect of gout flare in vivo.

Cucurbitacins, which constitute a group of tetracyclic triterpenes isolated from cucurbitaceous plants, have been demonstrated to have a variety of biological activities, such as anticancer, anti-inflammatory, antidiabetic, and antifertility activities (Chen et al. 2005, Kim et al. 2015). Of those cucurbitacins, CuB is one of the most widely distributed forms of cucurbitacins and has been widely used as an anticancer agent and anti-inflammatory agent. Recent studies have suggested that CuB is capable of inhibiting the growth of numerous human cancer cell lines via inhibiting different signaling pathways, including the STAT3 pathway, the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway, and MAPK pathways (Liu et al. 2008, Thoenissen et al. 2009, Zhang et al. 2009, Liu et al. 2010, Gao et al. 2014, Lv et al. 2015).

Limited literature has illuminated the anti-inflammatory activity of CuB. A previous study demonstrated CuB inhibited pulmonary inflammation by downregulating TNF-α and IL-6 in the bronchoalveolar lavage fluid (Hua et al. 2017). CuB could also significantly suppress releases of inflammatory cytokines (IL-5, IL-13, IL-6, and TNF-α) and improve liver fibrosis induced by concanavalin A through SIRT1/IGFBPPrP1/TGF β1 axis (Yang et al. 2020). A previous study also showed that CuB significantly inhibited imiquimod-induced inflammation of keratinocytes, via downregulation of NF-κB and STAT3 signaling pathway in human keratinocytes (Li et al. 2015).

Furthermore, CuB could attenuate the release of LPS-activated pro-inflammatory mediators (TNF-α, IL-1β, IL-6, IL-12, etc.), and suppress the LPS-activated expression of CD40, CD80, MHC II molecules in peritoneal macrophages (Kim et al. 2015). Although there is an increase in the amount of evidence indicating that CuB also has significant anti-inflammatory activity, whether CuB exhibits anti-inflammatory effects in gout and its exact molecular mechanism remains to be elucidated. The present study demonstrated, for the first time, the immunosuppressive characteristics of CuB on ATP or MSU-activated BDMDs and on arthritis in a MSU-induced mouse model of gout.
Cucurbitacin B is a NLRP3 inhibitor

Y Xue et al.

Journal of Molecular Endocrinology

Gout is a self-limiting acute inflammatory disease characterized by MSU crystal-induced gouty arthritis and hyperuricemia (Liu-Bryan 2010, Neogi 2016). Activation of the NLRP3 inflammasome and subsequent IL-1β secretion are crucial steps in the initiation of acute gout flares (Martinon et al. 2006, Weber et al. 2010). Thus, suppression of the NLRP3 inflammasome formation and activation has been considered as a potential therapeutic target for treating gouty arthritis (Schroder et al. 2010, Burns & Wortmann 2011, Dinarello 2011). As CuB has been known for its good anti-inflammatory function, we aimed to further illuminate its pharmacological effects and potential mechanisms in gouty arthritis. Our data reveal CuB has a therapeutic effect on gout flare in a MSU-induced mouse model of gout arthritis. Furthermore, we demonstrate that CuB exerts an anti-inflammatory effect via different mechanisms, then would reduce gout flares.

NLRP3 inflammasome activation has been reported to be involved in the pathologies of other autoimmune and autoinflammatory diseases, such as rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), gut microbiome, etc. (Lissner et al. 2015, Jenko et al. 2016, Pellegrini et al. 2017). Recent studies have shown that the expressions of NLRP3 inflammasome components were significantly increased in circulatory inflammatory cells (macrophages, monocytes, neutrophils, and dendritic cells) and synovial fluids of RA patient, which suggested that NLRP3 activation has an important role in the pathogenesis of RA (Rosengren et al. 2005, Choulaki et al. 2015, Ruscitti et al. 2015, Zhang et al. 2015). However, the exact mechanism by which the NLRP3 inflammasome is involved in the development of RA have not yet been fully elucidated. Current evidence has found the NLRP3 inflammasome plays an important role in the pathogenesis and progression of IBD. NLRP3, ASC and IL-1β expression were increased in the colon mucosa of IBD patients (Liu et al. 2017), and IL-1β secretion from macrophages was augmented along with the severity of the disease (Coccia et al. 2012). However, further experiments are still needed to develop effective therapies targeting NLRP3 inflammasome for IBS. Recent studies have indicated the importance of NLRP3 inflammasome on the regulation of the composition and quantity of the intestinal microflora in mouse models. The absence of inflammasome components was observed to promote dysbiosis, which was associated with colitis and tumorigenesis (Elinav et al. 2011, Levy et al. 2015,

Figure 7
Cucurbitacin B relieves MSU-induced arthritis in vivo (A-D) 8–12 weeks old male C57BL/6 mice were randomly divided into two groups (n = 8). The CuB group was given an intraperitoneal injection of CuB (2.5 mg/kg) in PBS, and the control group was given the same amount of DMSO in PBS. After 72 h, those two groups of mice were intra-articular injected with MSU crystals suspended in PBS (1 mg/50 μL) into the right hind footpad to build MSU-induced gouty arthritis. At the same time, PBS was injected into the left hind footpad as self-control. Eight hours after MSU crystal injection, joint swelling (A,B) and serum IL-1β levels (C) were examined. (A,B) Joint inflammation was measured as the ratio of the footpad thickness of right hind pad over the left hind pad. (C) Serum IL-1β levels were tested by ELISA. (D) Mice were sacrificed 8 h after MSU injection, and hind paw was used for H&E staining. Data are expressed as mean ± s.d., n = 8, *P < 0.05; **P < 0.01.

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Chen 2017). However, current evidence does not explain how the intestinal microflora of inflammasome-deficient mice lead to dysbiosis.

NLRP3 inflammasome also contributes to the onset and progression of metabolic disorders, including obesity and type 2 diabetes (T2DM) (Vandannmagar et al. 2011, Lee et al. 2013). NLRP3, ASC and caspase 1 expression were upregulated in adipocytes of obese patients (Stienstra et al. 2010, Yin et al. 2014). Several in vivo experiments were conducted to evaluate the association between the development of obesity and inflammasome activity. However, conflicting results were found in different studies. Caspase 1 deficient mice had reduced adipocyte size and decreased fat mass compared with controls (Stienstra et al. 2010, Stienstra et al. 2011), while in another study, more adipose tissue was observed in caspase 1-deficient mice than the controls (Wang et al. 2014a). The role of NLRP3 inflammasome and caspase 1 in obesity still needs to be clarified. Recent evidence has demonstrated the relationship between the NLRP3 inflammasome activation and type 2 diabetes development. Increased NLRP3 inflammasome activity in myeloid cells was found in patients with T2DM compared with healthy controls (Lee et al. 2013). In previous studies, insulin sensitivity and glucose tolerance were significantly improved in mice with inflammasome component deficiency after feeding with a high-fat diet (HFD) compared to controls (Stienstra et al. 2011, Vandannmagar et al. 2011, Wen et al. 2011, Stienstra et al. 2012). During the progression of T2DM, the accumulation of islet amyloid polypeptide (IAPP) triggers the NLRP3 inflammasome activation. In mice that overexpress human IAPP in β-cells, IL-1β was induced in pancreatic macrophages (Janson et al. 1996, Masters et al. 2010). The detailed mechanisms of the NLRP3 inflammasome activation in T2DM still remain to be well elucidated.

In addition, previous studies have illuminated that NLRP3 inflammasome activation may contribute to the immune/inflammatory responses in the onset and development of infectious disorders (Fusco et al. 2017, Gugliandolo et al. 2019, D’Amico et al. 2020). Mice with the absence of formyl peptide receptor 1 (Fpr-1) gene, which could modulate NLRP3 inflammasome signaling, were significantly less vulnerable to the development of bronchiolitis obliterans syndrome (BOS). That evidence demonstrated that NLRP3 inflammasome activation might play a crucial role in chronic obstructive pulmonary disease (D’Amico et al. 2020). Another study demonstrated that the NLRP3 inflammasome pathway could induce the release of inflammatory cytokines and amplify the inflammatory response to cause pleurisy (Fusco et al. 2017). In addition, previous researches have illustrated the relationship between the NLRP3 inflammasome and TLR4 pathway. TLR4, a key component in innate immune responses, could stimulate the upregulation of NLRP3 expression in macrophages (Qiao et al. 2012, Liao et al. 2013). TLR4 was also important for a rapid proinflammatory response to Pseudomonas aeruginosa (PA) infections and earliest control of bacterial replication by regulation of the NLRP3 inflammasome activation (Gugliandolo et al. 2019). Our evidence, well in line with the literature, suggested that TLR4 signaling may play an important role in the immune response of macrophages by modulation of the NLRP3 inflammasome complex.

Increasing evidence has revealed that the NLRP3 inflammasome is activated by two signals: the priming signal activated by LPS or PMA, and the second signal activated by a wide array of stimuli such as ATP, MSU or Nigericin (Martinon et al. 2006, Cruz et al. 2007, Bauernfeind et al. 2009, Schroder et al. 2010, Freigang et al. 2011, Heid et al. 2013, Rock et al. 2013, Mian et al. 2019). In our study, LPS was used as the stimulus of priming signal which was combined with the second stimulus (ATP or MSU crystals) to trigger the subsequent activation of NLRP3 inflammasome according to the previous studies (Mian et al. 2019). CuB effectively inhibited NLRP3 inflammasome formation and activation, and further decreased the generation of cleaved-caspase 1, leading to the reduction of IL-1β production in ATP or MSU crystal-stimulated macrophages. Our study suggests that CuB exerts an anti-inflammatory effect by inhibiting the activation of the NLRP3/IL-1β pathway in macrophages. Besides the anti-inflammatory effect in vitro, CuB could also play a role in the relief of gout arthritis in vivo. In mouse model of gouty arthritis, reduced foot swelling and inflammatory cell infiltration were observed in CuB-pretreated mice compared with controls. These results suggest that CuB has potential for the treatment of NLRP3 inflammasome-mediated diseases such as gouty arthritis.

Recent studies have revealed that the NLRP3 inflammasome could be regulated by key components of glycolysis, such as HIF-1α (Gupta et al. 2017) and HK1 (Moon et al. 2015, Hughes and O’Neill 2018). HIF-1α could serve as a key mediator in succinate-induced IL-1β production (Kim et al. 2006, Tannahill et al. 2013) and MSU-mediated IL-1β release (Mian et al. 2019), indicating that HIF-1α signaling is critically involved in glycolysis and is important for IL-1β production (Kim et al. 2006, Lee et al. 2011b, Tannahill et al. 2013). In our study, the expressions of HIF-1α and key enzymes in glycolysis, including HK1, HK2, PKM2, LDHA and PDH, were decreased by CuB, while...
pro-IL-1β production was also inhibited by CuB in various stimuli-activated macrophages. Therefore, our findings may, in part, explain the anti-inflammatory effects of CuB and its potential therapeutic effects in gout flare.

In conclusion, this study revealed that CuB effectively suppressed multiple stimuli-activated IL-1β secretion by interrupting NLRP3 inflammasome complex formation, inhibiting NLRP3 inflammasome activation and suppressing key enzymes of glycolysis in macrophages. Our findings suggest that CuB is an NLRP3 inflammasome inhibitor with the potential as an anti-inflammatory agent for the treatment of gout and other NLRP3 inflammasome-related diseases. Further studies are necessary for mouse model of gout to confirm the potential of CuB in treating gouty arthritis via its anti-inflammatory properties.

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**Supplementary materials**

This is linked to the online version of the paper at https://doi.org/10.1530/JME-20-0305.

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**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Data availability**

All data and material have been presented in the manuscript. No outliers were omitted from the main statistical analysis. Related information are available under request to the corresponding author.

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**Author contribution statement**

Y X performed the experiments, drafted the manuscript and revised the manuscript; R L, Z Q, Y F and Y Z coordinated the research; Y Z and K Q contributed to data interpretation; L L designed the study, performed the experiments, revised and prepared the final version of the manuscript. All authors read and approved the final manuscript.

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Y Xue et al. 2021

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