HSP60 regulates monosodium urate crystal-induced inflammation by activating the TLR4-NF-κB-MyD88 signaling pathway and disrupting mitochondrial function

Wei Gao
North Sichuan Medical University

Qiushi Huang
North Sichuan Medical University

Ting Qin
North Sichuan Medical College

Heng Mu
North Sichuan Medical College

Fan Long
The Fifth People’s Hospital of Nanchong

Long Ren
The Fifth People’s Hospital of Nanchong

Huan Tang
North Sichuan Medical College

Jianpin Liu
North Sichuan Medical College

Mei Zeng (zengmei123@gmail.com)
North Sichuan Medical college

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Abstract

Background

Acute gout is an inflammatory response induced by monosodium urate (MSU) crystals. HSP60 is a highly conserved stress protein that acts as a cellular “danger” signal for immune reactions. In this study, we aimed to investigate the role and molecular mechanism of HSP60 in the gout.

Methods

HSP60 expression was detected in peripheral blood mononuclear cells (PBMCs) and plasma of gout patients. The effect and mechanism of HSP60 in gout were studied in MSU crystals treatment macrophages and C57BL/6 mice. JC-1 probe and MitoSOX Red were used to measure the mitochondrial membrane potential (MMP) and mitochondrial reactive oxygen species (mtROS).

Results

HSP60 expression was significantly upregulated in the PBMCs and sera of patients with acute gout (AG) compared to those with inter-critical gout (IG) or healthy controls (HCs). MSU crystals induced expression and secretion of HSP60 in the macrophages. HSP60 knockdown or over-expression affects TLR4 and MyD88 expression, IκBα degradation and the nuclear localization of NF-κB in MSU crystal-stimulated inflammation. Further, HSP60 facilitates MMP collapse and mtROS production, and activates the NLRP3 inflammasome in MSU crystal-stimulated macrophages. In MSU crystal-induced arthritis and peritonitis mouse models pre-treated with HSP60 vivo-morpholino, paw swelling, ankle joint swelling, myeloperoxidase (MPO) activity and inflammatory cell infiltration significantly decreased.

Conclusion

Our study revealed that MSU crystal stimulates the expression of HSP60 which accelerates TLR4-MyD88-NF-κB signaling pathway and exacerbates mitochondrial dysfunction.

Background

Gout is one of the most common forms of inflammatory arthritis in adults and is triggered by MSU crystals deposited within the joints [1]. If left untreated, gout progression is usually characterized by the formation of uric acid deposits (tophi) in soft tissues and subsequent arthritis recurrences affecting multiple joints, ultimately leading to progressive joint destruction.

Gout treatment is difficult due to its inherent adverse reactions, contraindications and complications [2, 3]. Recently, MSU crystals have been shown to activate resident phagocytes and release IL-1β, driving up
the use of targeted IL-1β therapies [4–7]. IL-1β plays an important role in neutrophils recruitment, typical of acute gout, by binding to the IL-1 receptor type 1 (IL-1R1) [5, 6]. Currently, anti-IL-1β monoclonal antibody canakinumab and synthetic IL-1β antagonist anakinra are available clinically [5, 6], however, in addition to being ineffective in a large number of cases, these drugs require close patient monitoring for side effects and tolerance [2, 8].

It has been reported that HSP60 can regulate both IL-1β production and IL-1β-induced inflammation [9, 10]. IL-1β-mediated inflammation is an important aspect of gout inflammation. HSP60 is a key factor associated with inflammation and serum HSP60 levels may also be elevated in patients with inflammatory diseases such as Crohn's disease and ulcerative colitis [11]. However, whether HSP60 is involved in MSU crystal-induced inflammation and its molecular mechanism remains unclear. In the current study, we explored the role of HSP60 in MSU crystal-induced inflammation and its possible molecular mechanism.

**Methods**

**Patients**

All participants were gout patients from the Department of Rheumatology of The Affiliated Hospital of North Sichuan Medical College. Patients with acute gout (AG) must fall within the classification criteria of the American College of Rheumatology (ACR). Inter-critical gout (IG) is defined as complete remission of acute gout and normal C-reactive protein (CRP) or erythrocyte sedimentation rate. In addition, patients had no history of infection, hematopathy, cancer, nephropathy, or other autoimmune diseases. Age-matched males served as healthy controls (HC) for regular physical examinations at the Affiliated Hospital of North Sichuan Medical College during the same period. Patient characteristics are shown in Table 1. This study was approved by the Ethics Committee of the Affiliated Hospital of North Sichuan Medical College and all patients were required to provide informed consent in order to participate in the study.

**Preparation of MSU crystal, MSU Stimulation, and PBMC Harvest**

MSU crystals were prepared as previously described [12], after PBMCs were isolated from blood samples of gout patients or HCs through density gradient centrifugation and stored at −80°C for gene- and protein-related tests.

**RAW264.7 cells and THP-1-derived macrophage culture, transfection, MSU crystal stimulation, and cytokine measurement**

RAW264.7 cells were cultured with DMEM containing 10% FBS. THP-1 cells were seeded in 6-well culture plates with RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) and administrated with Phorbol Myristate Acetate (PMA, 100ng/ml) for 48 hours to obtain THP-1-derived macrophages. After HSP60 plasmid and mouse or human HSP60 gene siRNA were transfected into RAW264.7 cells or THP-1-derived
macrophages, the cells were treated with an MSU suspension for 24 h. In both cases, the supernatants were applied to measure IL-1β, TNF-α, IL-6 and PGE2 levels using ELISA (Neobioscience kit, Shenzhen, China) following the manufacturer's instruction.

**Western blot analysis**

Western blot was performed as previously described. RIPA buffer was applied to extract total protein from either macrophages or foot pad tissues. Anti-IL-1β, anti-Phospho-NF-κB p65(Ser536) and anti-Phospho-NF-κB p105/50(Ser933) were purchased from Cell Signaling Technology (CST, USA), and anti-COX-2, anti-IκBα, anti-MyD88, anti-SOD2, anti-Caspase-1, anti-TLR4, anti-NF-κB p65, anti-NF-kB p105/50 and anti-NLRP3 were obtained from HuaBio (Hangzhou, China).

**RNA Extraction and Quantitative Real-Time PCR**

Total RNA was extracted from macrophages using TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using reverse transcription reagents (TaKaRa). Real-time quantitative PCR (RT-qPCR) was performed using the SYBR Green I Two-Step qRT-PCR kit with ROX (Invitrogen), and β-actin as an internal reference. Target gene expression was analyzed through the \(2^{-\Delta\Delta CT}\) method. Primer sequences are shown in Table 2.

**Total superoxide dismutase (T-SOD) activity analysis**

The supernatant of RAW264.7 cell lysates was gathered for T-SOD measurement by T-SOD assay kit (Jiancheng Technology Company, Nanjing, China). The relationship between T-SOD activity and protein concentration was analyzed.

**MSU crystals induced mouse model of peritonitis and arthritis treatment with vivo morpholino**

All experimental procedures were approved by North Sichuan Medical College. HSP60 vivo-morpholino (HSP60-Mo) and control morpholino (Ctrl Mo) oligos were purchased from Gene Tools LLC (Philomath, OR, USA). Mice were pretreated with vivo-morpholino (foot pad or ankle joint injection, 15mg/kg of body weight or the same PBS volume as vivo morpholino) in the right foot pad. After 1 h, 1 mg of MSU (in 40 μl of PBS) was injected into the right ankle joint or foot pad and an equal PBS volume was simultaneously injected into the left ankle joint or foot pad as the control. The swelling index was expressed as the ratio of injected MSU joint to injected PBS joint. Paw and ankle joint swelling were measured 24h after MSU suspension injection, joint index evaluation is based on a previously described method [13]. The mice were sacrificed afterwards. Foot pad tissues were homogenized in RIPA buffer, and the supernatants were used to measure the related protein levels and myeloperoxidase (MPO) activity, in order to quantify neutrophil sequestration. This was done using an MPO activity assay kit (Jiancheng Technology Co, Nanjing, China) and foot pad tissue homogenates, according to the manufacturer's guidelines. Foot pad tissue sections were prepared for hematoxylin and eosin (H&E) staining and immunostaining.
Mice were intraperitoneally injected with vivo morpholino (15mg/kg of body weight of mice or same volume PBS as vivo morpholino). After 1 h, peritonitis was induced by intraperitoneal injection of MSU suspension (3 mg in 200 μl of sterile PBS). 6h later, mice were euthanized and peritoneal cavities were washed with 6 ml of cool PBS. Lavage fluids were detected for IL-1β production through ELISA and the total cell number in the lavage fluid was counted by hematocytometer.

**Mitochondrial potential and ROS staining detection**

Mitochondrial superoxide indicator (Mito-SOX red, 40778ES50) and Mito-Tracker Green (40742ES50) purchased from YEASEN (Shanghai, China). In brief, Nuclei were first stained with Hoechst 33342 (40731ES10) for 10mins at 37°C/5% CO2 in the dark and washed with PBS. Then cells were stained with the Mito-SOX red and Mito-Tracker Green for 30 min at 37°C/5% CO2 in the dark. Cells were subsequently washed with PBS to remove free probes. ROS quantification was represented by the ratio of Mito-SOX red to Mito-Tracker Green. Mitochondrial membrane potential (MMP) was evaluated by the MMP assay kit with JC-1 (Beyotime, Shanghai, China, C2006) following the manufacturer's guidelines protocol. Cells were washed with PBS and were then stained with JC-1 probe for 20 min at 37°C/5% CO2 in the dark. Subsequently, PBS was used to remove free probes. The red-to-green fluorescence ratio was used to analyze the changes of MMP.

**The quantification of mtDNA by quantitative PCR**

The quantification of mtDNA was performed as described previously [13]. The primers sequence was provided in the Table 2.

**Cell and tissue section immunofluorescence**

For mitochondria and HSP60 co-localization, the cells were first stained with Mito-Tracker Green and fixed at room temperature with 4% paraformaldehyde for 10 min, followed by incubation with rabbit anti-mouse or anti-human HSP60 antibody (1:150 HUABIO, Hangzhou, China) diluted in 1% BSA/ PBS (PBS, pH 7.6) overnight at 4°C. A secondary antibody (Alexa Fluor 488-conjugated Affinipure Goat) and DAPI staining solution were both purchased from Beyotime (Shanghai, China). Images were acquired using Olympus Laser Confocal Microscope. The synovia tissue from the patient with active gouty arthritis and mouse foot pad tissue section were analyzed by Immunofluorescence. Rabbit anti-HSP60 antibody (HUABIO), rabbit anti-Ly6G antibody (HUABIO) and rabbit anti-MPO antibody (Boster, Wuhan, China) were used.

**Statistics**

Statistical analysis was performed using GraphPad Prism 6 software. Data were expressed as the mean ± SEM. A one-way ANOVA with Student’s t-test with two or three repeats was used to determine the significant differences between groups. P<0.05 was considered statistically significant.
Results

HSP60 expression was up-regulated in gout patients

It has been firmly established that HSP60 is secreted extracellularly and can enter the blood stream. Serum HSP60 levels are elevated in certain inflammatory diseases [11, 14]. In this study, to confirm HSP60 expression in patients with gout, HSP60 expression levels in serum and PBMCs were detected. Serum HSP60 levels were significantly higher in patients with AG than that in IG or HC (Fig. 1a). In PBMCs, HSP60 protein levels in gout patients were higher compared to HCs (Fig. 1b).

MSU crystals promoted HSP60 expression, translocation to mitochondria and secretion in RAW264.7 cells and THP-1-derived macrophage

It has become clear that HSP60 is located not only in the mitochondria, but also in the cytoplasm that surrounding it. To investigate the effect of MSU crystal on the localization of HSP60 in the macrophage, RAW264.7 cells were exposed to varying dose of MSU crystals for 24 h, followed by an analysis of HSP60 distribution using double staining of MitoTracker and immunofluorescence. The double labeling of HSP60 and MitoTracker data revealed that HSP60 was predominantly located in the mitochondria and distributed through a small amount of cytoplasm in the macrophage; however, RAW264.7 cells exposed to MSU crystals, not only did HSP60 protein levels increase, but also it translocated to the mitochondria in almost its entirety (Fig. 1c). Next, we sought to examine the effect of MSU crystals on HSP60 secretion in RAW264.7 cells. Extracellular HSP60 levels were detected by ELISA in the culture supernatants, revealing an increased level of HSP60 secretion upon MSU crystals treatment (Fig. 1d). HSP60 expression levels were also assessed using western blot of protein lysates from RAW264.7 cells stimulated by different concentration of MSU crystals (Fig. 1e). MSU crystals dose dependently up-regulated the expression of HSP60 in RAW264.7 cells, and the expression of HSP60 peaked significantly in RAW264.7 cells treatment with 100μg/ml of MSU crystals. RAW264.7 cells were exposed to 100μg/ml of MSU crystals for all further assays. We further assessed the effect of different concentration of MSU crystals on the distribution, secretion and expression of HSP60 in the THP-1-derived macrophage. The results revealed that MSU crystals also had a great influence on the expression of HSP60, and HSP60 protein levels reached a peak in the THP-1-derived macrophage exposed to 50μg/ml of MSU crystals (Supplementary Fig. 1a, b and c). We chose to treat THP-1-derived macrophage with 50μg/ml of MSU crystals for all further experiments.

HSP60 regulates TLR4/MyD88/NF-κB signaling pathway in MSU crystal-stimulated inflammation

Previous studies have shown that HSP60 secretion into the extracellular medium activates TLR4 and induces an inflammation cascade [9, 15], and macrophages stimulated by MSU crystals also leads to the activation of the TLR4/MyD88 signaling pathway [16]. In our study, we set out to explore the effect of HSP60 on the TLR4/MyD88/NF-κB signaling pathway. We started by using specific siRNA to interfere with HSP60 expression in RAW264.7 cells for 48 h (Fig. 2a), followed by a 24 h MSU crystal treatment. Compared with control siRNA, the expression of TLR4 and its intracellular junction protein (MyD88) in RAW264.7 cells treatment with MSU crystals was inhibited due to down-regulation of HSP60 (Fig. 2b).
The MyD88 signaling pathway is closely related to IκBα degradation and the phosphorylation of NF-κB in the MSU crystal-induced inflammation. As a result of HSP60 knockdown, the IκBα degradation and the phosphorylation of NF-κB P65 showed a reduction in MSU crystals exposed to RAW264.7 cells (Fig. 2c). For *in vivo* HSP60 knockdown, mice were injected with HSP60-Mo or control-Mo in the foot pad. Consistent with *in vitro* data, after HSP60 reduction by HSP60-MO, protein levels of TLR4, MyD88, phosphorylated NF-κB P65 and IκBα degradation decreased significantly in the foot pad tissue of MSU suspensions injection mice (Supplementary Fig. 2a and b).

Phosphorylation of NF-κB induces its nuclear localization, which is important for regulating inflammatory gene regulation. Therefore, we sought to evaluate the impact of both HSP60 knockdown and HSP60 overexpression on the nuclear localization of phosphorylated NF-κB P65 using immunofluorescence in RAW264.7 cells. Nuclear translocation of phosphorylated NF-κB P65 triggered by MSU crystals was attenuated by HSP60 knockdown (Fig. 2d). RAW264.7 cells were transfected with HSP60 vector or empty vector for 24 h and then treated with MSU crystals for 24 h. Western blot analysis was used to demonstrate HSP60 overexpression (Fig. 2e). HSP60 overexpression further accelerated the nuclear localization of phosphorylated NF-κB P65 induced by MSU crystals (Fig. 2f). These results imply that HSP60 plays a significant role in the MSU crystal-stimulated nuclear localization of the phosphorylated NF-κB subunit.

**Both HSP60 knockdown and overexpression influence the production of downstream inflammatory cytokines of NF-κB induced by MSU crystals**

NF-κB activation impels the transcription of cytokines and prompts a complex inflammatory cascade response. As shown in Fig. 3a, reduction in endogenous HSP60 expression by specific siRNA in RAW264.7 cells blocked mRNA expression levels of MSU crystal-induced pro-inflammatory enzymes (COX-2, iNOS) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) (Fig. 3a). In accordance with the RT-PCR results, ELISA data indicated that lower IL-1β, TNF-α, IL-6 and PGE2 protein levels were found in the cell culture supernatants of HSP60 knockdown RAW264.7 cells in response to MSU crystals (Fig. 3b), and western blot analysis data also revealed that MSU crystal-induced iNOS and COX-2 protein levels were also suppressed in HSP60 knock-down RAW264.7 cells (Fig. 3c). To confirm this in human cells, we treated THP-1-derived HSP60 knockdown macrophages with MSU crystals. Consistently, HSP60 knockdown relieved the elevated mRNA expression of pro-inflammatory cytokines in response to MSU crystals (Supplementary Fig. 3). Inversely, we found that MSU crystals (100μg/ml) stimulated HSP60-overexpressing RAW264.7 cells significantly facilitating iNOS, COX-2, and pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) mRNA expression (Supplementary Fig. 4). All these data suggest that HSP60 has a positive effect on the production of MSU crystal-induced proinflammatory cytokines.

**HSP60 regulates NLRP3 inflammasome activation triggered by MSU crystals**

Previous studies have indicated that NLRP3 inflammasome activation is an important pathway by which MSU crystals lead to cellular inflammatory response, and nuclear localization of phosphorylated NF-κB promotes NLRP3 inflammasome activation by inducing transcription of the NLRP3 gene. Therefore, we
investigated the role of HSP60 in NLRP3 expression induced by MSU crystals using RT-PCR and western blot. MSU crystal-induced NLRP3 expression both at mRNA and protein levels was decreased in vivo and in vitro because of HSP60 knockdown (Fig. 4a and b, Supplementary Fig. 2a). NLRP3 inflammasome activation contributes to the cleavage of pro-Caspase-1 to Caspase-1 and the release of IL-1β. The cleavage of pro-Caspase-1 to Caspase-1 and the production of IL-1β were alleviated because of HSP60 knockdown in the MSU crystals stimulated RAW264.7 cells and THP-1-derived macrophage (Fig. 4c, 4d, 4e, 4f and Supplementary Fig. 5). Oppositely, pro-Caspase-1 cleavage into Caspase-1 and the production of IL-1β were observed to be alleviated in HSP60 overexpression RAW264.7 cells stimulated by MSU crystals (Fig. 4g, 4h, 4i and 4j).

**HSP60 aggravates mitochondrial dysfunction induced by MSU crystals**

When mitochondria are damaged, HSP60 expression increased. HSP60 is considered an indicator of mitochondrial stress [17]. Mitochondrial dysfunction, which includes mitochondrial membrane potential collapse, overproduction of mitochondrial reactive oxygen species (mtROS) and release of mitochondrial DNA, is crucial for the activation of the NLRP3 inflammasome; therefore, we sought to explore the role of HSP60 in the mitochondrial damage induced by MSU crystals. MitoSOX Red is a mitochondrial superoxide indicator that is inserted into mitochondrial DNA during oxidation and produces red fluorescence. MitoTracker was used to determine the total mitochondrial content. Red to green fluorescence ratio reflects the mtROS level. HSP60 knockdown resulted in decreased MitoSOX Red fluorescence in MSU crystal-stimulated RAW264.7 cells and THP-1-derived macrophage, supporting lower levels of superoxide production (Fig. 5a and 5b, Supplementary Fig. 6a). Furthermore, the protein levels of mitochondrial matrix protein SOD2 and the activity of total superoxide dismutase (T-SOD) were displayed to be blocked in HSP60 knockdown RAW264.7 cells exposed to the MSU crystals (Fig. 5c and 5d), which further indicated that HSP60 has the function of regulating the production of mtROS induced by MSU crystals. We also found a reduction in mitochondrial DNA release to cytosol in HSP60 knockdown RAW264.7 cells in response to MSU crystals (Fig. 5e). In the study, MMP was measured by JC-1 probe. When the mitochondrial membrane potential is high, JC-1 gathered in the mitochondrial matrix and formed a red fluorescent polymer (J-aggregates); whereas the mitochondrial membrane potential is low, JC-1 cannot be concentrated in the mitochondrial matrix. In this case, JC-1 is a monomer and can produce green fluorescence. The ratio of red to green fluorescence is used to calculate mitochondrial membrane potential and reflects levels of polarized functional mitochondria. Because of HSP60 knockdown, mitochondrial membrane potential were restored in MSU crystal-induced RAW264.7 cells and THP-1-derived macrophages, supporting enhanced mitochondrial function (Fig. 5f and 5g, Supplementary Fig. 6b). On the other hand, we noticed that MMP collapse and mtROS generation triggered by MSU crystal-stimulated RAW264.7 cells were aggravated due to HSP60 overexpression (Fig. 5h, 5i, 5j, 5k).

**HSP60 down-regulation alleviates the severity of MSU crystals induced mouse model of peritonitis and arthritis**
We proceeded to study the role of HSP60 in the mouse model of gouty arthritis triggered by MSU crystals. After mice were respectively injected with HSP60-MO or ctrl-MO into the right foot pad tissue and right ankle joint for 1 h, MSU suspensions were respectively injected into the right ankle joint or right foot pad of mice to simulate the etiology of human gouty arthritis. A significant decrease in paw swelling index was noted in MSU+HSP60-MO treated mice compared to MSU+ctrl-MO treated mice (Fig. 6a). Both immunostaining and western blot data indicated that HSP60 expression levels were significantly lower in MSU+HSP60-MO treated mice compared to MSU+ctrl-MO treated mice (Fig. 6b and 6c). Histological analyses of the foot pad tissue section revealed that HSP60 knockdown prevented the infiltration of inflammatory cells into the foot pad tissue (Fig. 6d). We further confirmed the effect of down-regulated HSP60 expression on neutrophils, myeloperoxidases and macrophages distribution in the foot pad tissue section through immunostaining. As depicted in Fig. 6e, a decreased number of neutrophils and macrophages in the footpad tissue section were observed in the MSU+HSP6-MO treated mice. Concomitantly, the amount of MPO containing cells was also significantly lower in the MSU+HSP6-MO treated mice compared to MSU+ctrl-MO treated mice (Fig. 6e). We further assessed the protein levels of important pro-inflammatory enzymes (MPO, iNOS, and COX2) in the foot pad tissue extracts using western blot. The MPO, iNOS and COX-2 protein levels from the foot pad tissue extracts in HSP60-MO treated mice were found to be greatly lower (Fig. 6f). Compare to MSU+ctrl-MO mice, the activity of MPO also revealed to be decreased in the MSU+HSP60-MO treated mice, reflecting lower levels of inflammation. HSP60-deficient mice had less ankle joint swelling compared to the ctrl vivo-morpholino treated group, which was consistent with the reduction in observed swelling of the foot pad (Fig. 6g). Mice were intraperitoneally injected with HSP60 for 1 h and then injected with MSU suspensions into the peritoneal cavity to establish a peritonitis model of gout. We found that total peritoneal cell numbers and IL-1β levels were lower in HSP60-MO treated mice (Fig. 6h and 6i). These results suggested that HSP60 knockdown relieved lymphocyte recruitment, alleviating MSU crystal-induced inflammation in vivo.

Discussion

In most cells, HSP60 is constitutively expressed. HSP60 is released from damaged cells during cellular stress or injury [18]. Although the exact mechanism by which HSP60 is secreted to the extracellular medium remains unclear, it is clear that extracellular HSP60 is a link between body tissues and the immune system [17]. HSP60 can guide the maturation and activation of innate immune cells and play an immunomodulatory role in various stress or injury situations [20–21]. Numerous studies have shown that people with type 2 diabetes have elevated HSP60 blood levels [22]. In the present study, our data indicates that the patient HSP60 serum level is higher than that in healthy individuals. In the THP-1-derived macrophage and RAW264.7 cells, MSU crystals can promote HSP60 secretion into the extracellular medium. These data imply that HSP60 may be released to modulate MSU crystal-induced inflammation.

Previous reports have shown that soluble HSP60 can activate macrophage through TLR4 [15]. When TLR4 is activated, it can coordinate multiple signaling cascades, including an increase in nitric oxide (NO) productions in cell culture, arthritis animal models and in patients with gout [23–26]. However, the role of
HSP60 in response to MSU crystal-induced inflammation is elusive. In the current study, we investigated the role of HSP60 in triggering the innate immune response to MSU crystals by HSP60 knockdown or overexpression in macrophage or mouse gout models. The results showed that HSP60 down-regulation inhibited MSU crystal-induced protein expression of TLR4 and MyD88, IκBα degradation and downstream NF-κB nuclear localization. However, HSP60 overexpression accelerated the activation of the TLR4/MyD88/NF-κB signaling pathway in MSU crystal-induced inflammation. The downstream effect included changes in the expression of pro-inflammatory enzymes (iNOS and COX-2) and pro-inflammatory cytokines (IL-1β, TNFα and IL-6). These results indicate that MSU crystals promote HSP60 secretion which further upregulates TLR4 expression and activates its downstream NF-κB signaling pathway.

IL-1β plays a pivotal role in initiation of gout flare, but production and release of IL-1β is a multi-step process. IL-1β gene transcription and generation of pro-IL-1β is induced by the activation of NF-κB signaling pathway. MSU crystals activate the NLRP3 inflammasome, resulting in activation of caspase-1, which enzymatically processes pro-IL-1β to bio-active IL-1β. The molecular mechanism by which MSU crystals activate NLRP3 inflammasome is still unclear. Most studies claim that mitochondrial dysfunction is closely associated with the activation of the NLRP3 inflammasome. Previous studies implied that elevated extracellular levels of HSP60 might be associated with mitochondrial stress [27]. According to our data, once the macrophages were activated by the MSU crystals, both the extracellular and in mitochondria HSP60 protein were elevated, while HSP60 protein located in the cytoplasm appears to be reduced. We hypothesize that HSP60 secretion, reduced HSP60 protein in the cytoplasm or excessive accumulation of HSP60 protein in the mitochondria may damage the latter and further activate the NLRP3 inflammasome. HSP60 knockdown in RAW264.7 cells or THP-1-derived macrophages dampened the reduction of mitochondrial membrane potential and the increase of mitochondrial ROS caused by MSU crystals. In contrast, mitochondrial dysfunction was exacerbated by HSP60 overexpression. More research should be performed to elucidate the molecular mechanisms of HSP60 involvement in regulation of mitochondrial dysfunction.

To further explore the effect of HSP60 on MSU crystal-induced inflammation in vivo, we established mouse models of gout arthritis and peritonitis induced by MSU crystals. This process triggers a series of inflammatory reactions, similar to acute gout arthritis [28]. Vivo-Morpholino can be injected directly into interested regions to achieve effective local delivery, so we directly injected it into the joint or abdominal cavity to suppress HSP60 expression. In MSU crystals injected into the foot pad and ankle joint, HSP60 knockdown relieved the swelling of the ankle joints and foot pad, and further MPO activity test, HE and immuno-histochemical staining showed that HSP60 knockdown alleviated the recruitment of neutrophils and macrophages to the foot pad. In line with the mouse model for arthritis, as a result of HSP60 knockdown, we also observed a decrease in the number of inflammatory cells in peritoneal lavage fluid induced by MSU crystals.

Conclusions
our study proposes that HSP60 activates the TLR4/MyD88/NF-κB signaling pathway and induces mitochondrial damage to trigger the activation of NLRP3 inflammasome, thereby exerting an effect akin to promoting gout arthritis.

**Abbreviations**

MSU: monosodium urate; TLR: toll-like receptor; NALP3: NLR family pyrin domain containing 3; PMA: phorbol myristate acetate; MPO: myeloperoxidase; PGE2: prostaglandin E2; MMP: mitochondrial membrane potential, ROS: reactive oxygen species; mtDNA: mitochondrial DNA; HSP60 MO: HSP60 Morpholino; Ctrl MO: control Morpholino;

**Declarations**

**Funding**

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**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

**Authors’ contributions**

MZ and JP-L initiated and designed this study. WG, QS-H, HM and TQ performed and analysed the majority of experiments. MZ and JP-L wrote the manuscript. FL and LR performed and analysed individual experiments. QS-H performed data curation. JP-L supervised the study.

**Ethics approval**

Handling of mice and experimental procedures were in accordance with requirements of the Institutional Animal Care and Use Committee and this study was granted permission by the Ethics Committee of the Affiliated Hospital of North Sichuan Medical College.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
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Table 1 Characteristics of gout patients and healthy controls

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Table 2 The primers used for quantitative PCR

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<tr>
<td>cytochrome c oxidase I for mouse mtDNA</td>
<td>GCCCCAGATATAGTCATCCC</td>
<td>GTTCATCCTGTTGCTGCTCC</td>
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<td>18S ribosomal RNA for mouse nDNA</td>
<td>TAGGGGACAAGTGGGCGTTC</td>
<td>CGCTGAGCCAGTCAGTGT</td>
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Supplemental Legends

**Supplementary Fig. 1** HSP60 expression, secretion and localization were affected by MSU crystals in THP-1 derived macrophage. a Representative images of double-labeling HSP60 and mitochondrial marker (MitoTracker), quantification of fluorescence intensity of HSP60 protein. Blue shows nuclei staining with DAPI. Scale bar: 20μm. Each experiment had six fields of view. b Influence of MSU crystals
on HSP60 secretion to the extracellular medium was analyzed by ELISA. c Influence of MSU crystals on HSP60 protein levels was tested by western blot. * In comparison with absence of MSU crystal treatment and # in comparison with 50μg/ml MSU crystal treatment. Values are the mean ± SEM of 3 independent experiments.

**Supplementary Fig. 2** HSP60 knockdown decreased NLRP3, TLR4, MyD88, p-P50 and p-P65 protein levels in foot pad tissue. a The protein levels of NLRP3, TLR4 and MyD88 in the foot pad tissue of HSP60-Mo or ctrl-Mo injection mice were measured by western blot. (n=4 per group, mean±SEM) b The protein levels of IkBa, P50, P65, phosphorylated P50 (p-P50) and phosphorylated P65 (p-P65) in the foot pad tissue of HSP60-Mo or ctrl-Mo injection mice were measured by western blot. (n=4 per group, mean±SEM). # Significantly different from absence of MSU crystals injection mice and * significantly different from MSU crystals and ctrl MO injection mice. Values are the mean ± SEM of 3 independent experiments.

**Supplementary Fig. 3** HSP60 knockdown decreased the mRNA expression of MSU crystal-stimulated cytokines in THP-1-derived macrophage. a The relative mRNA of IL-1β. b The relative mRNA of IL-6. c The relative mRNA of TNF-α. d The relative mRNA of COX-2. Experiments were repeated at least three times and data are shown as mean ± SEM. * P<0.05.

**Supplementary Fig. 4** HSP60 overexpression in RAW264.7 cells increased the mRNA expression of MSU crystal-induced cytokines. a The relative mRNA of IL-1β. b The relative mRNA of IL-6. c The relative mRNA of TNF-α. d The relative mRNA of COX-2. e The relative mRNA of iNOS. * P<0.05. Data are representative of mean ± SEM for three experiments.

**Supplementary Fig. 5** HSP60 knockdown blocked the expression of p20 subunit of Caspase-1 in MSU crystal-treated THP-1-derived macrophages. The p20 subunit of Caspase-1 protein levels were measured using western blot. Protein levels were normalized to α-Tubulin and then averaged. Values are the mean ± SEM of 3 independent experiments. * P<0.05.

**Supplementary Fig. 6** HSP60 knockdown alleviated mitochondrial membrane potential collapse and production mtROS in MSU crystal-stimulated THP-1-derived macrophages. a Representative images of MitoTracker green and MitoROS staining, mitochondrial ROS levels were determined using the MitoROS red indicator to Mitotracker green ratio. b MMP was stained using JC-1 probe, JC-1 red fluorescence to green fluorescence ratio was used to quantify the mitochondrial membrane potential. Blue shows nuclei staining with Hoechst33342. Scale bar: 20μm. For MMP and mtROS analysis, >40 individual cells were imaged per group from 3 culture dishes. Data are representative of mean ± SEM for three experiments. * P<0.05.

**Figures**
HSP60 expression was induced in gout and MSU crystal-stimulated macrophages. a The HSP60 plasma level from patients with AGA (n=31), IGA (n=33) and HC (n=43) was detected by ELISA. b HSP60 protein levels in PBMCs of patients with AGA, IGA, and HCs were measured by western blot (8 cases in each group). c Representative images of double-labeling HSP60 and mitochondrial marker (MitoTracker), quantification of immunofluorescence staining of HSP60. Blue shows nuclei staining with DAPI. d HSP60 secretion was analyzed by ELISA. e HSP60 protein level was tested by western blot. Data are represented as the mean ± SEM for three experiments. * In comparison with absence of MSU crystal treatment and # in comparison with 50μg/ml MSU crystal treatment.

Figure 1
Figure 2

HSP60 affects the TLR4/MyD88/NF-κB signaling pathway in MSU crystal-stimulated RAW274.7 cells. a HSP60 protein levels in RAW264.7 cells transfected with ctrl siRNA or HSP60 siRNA for 48 h. b-c Protein levels of TLR4, MyD88, IκBα, P50, P65, phosphorylated P50 (p-P50) and phosphorylated P65 (p-P65). d HSP60 knockdown inhibited MSU crystal-induced P65 nuclear localization in RAW264.7 cells and analyzed by immunostaining. Blue shows nuclei staining with DAPI. Scale bar: 10μm. Each experiment had six fields of view. e HSP60 protein levels in RAW264.7 cells transfected with control vector or HSP60 vector for 24 h. f HSP60 overexpression promoted MSU crystal-induced P65 nuclear localization in
RAW264.7 cells and analyzed by immunostaining. Blue shows nuclei staining with DAPI. Scale bar: 10μm. Each experiment had six fields of view. Values are the mean ± SEM of 3 independent experiments. * P<0.05.

Figure 3

HSP60 knockdown reduced the expression of MSU crystal-induced cytokines. Cells were transfected with ctrl siRNA or HSP60 siRNA for 48h and then stimulated with or without MSU crystals. a Quantification of RT-PCR analysis of IL-1β, IL-6, TNFα, COX-2 and iNOS mRNA expression. b The production of IL-1β, IL-6, TNFα and PGE2 in the culture supernatant was measured by ELISA. c Western blot analysis of COX-2 and iNOS protein levels. Data are represented as the mean ± SEM for three experiments. * P<0.05.
Figure 4

HSP60 affects the activation of the NLRP3 inflammasome in MSU crystal-treated RAW264.7 cells. a-b Effect of HSP60 knockdown on NLRP3 mRNA and protein levels. c and f Influence of HSP60 knockdown or overexpression on the expression of the p20 subunit of caspase-1. g and j Impact of HSP60 knockdown or overexpression on the expression of active p17 form of IL-1β. Protein levels were normalized to α-Tubulin and then averaged. Values are the mean ± SEM of 3 independent experiments. * P<0.05.
Figure 5
HSP60 knockdown alleviates mitochondrial dysfunction in MSU crystal-stimulated RAW264.7 cells. a and h Representative images of Mitotracker green and MitoROS staining in HSP60 knockdown or overexpression RAW264.7 cells treatment with MSU crystals. Blue shows nuclei staining with Hoechst33342. Scale bar: 10μm. For mtROS analysis, >40 individual cells were imaged per group from 3 culture dishes. c SOD2 expression in HSP60 knockdown RAW264.7 cells treatment with MSU crystals. d T-SOD activity in HSP60 knockdown RAW264.7 cells stimulated by MSU crystals. f and j MMP was stained using JC-1 probe in HSP60 knockdown or overexpression RAW264.7 cells treatment with MSU crystals. Blue shows nuclei staining with Hoechst33342. b and i Mitochondrial ROS levels were determined using the MitoROS red indicator to Mitotracker green ratio. e Mitochondrial DNA release was detected by Quantitative real-time PCR analysis in HSP60 knockdown RAW264.7 cells stimulated by MSU crystals. g and k JC-1 red fluorescence to green fluorescence ratio was used to quantify the mitochondrial membrane potential. Data are representative of mean ± SEM for three experiments. Each experiment had six fields of view. * P<0.05.
Figure 6
HSP60 downregulation relieved the severity of MSU crystal-induced inflammatory mouse model. a Mice injected with HSP60-MO presented decreased the paw swelling index (n=6 per group, mean±SEM). b Immunostaining was used to detect HSP60 expression in the foot pad tissue section (n=4 per group, mean±SEM). Blue shows nuclei staining with DAPI. Scale bar: 20μm. c HSP60 protein levels were analyzed by western blot in the foot pad tissue (n=4 per group, mean±SEM). d HE staining was used to observe the infiltration of inflammatory cells in the foot pad tissue sections (n=4 per group, mean±SEM). e Immunostaining of CD11b, Ly-6G and MPO in the foot pad tissue sections (n=4 per group, mean±SEM). Blue shows nuclei staining with DAPI. Scale bar: 40μm. f Protein levels of MPO, iNOS, and COX-2 in the foot pad tissue were measured by western blot (n=4 per group, mean±SEM). g The MPO activity of foot pad tissue (n=6 per group, mean±SEM). h Mice injected with HSP60-MO presented reduced ankle joint swelling index (n=6 per group, mean±SEM). i Mice were intraperitoneally injected with HSP60-MO or ctrl-MO for 1h and then injected with MSU suspensions into the peritoneal cavity of mice. The lavage fluid was harvested and detected for IL-1β production through ELISA. The total cell number in the lavage fluid was counted by hematocytometer (n=6 per group, mean±SEM). # Significantly different from absence of MSU crystals injection mice and * significantly different from MSU crystals and ctrl MO injection mice.

**Supplementary Files**

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- SFig.6.jpg
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