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Dehydroisohispanolone alleviates NLRP3-driven inflammation in gout arthritis

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ABSTRACT

Natural products constitute an important resource for drug discovery with hispanolone derivatives recognized as bioactive diterpenes due to their broad therapeutic potential. Dehydroisohispanolone (DIH) is an anti-inflammatory labdane diterpene, that acts as a multi-target agent through various inflammation pathways. Aberrant activation of the Nod-like receptor protein 3 (NLRP3) inflammasome is involved in several inflammatory diseases, including gout. This study explores the mechanisms underlying the effects of DIH on the NLRP3 inflammasome and pyroptosis *in vitro* and evaluates its therapeutic effects on gouty arthritis in mice. DIH suppresses interleukin (IL)-1β secretion and pyroptosis in lipopolysaccharide (LPS)-primed bone-marrow-derived macrophages (BMDMs) elicited by broad stimuli. DIH exerts its action by disrupting adaptor apoptosis-associated speck-like protein (ASC) oligomerization, thereby inhibiting the assembly of inflammasome complex. Notably, DIH specifically inhibits NLRP3 inflammasome activation in murine BMDMs without affecting the absent in melanoma-2 (AIM2) or NOD-like receptor family CARD domain containing 4 (NLRC4) inflammasomes. In animal models, DIH significantly mitigated monosodium urate (MSU)-induced joint inflammation, reducing cytokine levels and myeloperoxidase (MPO) secretion. Overall, this study identified DIH as a specific inhibitor of

Abbreviations: AIM2, absent in melanoma-2; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain CARD; ATP, adenosine triphosphate; BMDMs, bone marrow derived macrophages; BSA, bovine serum albumin; COX-2, cyclooxygenase-2; CXCL-1, C-X-C Motif Chemokine Ligand 1; DIH, dehydroisohispanolone diterpene; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; FBS, fetal bovine serum; GSDMD, gasdermin D; IL, interleukin; IMQ, imiquimod; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MPO, myeloperoxidase; MSU, monosodium urate crystals; MTT, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide; NACHT, nucleotide-binding oligomerization domain; NF-κB, nuclear factor-κB; Nig, nigericin; NLRC4, NOD-like receptor family CARD domain containing 4; NLRP3, NLR family pyrin domain containing protein 3; NOS-2, nitric oxide synthase-2; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; RLU, relative luminescence units; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TMB, tetramethylbenzidine; TNF, tumor necrosis factor; TLR, toll-like receptors; Vh, vehicle..

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the NLRP3 inflammasome, providing new insights into its potential as a therapeutic agent for NLRP3-mediated inflammatory diseases.

1. Introduction

Inflammation is a key immune response that protects the organism by removing injurious stimuli, including damaged cells, pathogens, or irritants. This process is initiated when immune cells, such as neutrophils and macrophages, recognize these signals and subsequently release a range of inflammatory mediators, including cytokines and chemokines. However, when inflammation is prolonged or dysregulated, it can contribute to the pathogenesis of various diseases, including cardiovascular diseases, cancer or arthritis, among others [1]. The inflammatory process is closely linked to the function of inflammasomes, which are intracellular multiprotein complexes also involved in the immune response [2]. The NOD-like receptor protein 3 (NLRP3) inflammasome, a pyrin domain-containing NOD-like receptor is composed of three key components: the NLRP3 protein, procaspase-1, and the adaptor apoptosis-associated speck-like protein (ASC). This inflammasome can be triggered by a variety of signals and cellular events, including ion influx, silica, pore-forming toxins as nigericin, extracellular danger signals such as adenosine triphosphate (ATP), reactive oxygen species (ROS) production and the deposition of urate crystals, a process observed in gouty arthritis [3,4]. Activation of the NLRP3 inflammasome by these diverse stimuli subsequently leads to the activation of caspase-1, which facilitates the processing of precursor forms of IL-1β and IL-18 into their mature active forms. In addition, NLRP3 inflammasome activation can induce caspase-1-dependent pyroptotic cell death [5]. The NLRP3 inflammasome represents a promising therapeutic target for novel anti-inflammatory therapies, given its crucial role in the pathogenesis of inflammatory diseases such as gout.

Gout, a common chronic inflammatory arthritis, has emerged as a significant public health issue, particularly in developed countries where its prevalence is rising. This condition is caused by hyperuricemia and monosodium urate (MSU) crystal deposition within joint and periarticular tissues, and is clinically characterized by edema, swelling, erythema, severe pain and inflammation [6,7]. The immune response in gout is driven by resident joint macrophages that phagocytize MSU crystals, leading to a localized inflammatory response. MSU crystals induce the activation of NRLP3 inflammasome which results in increased caspase-1 activity, IL-1β and IL-18 cytokine release and pyroptotic cell death. In addition, a significant accumulation of inflammatory cells is triggered to the site of urate crystal deposition [8].

Natural products have been a valuable source in the development of novel therapeutic agents for inflammation-driven diseases. The labdane diterpene Hispanolone, was isolated from the aerial parts of *Ballota* species. Hispanolone derivatives are bioactive diterpenes with notable biological activity, demonstrating anti-tumoral, cardioprotective and anti-inflammatory properties [9–14]. Our previous studies have shown that these compounds inhibit key inflammatory pathways, including activation of the transcription nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), leading to the down-regulation of pro-inflammatory enzymes such as nitric oxide synthase-2 (NOS-2) and cyclooxygenase-2 (COX-2) in macrophages [15,16]. More recently, some hispanolone derivatives have been described as potential selective inhibitors of the NLRP3 inflammasome [17]. Among them, the diterpene dehydroisohispanolone (DIH) has gained particular interest due to its multi-targeted anti-inflammatory effects. DIH has been reported to inhibit inflammasome activation through a dual mechanism of action by suppressing both the NF-κB and NLRP3 signalling pathways in cultured macrophages. Covalent docking studies further support the efficacy of DIH as an NLRP3 inflammasome inhibitor [18].

Considering the ongoing challenges in managing gouty arthritis and other inflammation-related conditions, the exploration of novel agents targeting NLRP3 is of great value. This study aims to investigate DIH as a potential pharmacological inhibitor of the NLRP3 inflammasome, with a specific focus on its applicability in treating gout-induced inflammation. Our findings demonstrate that DIH effectively reduces NLRP3-mediated inflammatory responses in primary bone-marrow-derived murine macrophages (BMDMs) and significantly alleviates inflammation in a mouse model of MSU-induced gouty arthritis.

2. Materials and methods

2.1. Material and reagents

Dehydroisohispanolone (DIH) (Fig. 1a) was obtained from hispanolone by addition of HCl in EtOH under reflux for 18 h, following the procedure described [19]. The spectroscopic data obtained were identical to those previously reported [17,18].

For cell culture, Dulbecco's modified Eagle medium (DMEM, D6429) and bovine serum albumin (BSA, A9418) were from Sigma-Aldrich (St. Louis, MO, USA); fetal bovine serum (FBS, 10500064) and penicillin/streptomycin (PenStrep, 15070063) from Thermo Fisher Scientific (Göteborg, Sweden). Lipopolysaccharide (LPS) from *Escherichia coli* O26:B6 (L2654), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M5655), poly (deoxyadenylic-thymidylic) acid sodium salt (Poly dA:dT) (P0883), tetramethylbenzidine (TMB) (T-8768), nigericin sodium salt (N7143), adenosine triphosphate (ATP) (A2383), MCC950 (PZ0280) and hydrogen peroxide (H1009) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pam3CysSerLys4 (Pam3CSK4) (tlrl-pms), flagellin from *Salmonella typhimurium* (tlrl-stfla) and imiquimod (R837), were purchased from InvivoGen (San Diego, CA, USA). Trichloroacetic acid (TCA) (10391351) and disuccinimidyl suberate (DSS) (21555) were acquired from Thermo Fisher Scientific (Göteborg, Sweden). Lipofectamine® 3000 transfection reagent (L3000008), from InvitroGen (Massachusetts, USA), Ac-Tyr-Val-Ala-Asp chloromethylketone (Ac-YVAD-CMK) (4018838.0005, Cambridge Bioscience, UK), silica (MIN-U-SIL 15, U.S. Silica, TX, USA). Isoflurane was from SomnoSuite (Kent Scientific, Torrington, CT, USA). ECL prime western blotting detection reagent was purchased from Cytiva (RPN2236, Amersham, UK) and MSU crystals (tlrl-msu-25) from InvivoGen (San Diego, CA, USA). Recombinant anti-pro Caspase-1 + p10 + p12 antibody (ab179515) and anti-GSDMD antibody (ab209845) were obtained from Abcam (Cambridge, UK). Anti-NLRP3/NALP3, mAb (Cryo-2) (AG-20B-0014, Adipogen, San Diego, CA, USA), mouse IL-1 beta /IL-1F2 antibody (AF-401-NA, R&D Systems, Minneapolis, USA), rabbit mAb anti-mouse ASC/TMS1 (D2W8U) (67824S, Cell Signaling Technology, Massachusetts, USA) anti-β-Actin-peroxidase mouse monoclonal antibody (A3854) from Sigma-Aldrich (St. Louis, MO, USA). All secondary antibodies were obtained from Agilent (CA, USA): Rabbit Anti-Goat Immunoglobulins/HRP (P044901-2), Goat Anti-Rabbit Immunoglobulins/HRP (P044801-2) and Rabbit Anti-Mouse Immunoglobulins/HRP (P026002- 2). ELISA kits were obtained from R&D Systems (Minneapolis, USA) for tumor necrosis factor (TNF)-α (DY410-05), IL-1β (DY401-05), C-X-C motif chemokine ligand (CXCL)-1 (DY453-05) and IL-6 (DY406-05). IL-18 ELISA kit (BMS618-3) was purchased from Thermo Fisher Scientific, (Göteborg, Sweden).

2.2. Cell culture of primary bone marrow derived macrophages

All animal care and experimental protocols adhered to the European Union guidelines (EEC Directive of 1986; 86/609/EEC) and the principles of the Declaration of Helsinki principles for Animal Care. BMDMs were isolated from the tibia and femur of adult male wild type C57BL/6

mice (n = 20, aged 8–20 weeks, mixed sex). Animals were bred and housed at the University of Manchester. Mice were maintained in groups under a standard 12-hour light–dark cycle (light phase: 07:00–19:00) with controlled conditions, as constant temperature of 21 °C, regulated humidity, *ad libitum* access to food and water, and environmental enrichment All experiments complied with UK Home Office regulations (PPL: P4035628) and were performed in accordance with ARRIVE guidelines for animal research. The University of Manchester Animal Welfare and Ethical Review Board approved the animal procedures. BMDMs were cultured in DMEM supplemented with 10 % (v/v) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 30 % L929 mouse fibroblast-conditioned medium. Cells were cultured for 7–10 days in a humidified incubator at 37 °C with 5 % CO_2. Afterwards, the cells were seeded (1×10^6 cells/well in 12-well plates or 1×10^5 cells/well in 96-well plates) in complete DMEM medium and incubated overnight before being used in experiments.

2.3. Activation of inflammasomes

For NLRP3 inflammasome activation, cells were primed for 4 h with 1 μg/ml LPS. Then, the medium was replaced with serum-free DMEM, and cells were treated with either vehicle (Vh) (dimethyl sulfoxide (DMSO) 0.1 %) or DIH (at the indicated concentrations) for 30 min. Canonical NLRP3 activation was induced using the following inflammasome activators: nigericin (Nig) (10 μM, 1 h), silica (300 μg/mL, 4 h), imiquimod (IMQ) (75 μM, 1 h) or ATP (5 mM, 1 h). BMDMs were primed with Pam3CSK4 (100 ng/mL, 4 h) to induce non-canonical NLRP3 activation. Subsequently, cells were incubated in serum-free DMEM with either vehicle or DIH for 30 min, followed by transfection with 2 μg/mL LPS using Lipofectamine 3000 for 24 h, according to the manufacturer's instructions.

Alternatively, to assess the effects of DIH on NOD-like receptor family CARD domain containing 4 (NLRC4) and absent in melanoma-2 (AIM2) inflammasomes, cells were primed with LPS (1 μg/mL, 4 h). After 30 min incubation with DIH, cells were transfected with either flagellin from *Salmonella typhimurium* (1 μg/mL) or poly(dA:dT) (1 μg/

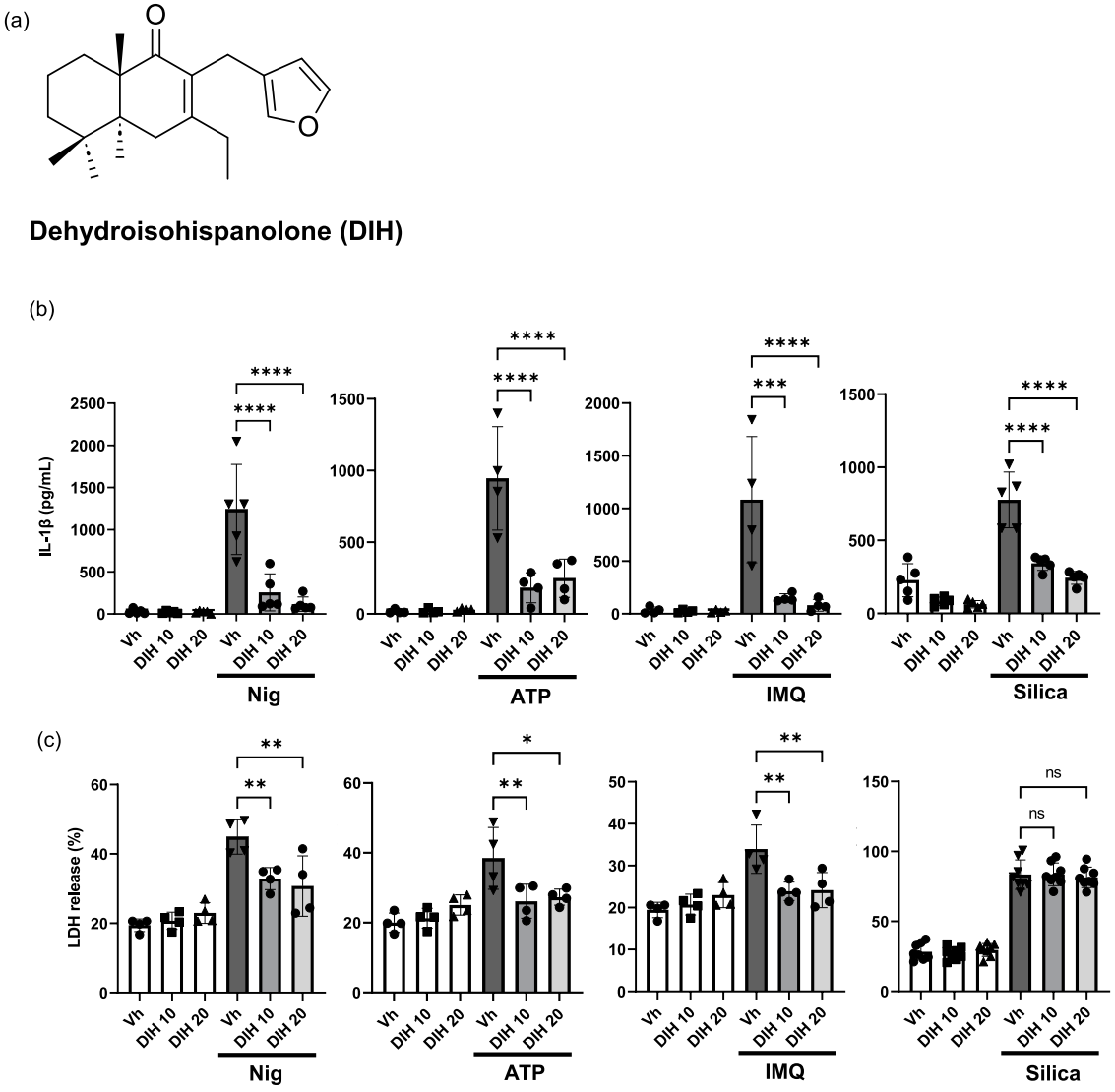

Fig. 1. DIH inhibits canonical NLRP3 inflammasome activation in primary macrophages. (a) Chemical structure of Dehydroisohispanolone (DIH). (b) LPS-primed (1 μg/mL, 4 h) BMDMs were preincubated with DIH (10, 20 μM) or Vh control for 30 min. Then, NLRP3 activator was added (Nig 10 μM, 1 h; ATP 5 mM, 1 h; Silica 300 μg/mL, 4 h or IMQ 75 μM, 1 h). Cell supernatants were analyzed for IL-1β released via ELISA. (c) Cells were treated as in b. Pyroptosis was assessed in supernatants using the CytoTox® kit. Data were expressed as a percentage of total LDH. Results were reported as mean ± SD of at least 4 independent experiments. ns, no significant, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 *vs* LPS + stimuli treatment.

mL) for 4 h, respectively using Lipofectamine 3000. To evaluate AIM2, cells were pretreated with MCC950 (10 μM), a reference NLRP3 inhibitor, before addition of DIH to avoid potential activation of the NLRP3 inflammasome triggered by cytosolic DNA, such as poly(dA:dT).

Supernatants were collected and used for ELISA assay and/or cell death evaluation. Alternatively, cells were lysed in-well for assessment of total protein content in combined cell lysate and supernatant for immunoblot assays.

2.4. Caspase-1 activity assay

For evaluation of caspase-1 activity, Caspase-Glo® 1 inflammasome Assay kit (Promega, G9951), was used. Relative luminescence units (RLU) were converted to percentage change, with 100 % set for LPS + nigericin treatment, and percent change was calculated for each DIH concentration.

2.5. Cell death and ELISA assays

Pyroptosis was measured in macrophages using a lactate dehydrogenase (LDH) assay kit (CytoTox® 96 Non-Radioactive Cytotoxicity Assay (Promega, G1780), according to the manufacturer's instructions.

ELISA kits were used to measure cytokine levels (IL-1β, TNF-α, IL-6, IL-18, CXCL-1) in cell culture supernatants, following the manufacturer's instructions.

2.6. ASC oligomerization assay

BMDMs (10^6 cells/ml) were plated at 12-well plates. Cells were pretreated with LPS for 4 h, DIH for 30 min and incubated with nigericin for 1 h. For cell lysates, 1 % v/v Triton x-100 and protease inhibitor cocktail were directly added in-well and centrifuged at 6800×*g* for 20 min at 4 °C. Then, the pellet containing the insoluble fraction was chemically crosslinked by adding disuccinimidyl suberate (DSS, 2 mM) for 30 min at room temperature in phosphate buffered saline (PBS) and spun at 6800×*g* for 20 min. A volume of 40 μL of Laemmli buffer (1×) was added for western blot analysis.

2.7. Western blotting

For protein determination in supernatants, cell lysates of macrophages and paw homogenates, Bradford reagent was used. For cell lysates, the Triton x-100 soluble fraction was precipitated with trichloroacetic acid (TCA) and then washed in acetone and centrifuged for 10 min (14,000×*g*, 4 °C). Samples were diluted in 2X Laemmli buffer and heated before loading into the gels. They were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes that were sealed with 5 % w/v milk in PBS-0.1 % Tween 20 (PBS-T) at room temperature for 1 h before addition of primary antibodies in BSA (5 % w/v in PBS-T) and incubated overnight at 4 °C. Afterwards, the membranes were washed and incubated (1 h, RT) with appropriate HRP-conjugated secondary antibodies. Chemiluminescence was visualized with Amersham ECL Prime Western Blotting Detection Reagent using a G:Box Chemi XX6 (Syngene).

Samples were assayed for NLRP3 (1:1000), pro-IL-1β (1:800), ASC (1:3000), mature IL-1β (1:500), pro-caspase-1 and caspase-1 p10 (1:3000), gasdermin D (GSDMD) (1:1000). β-Actin was used as a loading control. The secondary antibodies used were rabbit anti-mouse IgG (1.3 μg/mL in 1 % BSA/PBST), goat anti-rabbit IgG (250 ng/mL in 1 % BSA/PBST), or rabbit anti-goat IgG (500 ng/mL in 1 % BSA/PBST).

2.8. Experimental animals

Male C57BL/6 mice (Charles River, Écully, France), aged between 10 and 12 weeks (weighing 20–25 g), were randomly assigned to four groups and housed at 21 °C with a 12-hour light–dark cycle, having access to food and water *ad libitum* in the Central Service for Experimental Research (SCSIE-Burjassot) at the University of Valencia. The experimental protocol complied with the European regulations, the appropriate approvals and authorizations (protocol code GVRTE/2023/4772821).

2.9. MSU-induced gouty arthritis

DIH (5 mg/kg) was administered intraperitoneally to the corresponding groups (DIH with and without MSU crystals) in a volume of 250 μL per mouse. The other groups (vehicle and MSU control) received 250 μL of saline solution. After one hour, a single dose of 2 mg of MSU crystals (in 50 μL sterile PBS), was injected subcutaneously under the plantar surface of the left hind paw in the MSU and MSU + DIH groups [20]. The vehicle and DIH groups received 50 μL of sterile PBS in the same left hind paw.

A digital water plethysmometer (Panlab S.L.U., Barcelona, Spain) was used to measure paw edema at 1, 3, 6, and 24 h after MSU crystal injection. At the 24-hour time point, the animals were anesthetized with 4–5 % isoflurane and euthanized by cervical dislocation. The hind limbs were then removed, frozen at −80 °C, and stored for homogenization in liquid nitrogen for western blot analysis and assessment of inflammatory mediators.

2.10. Determination of mediators in paw homogenates

Frozen limbs were homogenized in liquid N_2 with 1 mL of buffer A, pH 7.4 containing 10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na_3VO_4, 10 mM Na_2MoO_4, 0.1 mg/mL aprotinin, 1 mg/mL leupeptin and 0.5 mM phenylmethylsulfonyl fluoride. The tissue homogenates were then sonicated (3 × 10 s) on ice, incubated at 4 °C for 10 min and then centrifuged at 1500×*g* at 4 °C for 5 min. Collected supernatants were further centrifuged at 10,000×*g* at 4 °C for 5 min. These final supernatants were used to measure cytokines (TNF-α, IL-1β, IL-6, IL-18, CXCL-1), caspase-1 protein expression and myeloperoxidase (MPO) activity.

2.11. Myeloperoxidase activity assay

Supernatants from paw homogenates were incubated with PBS (pH 7.4) and phosphate buffer pH 5.4, containing 0.09 % Na_2HPO_4 and 1.15 % NaH_2PO_4 and hydrogen peroxide (0.05 %) for 5 min. Then, TMB (18 mM) previously dissolved in dimethylformamide (8 % in distilled water) was added and incubated at 37 °C for 10 min. Sulfuric acid (2 N) was added to stop the reaction, and absorbance was measured using a Wallac 1420 VICTOR3TM spectrophotometer (PerkinElmer, Turku, Finland) at 450 nm [21].

2.12. Statistical analysis

Statistical analyses were conducted using GraphPad Prism Software 9 (CA, USA). All values are presented as means ± standard deviation (SD) from a minimum of three independent experiments, along with individual data points when available. Normality was assessed using the Shapiro-Wilk test. Statistical significance was determined using one-way ANOVA followed by Dunnett's post-hoc test. In the MSU model, two-way ANOVA analysis was performed considering edema volume respect two independent variables (time and treatment). In this case, a post-hoc Bonferroni test for multiple group comparisons was used. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. DIH supresses NLRP3 inflammasome activation in bone marrow-derived primary macrophages

NLRP3 activation occurs in response to diverse stimuli. To investigate whether DIH could inhibit the activation of the NLRP3 inflammasome, BMDMs were treated with DIH after the cells were primed with LPS. Subsequently, macrophages were exposed to different NLRP3 activators: nigericin (Nig), ATP, imiquimod (IMQ) and silica crystals, via the canonical pathway. ELISA analysis revealed that DIH significantly reduced IL-1β secretion in cell supernatants with all the NLRP3 activators tested (Fig. 1b). Furthermore, DIH did not compromise BMDM viability at the concentrations tested [18].

Pyroptosis, a form of inflammatory cell death, serves as a hallmark of inflammasome activation. The effects of DIH on pyroptosis were then evaluated using a lactate dehydrogenase (LDH) release assay. Stimulation of LPS-primed BMDMs macrophages with nigericin, ATP, imiquimod or silica led to increased LDH into the supernatant, indicating pyroptotic cell death. As shown in Fig. 1c, DIH treatment significantly reduced LDH release in response to these activators, with the exception of silica.

IL-1β release requires previous caspase-1 activation, so we next investigated whether the regulation of NLRP3 activation by DIH was attributable to a direct inhibition of this enzyme. For these experiments, nigericin was used as the NLRP3 activator. Since caspase-1 activity can be detected in the cell supernatant following pyroptosis, cell-free supernatants were collected and incubated with DIH or the caspase-1 inhibitor Ac-YVAD-CFK (YVAD), prior to being assayed for caspase-1 activity. As shown in Fig. 2, nigericin treatment significantly enhanced caspase-1 activity, which was fully inhibited after YVAD treatment but not by DIH. These findings confirm that DIH clearly disrupts NLRP3 inflammasome complex assembly rather than directly inhibiting caspase-1.

3.2. DIH disrupts ASC oligomerization

ASC oligomerization, a critical step in inflammasome activation, enables NLRP3 assembly and subsequent caspase-1 activation [22,23]. ASC forms oligomers in response to NLRP3 activators. Thus, the effects of DIH on this process were determined by immunoblotting. DIH treatment significantly suppressed nigericin-induced ASC oligomerization in BMDMs, indicating that DIH disrupts NLRP3 inflammasome assembly (Fig. 3). Consequently, treatment with DIH also produced lower expression of cleaved GSDMD fragment (GSDMD-N), the active subunit of caspase-1 (Casp-1 p10) and cleaved IL-1β (cIL-1β), while the levels of the precursor components (GSDMD, Casp-1 p45 and pro-IL-1β) remained unaffected. These findings suggest that DIH does not inhibit ASC directly but likely interferes with ASC oligomerization by modulating a step specific to NLRP3 inflammasome assembly.

3.3. DIH inhibits non-canonical NLRP3 activation

In addition to the canonical pathway, NLRP3-dependent IL-1β processing can also be triggered indirectly via a non-canonical pathway downstream of caspase-11 in mice [24,25]. Given the effectiveness of DIH in inhibiting the canonical activation of NLRP3 inflammasome, we next examined its effect on the non-canonical pathway. To test this, BMDMs were first primed with Pam3CSK4 (a toll-like receptor (TLR)2/TLR1 ligand) and then pretreated with DIH before stimulation with cytosolic LPS (cLPS). DIH treatment, administered between Pam3CSK4 priming and cLPS transfection, significantly reduced IL-1β release at the highest concentration tested, indicating that DIH also impacts the non-canonical pathway. However, DIH did not significantly inhibit c-LPS induced pyroptosis (Fig. 4).

3.4. DIH does not affect AIM2 and NRLC4 inflammasomes

Finally, we explored whether DIH could suppress the activation of other inflammasome complexes to determine if its inhibitory effects were specific to the NLRP3 inflammasome. To this end, we assessed the effects of DIH on NLRC4 and AIM2. LPS-primed BMDMs were pretreated with DIH and then stimulated with poly(dA:dT) (an AIM2 activator) or flagellin from *S. typhimurium* (an NLRC4 activator). DIH pretreatment did not reduce IL-1β release or pyroptosis (measured by LDH release) in response to either activator (Fig. 5). Collectively, these findings strongly suggest that DIH specifically inhibits NLRP3 inflammasome activation in murine BMDMs without affecting the AIM2 or NLRC4 inflammasomes.

3.5. DIH mitigates MSU-induced gouty arthritis

To evaluate the anti-inflammatory effects of the diterpene DIH in vivo, we used an MSU-induced gouty arthritis model, where gout was simulated by subcutaneous injection of MSU crystals in mice.

Edema formation following MSU crystal injection (2 mg) was measured at specific intervals (1, 3, 6, and 24 h) to evaluate the temporal progression and magnitude of the inflammatory response. Experimental scheme is shown in Fig. 6a. MSU injection caused pronounced paw edema, as measured using a digital plethysmometer (Fig. 6b, c). However, compared with MSU group, animals treated with DIH (5 mg/kg,

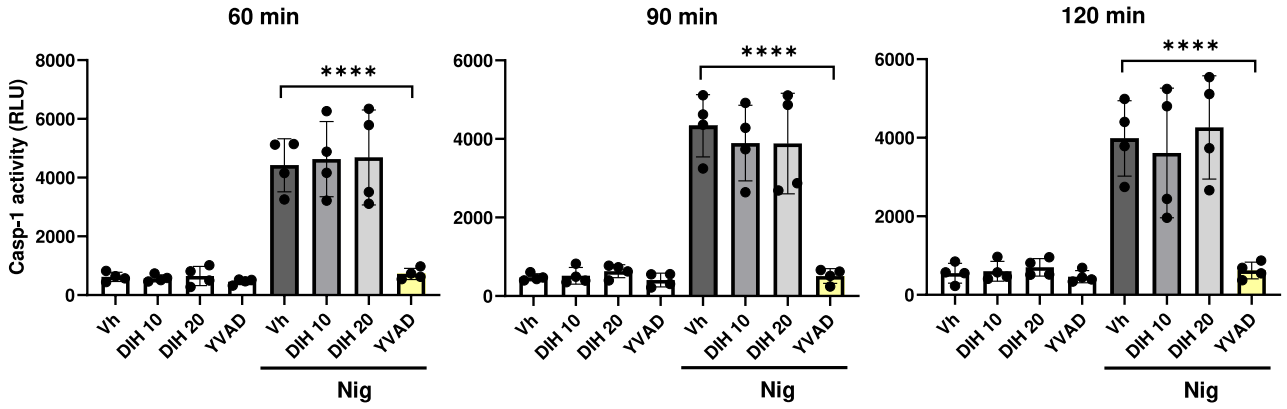

Fig. 2. DIH does not affect caspase-1 activation. BMDMs were primed with LPS (1 μg/mL, 4 h) and then stimulated with Nig (10 μM) for 1 h. Following caspase-1 activation, cell supernatants were collected and incubated for 15 min with either DIH (10, 20 μM), the caspase-1 inhibitor Ac-YVAD-CMK (YVAD) (100 μM), or vehicle (Vh). Measurement of Caspase-1 activity was performed in the supernatants using the Casp-Glo® 1 kit after 60, 90 and 120 min. Results were expressed as mean ± SD of relative luminescence units (RLU) (n = 4 independent experiments). ****$p < 0.0001$ *vs* LPS + Nig treatment.

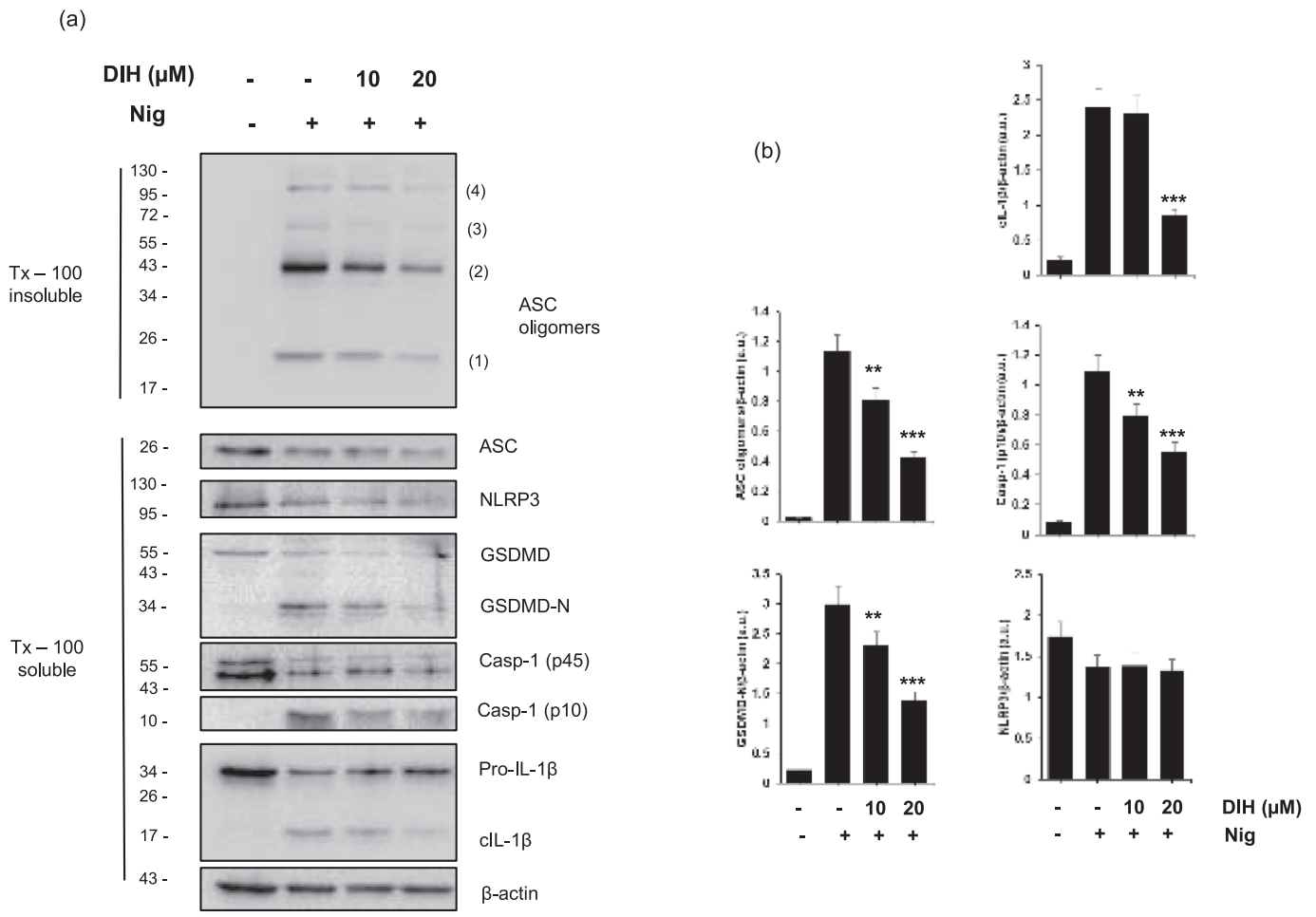

Fig. 3. Suppression of inflammasome activation by DIH is dependent on ASC. LPS-primed (1 μg/ mL, 4 h) BMDMs were preincubated with DIH (10, 20 μM) for 30 min before stimulation with Nig (10 μM) for 1 h. Immunoblot analysis (a) and densitometric quantification (b) of crosslinked ASC oligomers in Triton x-100 insoluble pellet, and soluble total cell lysates probed with appropriated antibodies (ASC, NLRP3, GSDMD, caspase-1 and IL-1β). β-actin was used as a loading control. Data is representative of four independent experiments. **p < 0.005 and ***p < 0.001 *vs* LPS + Nig treatment.

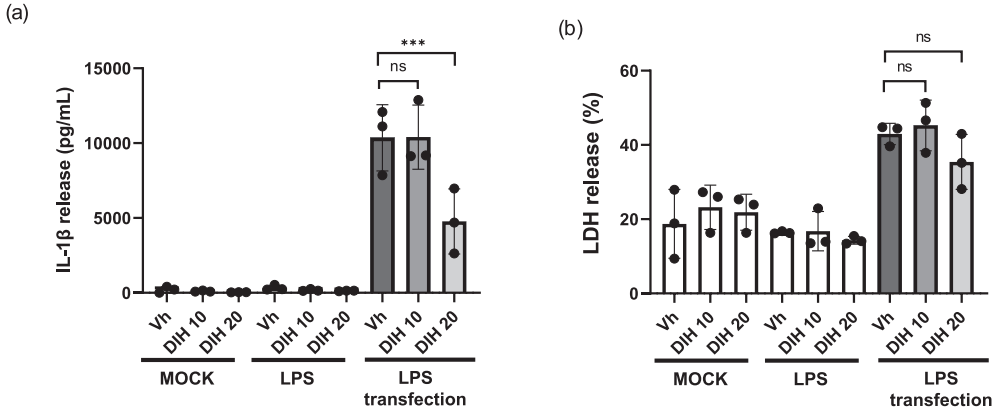

Fig. 4. DIH impairs non-canonical NLRP3 activation. Pam3CSK4-primed (100 ng/mL, 4 h) BMDMs were preincubated with DIH (10, 20 μM) or Vh for 30 min. Then, cells were stimulated with cytosolic LPS (cLPS, 2 μg/mL) for 24 h. (a) ELISA analysis of IL-1β levels in cell supernatants. (b) Pyroptosis was assessed in terms of LDH release in supernatants using the CytoTox® kit and expressed as percentage of total LDH. Data are reported as mean ± SD (n = 3 independent experiments). Ns, no significant and ***p < 0.001 *vs* Pam3CSK4 + cLPS.

intraperitoneal), showed a significant reduction in paw edema, with the most notable effects at 6 and 24 h post-injection (Fig. 6c, d).

To further assess the inflammatory response, myeloperoxidase (MPO) activity was measured in paw homogenates as an indicator of neutrophil recruitment. While MPO plays a crucial role for controlling infection, it has also been linked to tissue damage and chronic inflammation, contributing to gout pathogenesis [26]. MPO activity significantly increased 24 h after MSU injection but was markedly reduced in DIH-treated mice (Fig. 6d).

The effect of DIH as NLRP3 inflammasome inhibitor was validated by

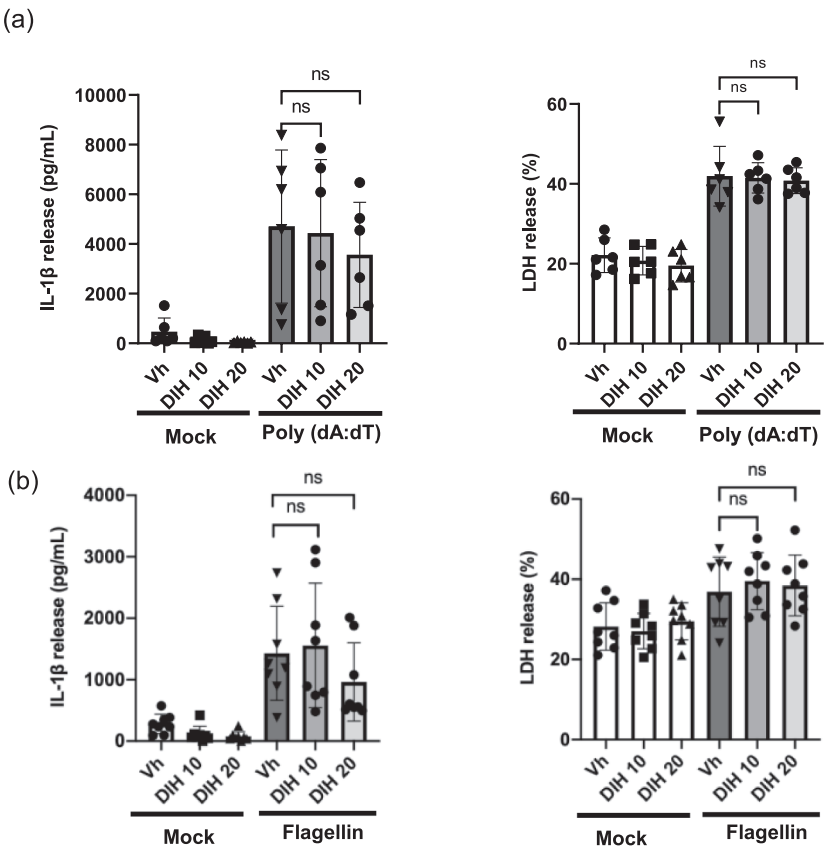

Fig. 5. DIH does not affect activation of other inflammasomes (NLRC4 or AIM2). LPS-primed (1 μg/mL, 4 h) BMDMs were pretreated with diterpene DIH (10, 20 μM) or Vh for 30 min followed by stimulation with 1 μg/mL cytosolic poly (dA:dT) (a) or 1 μg/mL flagellin (b) for 4 h. Determination of IL-1β and LDH release was performed in cell supernatants. Results are reported as mean ± SD of at least six independent experiments. Ns, no significant.

measuring IL-1β and IL-18 secretion in paw tissues 24 h after MSU injection. As shown in Fig. 7a, DIH treatment significantly suppressed the elevated levels of both cytokines induced by MSU crystals. Furthermore, caspase-1 expression was also determined by western blot analysis of paw homogenates. MSU crystals induced an increased expression of caspase-1 subunits p20 and p10 (Fig. 7b). In contrast, the DIH-treated group exhibited a marked decrease in these subunits, indicating reduced caspase-1 activity. This result aligns with the observed decrease in IL-1 β and IL-18 levels.

Furthermore, macrophages activated by MSU crystals initiate a cascade of signalling events that lead to the release of proinflammatory mediators, amplifying the inflammatory response characteristic of gouty arthritis. MSU injection significantly augmented other key pro-inflammatory mediators, including IL-6, TNF-α and CXCL-1, which were significantly diminished by DIH treatment (Fig. 7a). This reduction confirms the anti-inflammatory properties of DIH. Collectively, these results indicate that the diterpene could act against gout arthritis though the inhibition of NLRP3 inflammasome activation.

4. Discussion

The present study provides novel insights into the anti-inflammatory properties of the diterpene DIH, highlighting its efficacy in preventing acute gout by targeting NLRP3 inflammasome activation. Dysregulated activation of NLRP3 inflammasome has been linked to the onset or progression of a variety of inflammation-driven diseases that have a significant impact on public health, including gout [27,28]. Targeting the NLRP3 inflammasome represents a therapeutic promising approach, and the development of potential NLRP3 inhibitors is a clinical priority, given that no NLRP3 inhibitors have yet been approved for clinical use [2].

Macrophages are cells of the innate immune system that are crucial in the inflammatory response mediated by NLRP3 inflammasome [28]. The canonical activation of NLRP3 inflammasome is dependent on caspase-1 activation, which promotes the maturation of pro-IL-1β / pro-IL-18 and the release of inflammatory mediators (IL-1β and IL-18). These are associated with both inflammation and pyroptosis [29]. Activation signals, including ATP, bacteria, and uric acid crystals, lead to the activation of NLRP3 inflammasomes in macrophages [30].

Experimental data confirm that DIH significantly inhibited canonical NLRP3 inflammasome activation, thereby reducing IL-1β release in response to a variety of stimuli including Nig, ATP, IMQ and silica in LPS-primed macrophages. This inhibitory effect is not dependent on K^+ efflux, as it was also exerted when using IMQ, a K+ efflux-independent stimuli. DIH did not directly inhibit caspase-1 activity but instead interfered with the assembly of the NLRP3 inflammasome complex, which is likely achieved by targeting ASC oligomerization. This finding is consistent with our previous molecular docking studies, which described that DIH binds within the ATP-binding pocket of NLRP3, a region essential for ASC recruitment and subsequent oligomerization required for inflammasome assembly [18]. In the present study, the observed reduction in ASC oligomer formation, along with decreased expression of downstream effectors (such as cleaved GSDMD, caspase-1 p10, and IL-1β), support the hypothesis that DIH disrupts NLRP3-ASC interaction, thereby preventing further oligomerization through direct inhibition of NLRP3. Therefore, the current *in vitro* findings align with our *in silico* data, reinforcing the conclusion that DIH specifically inhibits NLRP3 inflammasome activation by blocking the assembly of the inflammasome complex.

Furthermore, DIH also suppressed pyroptotic cell death, as evidenced by a significant reduction in LDH levels, a key marker of pyroptosis. Once activated, inflammasome assembly involves

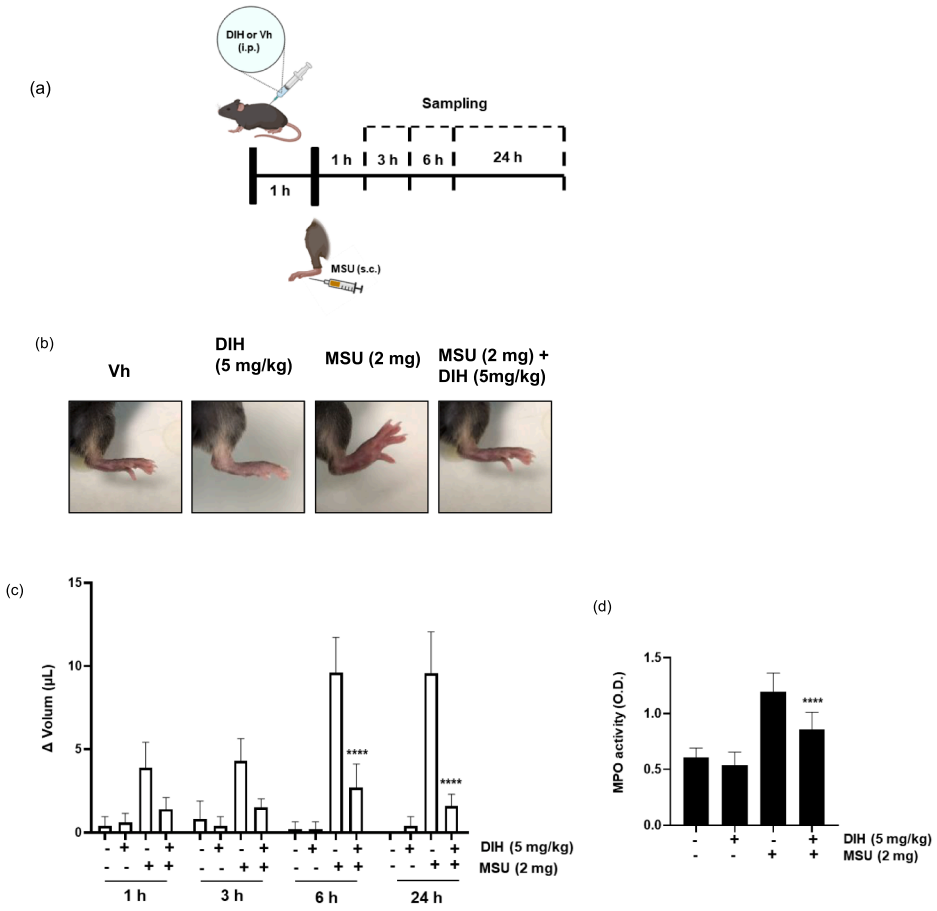

Fig. 6. Pretreatment with DIH alleviates MSU-induced gouty arthritis in mice. (a) Experimental scheme of the treatment protocol. DIH (5 mg/kg) or vehicle (Vh) were i.p. administered. After 1 h, MSU crystals (2 mg) were injected into the left hind paw. Mice were randomly divided into four groups (Vh and DIH: n = 5/group; MSU and MSU + DIH: n = 10/group). (b) Representative images of left hind paw at 24 h post-MSU injection. (c) Edema assessment at various time points (1, 3, 6, and 24 h post-MSU administration). (d) MPO activity was spectrophotometrically measured in paw homogenates at 24 h (absorbance at 450 nm). The values represent the means ± SD. ****p < 0.0001 MSU + DIH *vs* MSU.

interactions between the inflammasome sensor and either inflammatory caspase-1 or non-canonical caspase-11, with or without the co-binding of the adapter protein ASC [23]. According to this, our findings demonstrate that DIH disrupts ASC oligomerization, thereby inhibiting inflammasome-mediated responses. The non-canonical inflammasome activation pathway in mice is a caspase-11–dependent mechanism triggered by the recognition of cytosolic LPS, leading to cell lysis and release of proinflammatory cytokines [25,31,32]. Notably, DIH treatment also impaired non-canonical NRLP3 activation, as demonstrated by a significant reduction of IL-1β levels in macrophages. These findings further support the dual inhibitory effects of DIH on both canonical and non-canonical inflammasome activation pathways highlighting its therapeutic potential as regulator of the NLRP3 inflammasome.

It is also noteworthy that DIH specifically inhibited NLRP3 inflammasome activation without affecting AIM2 or NLRC4 inflammasomes in BMDMs, underscoring a relevant feature for this diterpene. This selectivity is especially significant given that both AIM2 and NLRC4 inflammasomes also require ASC for signalling. The lack of effect on these inflammasomes strongly suggests that ASC is not the direct molecular target. Rather, the data support a mechanism in which DIH disrupts a structural or conformational element specific to NLRP3. Indeed, these findings are in line with previous molecular docking studies, which showed that DIH binds directly to the nucleotide-binding oligomerization (NACHT) domain of NLRP3, making a covalent bond with Cys415, a region critical for ATP binding, ASC recruitment, and subsequent inflammasome assembly [18]. By targeting this domain, DIH likely disrupts NLRP3-mediated ASC oligomerization without interfering with ASC itself. This selective mechanism resembles the action of MCC950, a well-characterized and specific NLRP3 inhibitor. MCC950 has been shown to inhibit both canonical and non-canonical NLRP3 inflammasome activation as well as suppress IL-1β secretion by blocking NLRP3-induced ASC oligomerization in macrophages. Interestingly, like DIH, MCC950 does not interfere with the activation of AIM2, NLRC4, or NLRP1 inflammasomes [33].

Gout is a form of inflammatory arthritis characterized by the deposition of MSU crystals within the joints [34]. The injection of MSU crystals induces swelling, pain, and the release of inflammatory cytokines and chemokines, along with neutrophils infiltration and NLRP3 inflammasome activation. This process triggers caspase-1 activation, followed by the release of IL-1β and IL-18 cytokines. In particular, IL-1β plays a crucial role in the development of MSU-induced inflammation *in vivo* by promoting neutrophil recruitment into the synovium and joint fluid [35,36]. Additionally, MSU crystals stimulate neutrophils to release IL-6 and TNF-α, further amplifying inflammation [37]. Previous results demonstrated that DIH inhibited MSU-induced NLRP3 inflammasome activation in macrophages [18]. In this study, we report that DIH effectively mitigated acute gout by blocking NLRP3 inflammasome activation in an MSU-induced gouty arthritis model. DIH treatment significantly reduced MSU-induced swelling and neutrophil infiltration, as evidenced by decreased MPO release.

NF-κB is a major transcription factor involved in the production of pro-inflammatory mediators in response to MSU [38]. Our previous

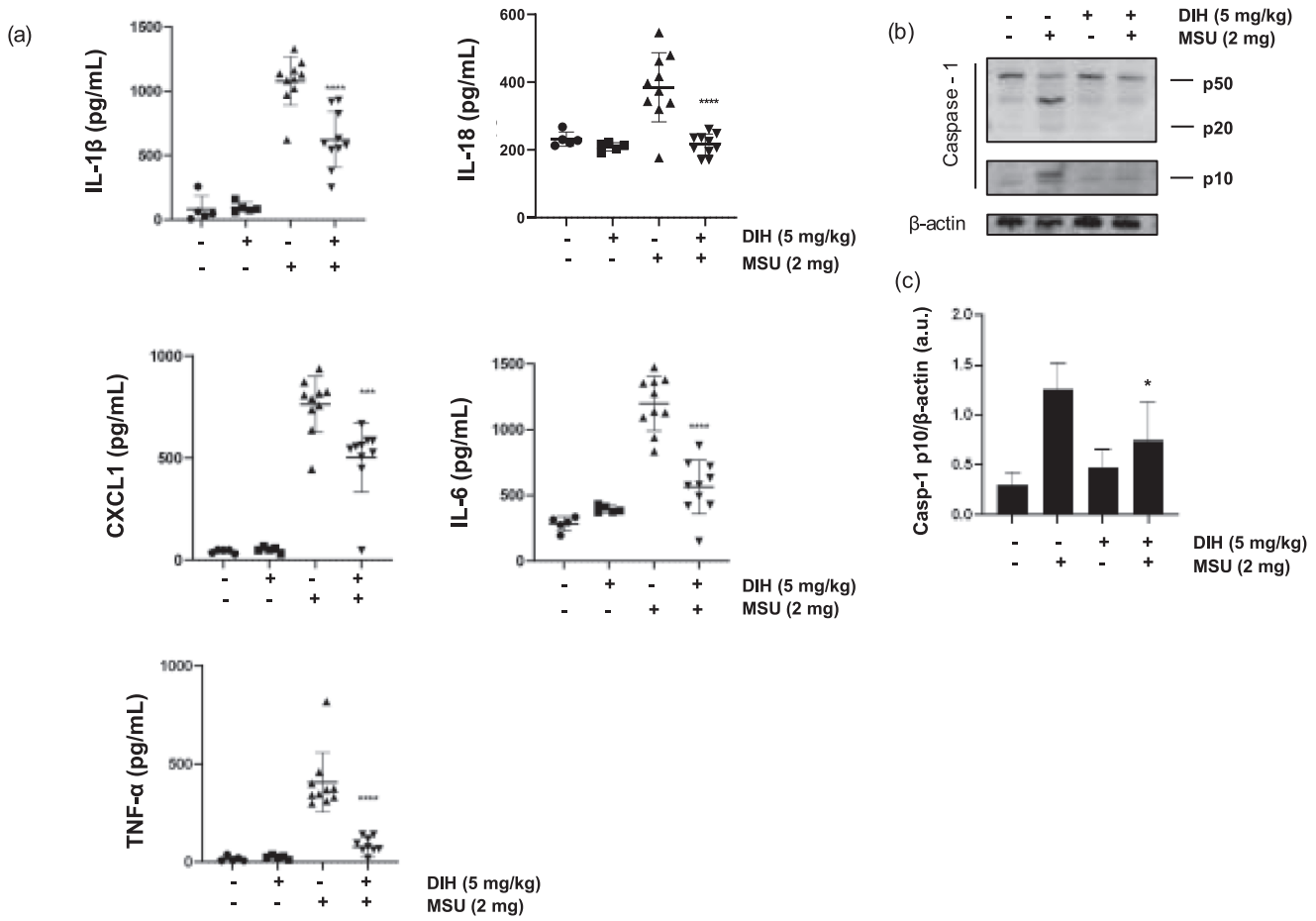

Fig. 7. DIH inhibits the production of pro-inflammatory mediators in paw homogenates. DIH (5 mg/kg) or vehicle were i.p. administered. After 1 h, MSU crystals (2 mg) were injected into the left hind paw. (a) The levels of IL-1β, IL-18, IL-6, TNF-α and CXCL-1 were measured by ELISA. Data are expressed as the mean ± SD of n = 5 mice in Vh (−) and DIH groups and n = 10 mice in MSU and MSU + DIH groups. (b) Immunoblotting for Caspase-1. β-actin was used as a loading control. A representative experiment of four performed is shown. (c) Densitometric quantification of the bands from four experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 MSU + DIH *vs* MSU. All data are presented as the mean ± SD.

research have shown that DIH suppressed NF-κB activation [15], a finding further supported by its modulation of inflammasome-independent cytokine and chemokines (IL-6, TNF-α, CXCL-1) in the *in vivo* model, thereby reinforcing its comprehensive anti-inflammatory profile.

These findings highlight the potential of DIH as a therapeutic agent for NLRP3-mediated diseases, including acute gout, and provide a foundation for its further development as a selective inflammasome inhibitor.

5. Conclusion

Collectively, the results of this study suggest that DIH exert its anti-inflammatory effects through inhibition of NLRP3 inflammasome activation, which was clearly demonstrated in BMDMs macrophages and *in vivo* models. Therefore, DIH may be a novel therapeutic agent targeting NLRP3 for the treatment of acute gouty based on its anti-inflammatory properties.

CRediT authorship contribution statement

Laura González-Cofrade: Formal analysis, Methodology, Data curation, Writing – review & editing, Investigation. **Jack P. Green:** Conceptualization, Validation, Writing – review & editing, Methodology. **Beatriz de las Heras:** Writing – original draft, Conceptualization, Writing – review & editing, Supervision. **María Carmen Terencio:** Writing – original draft, Writing – review & editing, Investigation. **Sonsoles Hortelano:** Writing – review & editing, Supervision, Resources, Writing – original draft, Funding acquisition. **David Brough:** Writing – review & editing, Validation, Supervision. **Ana Estévez-Braun:** Project administration, Writing – review & editing, Funding acquisition. **María Luisa Ferrándiz:** Writing – review & editing, Investigation, Writing – original draft, Conceptualization. **Irene Cuadrado:** Writing – review & editing, Data curation, Conceptualization, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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