1 **SUPPLEMENTARY MATERIALS**

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3

4 **Title: The mechanosensitive TRPV4 is required for crystal-induced inflammation**

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6 Zhou Lan[1, 2, 3, *], Lvyi Chen[1, 2, 4, *], Jing Feng[1, 2], Zili Xie[1, 2], Zhiyong Liu[5], Fang
7 Wang[2, 6], Peng Liu[4], Xueping Yue[1, 2], Lixia Du[1, 2], Yonghui Zhao[1, 2], Pu Yang[1, 2], Jialie
8 Luo[1, 2], Zhe Zhu[7], Xueming Hu[1, 2], Liang Cao[1, 2], Ping Lu[1, 2], Rajan Sah[8], Kory
9 Lavine[8], Brian Kim[1, 2, 6, 9], Hongzhen Hu[1, 2, 6]

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11 [1] Department of Anesthesiology, Washington University School of Medicine in St.
12 Louis, St Louis, Missouri

13 [2] Center for the Study of Itch and Sensory Disorders, Washington University School
14 of Medicine in St Louis, St Louis, Missouri

15 [3] School of Pharmacy, Hubei University of Chinese Medicine, Wuhan, China

16 [4] School of Pharmaceutical Sciences, South-Central University for Nationalities,
17 Wuhan, China

18 [5] Renmin Hospital of Wuhan University, Wuhan, China

19 [6] Division of Dermatology, Department of Medicine, Washington University School
20 of Medicine in St Louis, St Louis, Missouri

21 [7] Herbert Irving Comprehensive Cancer Center, Columbia University, 1130 St.
22 Nicholas Avenue New, York, NY

23 [8] Department of Internal Medicine, Cardiovascular Division, Washington University
24 School of Medicine in St Louis, St Louis, Missouri

25 [9] Department of Pathology and Immunology, Washington University School of
26 Medicine, St. Louis, Missouri

27 Correspondence to: Lvyi Chen (clyhappy05@163.com) and Hongzhen Hu
28 (hongzhen.hu@wustl.edu)

29 * These authors contributed equally: Zhou Lan, Lvyi Chen

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31

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Methods

Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee at Washington University School of Medicine and were in accordance with guidelines provided by the National Institutes of Health and the International Association for the Study of Pain. All human patient-related experiments were performed under the guidance of the Helsinki Declaration and approved by the Institutional Review Board (IRB) at Renmin Hospital of Wuhan University. The study was conducted with fully informed consent of the participants.

Mice

Adult C57BL/6J (Jackson Laboratories), $Trpv4^{-/-}$ (Suzuki et al., 2003),[1] $NLRP3^{-/-}$ and $caspase\text{-}1^{-/-}$ (Shanghai Model Organisms Center, Inc.), $Trpv4^{eGFP}$ (Mutant Mouse Regional Resource Centers), $Pirt^{GCamp3/+}$ (kindly provided by Qin Liu), and $Trpv4^{f/f}$ mice (Luo et al., 2018)[2] were used in this study. $Trpv4^{f/f}$ mice were crossed with $Cx3cr1^{CreERT}$, $Col2A^{CreERT}$, $S100A8^{Cre}$ and $Trpv1^{Cre}$ mice to generate $Cx3cr1^{CreERT}$; $Trpv4^{f/f}$ mice (macrophage-specific *Trpv4 cKOs*), $Col2A^{CreERT}$; $Trpv4^{f/f}$ mice (articular chondrocytes-specific *Trpv4 cKOs*), $S100A8^{Cre}$; $Trpv4^{f/f}$ mice (neutrophils-specific *Trpv4 cKOs*) and $Trpv1^{Cre}$; $Trpv4^{f/f}$ mice (nociceptive sensory neuron-specific *Trpv4 cKOs*), respectively. Mice were randomly allocated to different experimental groups by the lab managers who were blinded to the experimental design. No sex difference in pain and inflammation responses were observed. For DRG neuron isolation, 4–6-week-old mice were used. For other experiments, 8–12-week-old mice were

61 used.

62

63 **Patients**

64 According to the 2015 ACR/EULAR diagnostic criteria of primary gout,[3] thirteen
65 patients who met the following criteria were enrolled in our studies: (1) 25-64 years
66 old male; (2) have been admitted within 72 h of the onset of an acute gout attack, and
67 (3) serum uric concentration ≥ 420 µmol/L. These patients had no other inflammatory
68 conditions and had not taken any anti-inflammatory drugs during this period. Eight
69 males (25-55 years old) were included as the healthy controls. Informed consent was
70 obtained from each subject before all procedures. The clinical characteristics of the
71 patients and healthy control subjects are provided in Supplementary Table E1.

72

73 **Human PBMCs**

74 The human PBMC studies were conducted with fully informed consent of all
75 participants and performed under the guidance of the Helsinki Declaration and
76 approved by the Institutional Review Board (IRB) at Renmin Hospital of Wuhan
77 University. Under the fasting state in the early morning of all enrolled patients and
78 healthy controls, 10 ml of fresh disposable venous blood was drawn and placed in a
79 sterile blood collection tube containing sodium citrate anticoagulant, and the
80 monocytes in the peripheral blood were separated by using a human lymphocyte
81 separation medium (catalog no. 17-829E, Lonza). PBMCs were cultured overnight
82 before stimulation in RPMI 1640 medium.

83

84 **MSU Crystal-induced gouty arthritis**

85 The amount of endotoxin presents in the MSU crystals injected was < 5 pg, as
86 assessed using a Chromogenic Limulus amebocyte lysate assay (Endosafe). 0.8 mg
87 MSU (dissolved in 20 μL PBS) was intraarticularly administrated to induce the
88 arthritis in mice. The index of ankle oedema, reflexive mechanical and thermal
89 pain-related responses were evaluated at different time points after injection of the
90 MSU crystals. 6 hours after intra-articular injection of MSU, some mice were
91 sacrificed, and the joint cavity was washed with PBS for immune cell counting.
92 Peri-articular tissue was removed from the joint, homogenized and assessed for MPO
93 expression by ELISA.

94

95 **Pharmacologic inhibition of TRPV4 function**

96 To determine the preventive and acute therapeutic effects of GSK219 on
97 TRPV4-mediated gouty arthritis, either daily I.P. injections of the GSK219 for 3
98 consecutive days before IA injections of MSU crystals or acute administration of
99 GSK219 5 hours after IA injections of MSU crystals was used. Colchicine (I.P., 1
100 mg/kg in 100 μL of PBS; Sigma-Aldrich) applied for 3 consecutive days before MSU
101 crystal injections was used as a positive control in our gout arthritis model.

102

103 ***In vivo* silica model**

104 To establish the silica model, 40 μl aqueous suspensions prepared by dissolving 200

105 μg silica crystals (MIN-U-SIL-15) in PBS was applied to mouse through orotracheal
106 instillation. 16–18 hours later, bronchoalveolar lavage fluid was collected for further
107 analysis.[4]
108

109 **Mouse model of crystal-induced peritonitis**

110 Mice were intraperitoneally injected with 100 μg alum adjuvant, [5] 1 mg CPPD, [6] or
111 1 mg MSU crystals in 500 μl sterile PBS to generate crystal-induced peritonitis. 6-12h
112 after the challenge, mice were killed by CO_2 exposure, and their peritoneal cavities
113 were washed with 10 ml PBS containing 3 mM EDTA and 10 U/ml of heparin. Total
114 peritoneal exudate cells and neutrophils were analyzed as described above.
115

116 **von Frey test**

117 The mechanical threshold was determined before and after IA joint injection of MSU
118 crystals with flexible nylon von Frey filaments in logarithmic increments of force
119 (0.04–2 g) using the up-and-down paradigm. The 50% mechanical paw withdrawal
120 threshold response (g) was assessed.[7]
121

122 **Hargreaves test**

123 Hargreaves test was performed to determine the reflexive thermal pain behavior in
124 response to radiant heat. A radiant light beam generated by a 60-W lightbulb in a
125 Plantar Test apparatus (Ugo Basile, Varese, Italy) was directed into the right hind paw
126 to determine the paw withdrawal latency at 10 min intervals (the time spent to remove

127 the paw from the stimulus) before and after IA injections of MSU crystals. The
128 latency of paw withdrawal was tested 3 times per animal and averaged for analysis.
129

130 **Tamoxifen administration**

131 To induce robust Cre activity in *Cx3cr1*^{CreERT} *and Col2A*^{CreERT} line, tamoxifen (Sigma,
132 St. Louis, MO, USA) was dissolved in corn oil and made fresh daily before use. Both
133 Cre^{-} and Cre^{+} mice received intraperitoneal injection of tamoxifen at 100 mg/kg body
134 weight for 5 consecutive days. In vivo and ex vivo experiments were performed
135 between 7 and 14 days after tamoxifen injection.
136

137 **Pharmacological ablation of TRPV1-positive sensory nerves**

138 Resiniferatoxin (Sigma-Aldrich), a potent TRPV1 agonist, was injected
139 subcutaneously into the flank of 4-week-old mice in three escalating (30, 70, and 100
140 μg/kg) doses on three consecutive days. Control mice were treated with vehicle
141 solution (DMSO in PBS). Mice were rested for 6 weeks before behavioral test. Paw
142 withdrawal threshold and latency was tested to confirm the efficacy of chemical
143 ablation of TRPV1-positive sensory neurons.
144

145 **Ankle oedema**

146 Ankle oedema was measured with an engineer's micrometer (Moore and Wright,
147 Sheffield, England) with 0.1mm accuracy, before and after IA joint injection of
148 crystals. Data were presented as % increase of ankle thickness compared to the initial

149 pre-challenge values.

150 **Isolation of synovial macrophages from mice**

151 Synovial macrophages were isolated as described previously.[8] In brief, hind limbs
152 were dissected and placed in a 60-mm Petri dish containing 2 ml of DMEM. Under a
153 stereomicroscope, the muscle inside the popliteal fossa was cut open with
154 microsurgery scissors and forceps to harvest the synovium. Subsequently, the
155 synovium was transferred to another 60-mm Petri dish containing 2 ml of DMEM. To
156 harvest the intra-articular synovium, the articular cavity of the knee was cut open
157 along both sides of the patella under a stereomicroscope and the intra-articular
158 synovium was isolated carefully. The synovium was then transferred into a 1.5 ml
159 Eppendorf tube containing 0.5 ml DMEM and 0.5 ml 1% type IV collagenase and the
160 tissues were separated into 1-mm^3 blocks with microsurgery scissors. The Eppendorf
161 tube was incubated at 37°C in an orbital shaker incubator (200 rpm) for 60 min and
162 the sample was then vortexed vigorously for 1.5 min to release the cells. The sample
163 was centrifuged for 5 min at 300 g and resuspended with DMEM supplemented with
164 10% FBS and 1% penicillin-streptomycin. The cells were seeded on a 75 cm^2 flask
165 and placed in a humidified tissue culture incubator (37°C, 5% CO_2). Single-cell
166 suspensions were used for subsequent live-cell Ca^{2+} imaging, flow cytometry, and
167 qRT-PCR experiments.

168

169 **Preparations of mouse BMDMs, human THP-1 cells and human PBMCs**

170 BMDMs were isolated from the marrow of the femurs and tibias of 6-8 weeks-old

171 mice. The marrow was flushed out into cold PBS with 2% heat inactivated Fetal
172 Bovine Serum (FBS). The cell suspension was filtered through a nylon mesh filter (40
173 μm; Corning Cell Strainer, USA) into a sterile tube and then cells were centrifuged at
174 400 g for 5 min at 4 °C. The pellet was resuspended in red blood cell lysis buffer
175 (Roche, USA) and then centrifuged (400×g, 5min, 4°C). The cell pellet was
176 resuspended, and cells were cultured for 7 days in Iscove's Modified Dulbecco's
177 Medium (IMDM) supplemented with 10% FBS, 10 ng/mL M-CSF, 100 U/mL
178 penicillin and 100 mg/mL streptomycin.
179 Human THP-1 cells were grown in RPMI 1640 medium, supplemented with 10%
180 FBS and 50 mM 2-mercaptoethanol. THP-1 cells were differentiated for 3 hours in
181 the presence of 100 nM phorbol-12-myristate-13-acetate (PMA).[9]
182 The human PBMC studies were conducted with fully informed consent of all
183 participants and performed under the guidance of the Helsinki Declaration and
184 approved by the Institutional Review Board (IRB) at Renmin Hospital of Wuhan
185 University. Under the fasting state in the early morning of all enrolled patients and
186 healthy controls, 10 ml of fresh disposable venous blood was drawn and placed in a
187 sterile blood collection tube containing sodium citrate anticoagulant, and the
188 monocytes in the peripheral blood were separated by using a human lymphocyte
189 separation medium (catalog no. 17-829E, Lonza). PBMCs were cultured overnight
190 before stimulation in RPMI 1640 medium.[10]
191
192 **Activation of NLRP3 inflammasome**

193 To activate NLRP3 inflammasome, 5×10^5/mL BMDMs were plated in 96-well plates.
194 The cells were primed with 100 ng/ml LPS for 3 h and then treated with GSK 101
195 (300 nM, for 30 min), MSU (150 µg/mL, for 6 h), CPPD (100 µg/mL for 6 h), Alum
196 (200 µg/mL for 6 h), SiO_2 (100 µg/mL for 6 h), ATP (5 mM for 30 min), Nigericin (5
197 µM for 90 min), Gramicidin A (0.5 µM for 90 min) or Hypotonic solution (90 mOsm
198 for 40 min) for NLRP3 inflammasome activation. For pharmacological inhibition
199 studies, LPS-primed cells were treated with GSK219 for 30 min followed by
200 stimulation with various NLRP3 inflammasome activators. None of the reagents
201 induced cytotoxicity, which were confirmed by LDH assay (K311-400, BioVision).
202

203 ***In situ* hybridization**

204 In situ hybridization was performed using an RNAscope® Multiplex Fluorescent
205 Reagent Kit v2 (cat. no. 322300-USM; Advanced Cell Diagnostics) according to the
206 manufacturer's instructions. The RNA probes (Mm-Trpv1-C2, Cat No. 313338-C2;
207 Mm-Trpv4-C2, Cat No. 406071-C2) were complementary to the target mRNAs. In
208 each experiment, we used Hs-ppib (human peptidylprolyl isomerase B) and Dapb
209 (bacterial dihydrodipicolinate reductase) probes as positive and negative controls,
210 respectively. Fixed frozen samples (L4-L6 DRGs) were sliced with a microtome
211 (Leica 2000, Germany) at 10 µm thickness. Samples were incubated with hydrogen
212 peroxide for 10 min at room temperature and then boiled with target retrieval reagent
213 for 5 min. Slides were incubated with protease III at 40 °C for 30 min. Hybridization
214 of the probes to the RNA targets was performed by incubation in the HybEZ Oven for

215 2 hr at 40°C. Then the slides were processed for standard signal amplification steps
216 and fluorophores staining steps.
217
218 **Flow cytometry and cell sorting**
219 The cells isolated from the articular cavity fluid from *Trpv4*^{eGFP} mice were first
220 stained with Zombie UV™ Fixable Viability Kit (Biolegend) according to the
221 manufacturer's instructions. Cells were then stained with a standard panel of
222 immunophenotyping antibodies, including anti-mouse CD11b (1:300; Biolegend),
223 anti-mouse Ly-6G (1:300; Biolegend), and anti-mouse CD45 (1:300; BD
224 PharMingen), for 30 minutes at room temperature. After staining, cells were washed
225 with PBS+2% FBS buffer, centrifuged at 400 g for 5 min at 4 °C, and resuspended
226 with PBS+2% FBS. Compensation was performed on a BD LSRFortessa ™ X-20
227 flow cytometer (BD Biosciences) at the beginning of each experiment. Data of
228 samples were analyzed using FlowJo software (V10, Tree Star). Cell sorting was
229 performed on a FACSAria II (BD Biosciences) cell sorter.
230
231 **Live-cell Ca^{2+} imaging**
232 Fura-2-based radiometric measurement of $[Ca^{2+}]_i$ was performed as described
233 previously.[2] Freshly isolated ankle-resident macrophage cells and PBMCs were
234 loaded with 4 μM Fura-2 AM (Life Technologies) in culture medium at 37°C for 60
235 min. Fluorescence at 340 and 380 nm excitation wavelengths was recorded on an
236 inverted Nikon Ti-E microscope equipped with 340, 360 and 380 nm excitation filter

237 wheels using NIS-Elements imaging software (Nikon Instruments Inc., Melville, NY,
238 USA). Fura-2 ratios (F340/F380) were used to reflect changes in $[Ca^{2+}]_i$ levels upon
239 stimulation. Values were obtained from 100–250 cells in time-lapse images from each
240 coverslip. Threshold of activation was defined as 3 standard deviations above the
241 average (~20% above the baseline).
242

243 **Whole-cell patch-clamp recording**

244 Whole-cell patch-clamp recordings were performed with either an EPC 10 amplifier
245 (HEKA Elektronik, Lambrecht/Pfalz, Germany) or multiclamp 700B amplifier
246 (Molecular Devices, Sunnyvale, Calif) at room temperature (22°C to 24°C) on the
247 stage of an inverted phase-contrast microscope equipped with a filter set for green
248 fluorescent protein (GFP) visualization. Data were acquired with PatchMaster (HEKA
249 Elektronik, Lambrecht/Pfalz, Germany) or Clampex 10 (Molecular Devices,
250 Sunnyvale, Calif). Currents were filtered at 2 kHz and digitized at 10 kHz. Data were
251 analyzed and plotted with Clampfit 10 (Molecular Devices).
252

253 **Immunofluorescence**

254 Immunofluorescent staining was performed as previously described.[38] Tissues were
255 incubated with the primary antibodies (Table E2) diluted in blocking solution
256 overnight at 4°C. After washing with PBS for 3 times, the secondary antibodies
257 (including Donkey anti-rat Cy3, Donkey anti-rabbit Cy3, Donkey anti-mouse Cy3,
258 and Alexa Fluor 488 Donkey anti-chicken IgG) were incubated with tissues for 30

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259 min at room temperature. All preparations were examined with Nikon C1 Confocal
260 Laser Microscope System equipped with NIS-Elements software.
261
262 **Real-time RT-PCR**
263 Total RNA was extracted from macrophages in synovial cavity of mice or PBMCs
264 from human blood using Trizol reagent (Invitrogen) according to the manufacturer's
265 instructions. The cDNA was synthesized *in vitro* using ThermoScript® RT-PCR
266 System kit (Invitrogen). The sequences of the primers were summarized in Table E2.
267 Reactions were carried out in a volume of 20 μl per reaction containing 10 μl SYBR
268 Green master mix (2 ×) (Applied Biosystems), 0.5 μl cDNA, 5 μl 0.4 μM primer mix,
269 and 4.5 μl water using Step One Plus real-time PCR system (Applied Biosystems,
270 Foster City, CA, USA) with the following PCR protocol: 95°C for 3 min, 40 cycles of
271 30 s at 95°C, 30 s at 52°C and 60 s at 72°C. The generated Ct (cycle threshold) value
272 of human TRPV4 was normalized to its respected C_t value of GAPDH (ΔC_t). The
273 value of ΔC_t for GA group was further normalized to that for control group to yield
274 the $\Delta\Delta C_t$. The data were expressed as $2^{-\Delta\Delta Ct}$.
275
276 **Western blot assay.**
277 Cell lysates and culture supernatants were denatured in 2×Laemmli buffer (150 mM
278 Tris–HCl pH 6.8, 20% glycerol, 10% SDS, 10% dithiothreitol, 4% β-mercaptoethanol
279 and bromophenol blue). The protein samples were subsequently boiled at 95 °C for
280 10 min and separated by SDS-PAGE. Separated proteins were transferred to PVDF

281 membranes. The immunoblots were incubated overnight with primary antibodies and
282 anti-rat secondary antibody was used to detect proteins by enhanced
283 chemiluminescence (GE Healthcare, RPN2236).
284

285 **ELISA**

286 The concentrations of TNF-α and IL-1β in supernatant samples from cultured
287 BMDMs were detected by ELISA assay according to the manufacturer's instructions
288 (R&D Systems, USA). The plates were read at 450 nm and 570 nm using a microplate
289 reader (Synergy H1 Hybrid Reader, BioTek, USA). The presence of neutrophils in the
290 synovial lavage fluid was evaluated through the measurement of MPO activity. The
291 MPO activity were detected by ELISA according to the manufacturer's instructions
292 (R&D Systems, USA).
293

294 **Histology**

295 Formalin-fixed, EDTA-decalcified, paraffin-embedded mouse tissue specimens were
296 sectioned and stained with haematoxylin and eosin. The stained joint sections were
297 semi-quantitatively and blindly evaluated for the synovial inflammation, as described
298 previously.[11]
299

300 **Reagents**

301 All reagents were listed in Table E2. The MSU crystals were prepared as previously
302 described.[10]

303 **Quantification and Statistical Analysis**

304 All data are presented as mean ± SEM for n independent observations. Student's *t*-test
305 was used to analyze statistical significance between two groups. ANOVA and repeated
306 measures tests were used to test hypotheses about effects in multiple groups. $p<0.05$
307 was considered significantly different.

308

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339 **ONLINE SUPPLEMENTAL FIGURES**
340
341 **Online supplemental figure 1. Acute administration of GSK219 suppresses MSU**
342 **crystal-induced inflammation and reflexive pain-related responses. (a, b)**
343 GSK219 (5 mg/kg, i.p.) was applied 5 hr after IA injections of MSU crystals
344 (0.8 mg/site). Both reflexive mechanical (**a**) and thermal (**b**) pain-related responses
345 were evaluated at various time points (0, 3, 6, 9, 12, 15 and 24 hours) after injections
346 of MSU crystals. **(c)** The diameter of the tibio-tarsal joint was measured using a
347 digital caliper, and the swelling rate was evaluated at the same time points after
348 injections of the MSU crystals, n=9-10 per group. **(d, e)** Leucocyte infiltration (**d**) and
349 MPO activity (**e**) were evaluated 6 hr after IA injections of MSU crystals (0.8 mg/site).
350 n=8 for all groups. Statistical significance was determined using two-way ANOVA
351 followed by Bonferroni's post-hoc test (**a**-**c**), and Student's *t* test (**d**, **e**), **P<.05*,
352 ***P<.01*, ****P<.001*.
353
354 **Online supplemental figure 2. TRPV4 expression and function are absent from**
355 **DRG neurons and sensory nerve endings. (a)** TRPV4-eGFP signals were detected
356 in the skin resident cells but not nerve terminals in the skin although both structures
357 were in close proximity. **(b)** No TRPV4-eGFP signals were detected in DRG cell
358 bodies. TRPV1-immunioreactivity was used as positive control. n=3 independent
359 repeats with similar results for **a** and **b**. **(c-f)** Representative RNAscope *in situ*
360 hybridization (ISH) images show that the TRPV1 (**e**) but not TRPV4 mRNA
361 transcripts (**f**) were abundantly expressed in DRG neurons. DapB (**c**) and PPIB (**d**)

362 were negative and positive controls, respectively. n=6 independent repeats with
363 similar result. **(g, h)** Representative confocal images (**g**) and traces (**h**) show the
364 changes in gCaMP3 signals in a DRG explant from a *Pirt*^{GCaMP3} mouse in response to
365 GSK101 (3 μM), Cap (1μM), and KCl (50 mM). **(i)** Proportions of responsive
366 neurons in DRG explants treated with GSK101, Cap, and KCl. n=6 independent
367 repeats.

368

369 **Online supplemental figure 3. Upregulation of TRPV4 expression in the synovial**
370 **MΦs of MSU crystal-treated mice and PBMCs from patients with acute gout**
371 **flares.** **(a)** Schematic diagram of gating strategy for purifying
372 TRPV4-eGFP^{+}CD45^{+}CD11b^{+}F4/80^{+} synovial MΦs by FACS. Abbreviations: sym,
373 synovial membrane; c, cartilage. **(b)** Representative double labeling images showing
374 co-localization of TRPV4-eGFP with CD68 (left), Cx3cr1 (middle) and F4/80 (right)
375 in synovial MΦ isolated from the *Trpv4*^{eGFP} mice. Scale bar=50 μm. **(c)**
376 Representative double labeling images showing that the number of F4/80^{+}/eGFP^{+}
377 MΦs was markedly increased in the ankle sections from the *Trpv4*^{eGFP} mice subjected
378 to IA injections of MSU crystals (bottom) compared with vehicle (PBS) (top).
379 Magnified images on the right were taken from the framed area in the images on the
380 left. Abbreviations: b, bone marrow; c, joint cavity; s, synovial lining. Bar=50 μm,
381 n=3 independent repeats with similar result. **(d)** Bar charts illustrate the levels of
382 mRNA transcripts for TRPV4, TRPV1, TRPA1 and TRPM8 in patients with acute
383 gout flares (n=13) and healthy control subjects (n=8). **(e)** Representative traces of 100

384 nM GSK101-elicited $[Ca^{2+}]_i$ responses in PBMCs isolated from healthy controls and
385 patients with acute gout flares. **(f)** Quantification of GSK101-elicited $[Ca^{2+}]_i$
386 responses in PBMCs isolated from healthy controls and patients with acute gout flares.
387 Data were collected and averaged from 5 independent repeats and n=568 cells from
388 the healthy control group, n=523 cells from the gout patient group). Statistical
389 significance was determined using Student's *t* test (**d and f**), **P<.05*, ***P<.01*,
390 ****P<.001*.

391

392 **Online supplemental figure 4. MΦ-specific *Trpv4 cKOs* display reduced ankle**
393 **swelling and reflexive pain-related responses in MSU crystal-induced gouty**
394 **arthritis 4 weeks after tamoxifen induction of Cre expression**. **(a-c)** Time courses
395 for reflexive mechanical (**a**) and thermal (**b**) pain-related responses as well as joint
396 swelling (**c**) induced by IA injections of MSU crystals (0.8 mg/site) in both Cre^+ and
397 Cre^- $Cx3cr1^{CreERT}$; $Trpv4^{f/f}$ mice. Experiments were performed 4 weeks after the last
398 tamoxifen administration. Tamoxifen was applied by intraperitoneal administration
399 for 5 consecutive days at 100 mg/kg in 0.2 mL of corn oil. Statistical significance was
400 determined using two-way ANOVA followed by Bonferroni's post-hoc test. **P<.05*,
401 ***P<.01*, ****P<.001*. n=8-11 for each group.

402

403 **Online supplemental figure 5. Neutrophil-, articular chondrocytes- and sensory**
404 **nociceptor- expressed TRPV4 does not contribute to MSU crystal-induced gouty**

405 **pain and inflammation**. **(a)** Representative double labeling images showing
406 colocalization of TRPV4-eGFP with Ly6G-immunoreactivity in ankle sections from
407 the *Trpv4*^{eGFP} mice treated with either vehicle (PBS) (top) or MSU crystals (bottom).
408 Magnified images on the right were taken from the framed area in the images on the
409 left. Bar=50 μm, n=3 independent repeats with similar result. b, bone marrow; c,
410 articular cavity; s, synovial lining. **(b)** Representative flow cytometry analysis
411 showing the proportion of the TRPV4-eGFP^{+}/Ly6G^{+} cells in response to vehicle or
412 MSU crystals after gating by CD45^{+}/CD11b^{+}. **(c)** Quantification of the number and
413 the percentage of TRPV4-eGFP^{+}/Ly6G^{+} synovial neutrophils in response to vehicle or
414 MSU crystals. n=5 per group. **(d-f)** Reflexive mechanical (**d**) and thermal (**e**)
415 pain-related responses and joint inflammation (**f**) in the *Cre*$^{+}$ neutrophil-specific
416 *Trpv4 cKO*s (*S100A8*^{Cre}*; Trpv4*$^{f/f}$) and *Cre*$^{-}$ littermates. **(g-i)** Reflexive mechanical (**g**)
417 and thermal (**h**) pain-related responses and joint inflammation (**i**) in the *Cre*$^{+}$ cartilage
418 chondrocyte-specific *Trpv4 cKOs* (*Col2A*^{CreERT}*; Trpv4*$^{f/f}$) and *Cre*$^{-}$ littermates. **(j-l)**
419 Reflexive mechanical (**j**) and thermal (**k**) pain-related responses and joint
420 inflammation (**l**) in the *Cre*$^{+}$ nociceptor-specific *Trpv4 cKOs* (*Trpv1*^{Cre}*; Trpv4*$^{f/f}$) and
421 *Cre*$^{-}$ littermates. n=6-7 for each group. Statistical significance was determined using
422 Student's *t* test (**c**) and two-way ANOVA followed by Bonferroni's post-hoc test (**d-l**),
423 ****P<.001*.
424

425 **Online supplemental figure 6. TRPV4-dependent NLRP3 inflammasome**
426 **activation in mouse BMDMs, human THP-1 cells and PBMCs induced by MSU**

427 **crystals.** **(a)** Representative Western blot analysis of cleaved IL-1β, caspase-1 in
428 culture supernatants (SN) and pro-IL-1β, pro-caspase-1 in lysates (Input) in
429 LPS-primed BMDMs cells isolated from *Trpv4*$^{+/+}$ or *Trpv4*$^{-/-}$ mice treated with MSU
430 (200 µg/mL, for 6 h) or GSK101 (10, 100 or 300 nM, for 30 min). **(b)** Representative
431 western blot analysis of cleaved IL-1β, activated caspase-1 in culture supernatants
432 (SN) and pro-IL-1β, pro-caspase-1 in lysates (Input) from PMA-differentiated THP-1
433 cells treated with various doses of GSK219 (0.5, 1.0 or 5.0 μM) followed by MSU
434 crystals (200 µg/mL) stimulation. **(c)** Representative western blot analysis of cleaved
435 IL-1β, activated caspase-1 in culture supernatants (SN) and pro-IL-1β, pro-caspase-1
436 in lysates (Input) from PBMCs treated with various doses of GSK219 (0.5, 1.0 or 5.0
437 μM) followed by MSU crystals (200 µg/mL) stimulation.

438

439 **Online supplemental figure 7. TNF-α production in LPS-primed BMDMs is**
440 **TRPV4-independent**. **(a)** TNF-α production from the LPS-primed *wt* BMDMs in the
441 presence of escalating concentrations of GSK101. **(b)** MSU crystal-induced TNF-α
442 production from the LPS-primed *wt* BMDMs in the presence of various escalating
443 concentrations of GSK219. **(c)** TNF-α production induced by LPS, MSU crystals, and
444 LPS+MSU crystals in the BMDMs from *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. **(d)** TNF-α
445 production induced by LPS, MSU crystals, and LPS+MSU crystals in BMDMs from
446 the *Cre*$^{+}$ and *Cre*$^{-}$ *Cx3cr1*^{CreER}*; Trpv4*$^{f/f}$ mice. Statistical significance was determined
447 using one-way ANOVA followed by Tukey post hoc tests (**a** and **b**) and Student's *t*
448 test (**c** and **d**). n.s., not significant versus control group. n=6 per group for **a-d**.

449

450 **Online supplemental figure 8. TRPV4 is required for MΦ phagocytosis.**

451 **(a)** Representative immunofluorescent image showing phagocytosis (10 min) of
452 pHrodo™ Red *E. coli* bioparticles® by *wt* BMDMs in the presence of vehicle (0.5%
453 DMSO) (**ii**), GSK219 (1 μM) (**iii**) or cytochalasin D (10 μM) (**iv**). **(b)** Representative
454 immunofluorescent showed phagocytosis (10 min) of pHrodo™ Red *E. coli*
455 bioparticles® by BMDMs isolated from the *Trpv4*$^{+/+}$ (**ii**) and *Trpv4*$^{-/-}$ (**iii**) mice.
456 Representative images in **a i** and **b i** are taken from the control experiments without
457 treatment of bioparticles. Bar=5 μm.

458

459 **Online supplemental figure 9. TRPV4 is not involved in NLRP3 inflammasome**
460 **activation induced by the classic NLRP3 inflammasome activators ATP, nigericin**
461 **and gramicidin. (a-c)** Pharmacological inhibition with GSK219 (1 μM) and genetic
462 ablation of TRPV4 function had no effect on IL-1β production from LPS-primed
463 BMDMs stimulated with ATP (5 mM) (**a**), nigericin (5 μM) (**b**) or Gramicidin A (0.5
464 μM) (**c**). Statistical significance was determined using Student's *t* test. n.s., not
465 significant versus control group. n=6 for all groups.

466

467 **Online supplemental figure 10. TRPV1-positive primary nociceptors are required**
468 **for MSU crystal-induced joint inflammation and reflexive pain-related responses**.
469 **(a-d)** Effects of resinifertoxin (RTX) treatment on reflexive mechanical (**a**) and thermal
470 (**b**) pain-related responses, joint swelling (**c**), and IL-1β release (**d**) at 6 h post IA

471 injections of MSU crystals (0.8 mg/site), n=7 per group. **(e)** Representative
472 immunofluorescent images revealed F4/80-positive (red, macrophages) and
473 Ly6G-positive (green, neutrophils) innate immune cells in ankle sections from mice
474 treated with either vehicle (PBS) (top) or RTX (bottom). Bar=100 µm, n=5 independent
475 repeats with similar result. b, bone marrow; c, articular cavity; s, synovial lining. **(f)**
476 Representative flow cytometry analysis showing the proportion of the Ly6G$^+$ cells (left)
477 and F4/80$^+$ (right) in response to vehicle or RTX after CD45$^+$/CD11b$^+$ gating. n=5 per
478 group. **(g)** Myeloperoxidase activity at 6 h after IA injections of MSU crystals (0.8
479 mg/site) in response to vehicle or RTX, n=7 per group. **(h)** TRPV4 mRNA expression
480 in the synovial cavity of mice treated with vehicle or RTX. n = 5 per group. Data were
481 presented as mean ±S.E.M. One-way ANOVA followed by Bonferroni post-hoc test
482 (a-d). Student *t* Test, (f-h). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

483

484 **Online supplemental figure 11. TRPV4 is required for hypotonicity-induced**
485 **IL-1β production in LPS-primed BMDMs.** Pharmacological inhibition with
486 GSK219 (1 µM) and genetic ablation of TRPV4 function using both global and
487 MΦ-specific *Trpv4 cKOs* significantly reduced IL-1β production in LPS-primed
488 BMDMs stimulated with a hypotonic solution (90 mOsm) for 40 min. The hypotonic
489 solution was made by diluting isotonic physiological buffer with distilled sterile water.
490 Statistical significance was determined using Student's *t* test. $^{***}P<.001$. n=6 for all
491 groups.

492

493 **TABLES**

494 **Table E1. Characteristics of patients with Gouty arthritis (GA) and healthy**
495 **subjects**

Characteristics	**GA** (n=13)	**Healthy subjects** (n=8)
Age (years)	42.2 ± 10.4	42.1±9.8
Gender (male/female)	13/0	8/0
Serum uric (µmol/L)	538.1 (438.3-647.3)	316.2 (268.3-390.2)
Disease duration (years)	3.7 (0-10)	-

Data are presented as Mean ± SD (for normally distributed variables) or median with interquartile range (IQR) (for non-normally distributed variables).

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509 **Table E2. Reagents and products used in this study**
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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD45, eVolve605	eBioscience	Cat# 83045142; RRID: AB_2574712
Anti-mouse CD45, PE-Cy7	BD PharMingen	Cat# 552848; RRID: AB_394489
Anti-mouse CD11b, BV51	BioLegend	Cat# 101245; RRID: AB_2561390
Anti-mouse CD11b, APC	eBioscience	Cat# 17011282; RRID: AB_469343
Anti-mouse CD206, APC	BioLegend	Cat# 141707; RRID: AB_10896057
Anti-mouse CD64, PE-Cy7	BioLegend	Cat# 139313; RRID: AB_2563903
Anti-mouse MHC II, BV421	BioLegend	Cat# 107631; RRID: AB_10900075
Anti-mouse MHC II, Alexa 700	eBioscience	Cat# 5016917; RRID: AB_494009
Anti-mouse Ly6C, APC-Cy7	BioLegend	Cat# 128025; RRID: AB_10643867
Anti-mouse F4/80, PE-TexasRed	Invitrogen	Cat# MF48017; RRID: AB_10372055
Anti-mouse CX3CR1, PE	BioLegend	Cat# 149005; RRID: AB_2564314
Anti-mouse CD206, Purified	AbD Serotec	Cat# MCA2235; RRID: AB_324622
Anti-mouse F4/80, Purified	BioLegend	Cat# 123101; RRID: AB_893504
Anti-mouse PGP9.5 Purified	UltraClone	Cat# RA95101; RRID: AB_2313685
Anti-mouse GFP, Purified	Aves Labs Inc	Cat# GFP-1020; RRID: AB_10000240
PerCP-Cyanine5.5 anti-Mo CD11b	Invitrogen	Cat# 45-0112-82; RRID: AB_953558
Brilliant Violet 605 anti-mouse Ly-6G	Biolegend	Cat# 127639; RRID: AB_2565880
APC anti-mouse CD45	Biolegend	Cat# 103111; RRID: AB_312976

Anti-rat IgG, Cy3	Jackson ImmunoResearch	Cat# 712165153; RRID: AB_2340667
Anti-chicken IgY, Alexa 488	Jackson ImmunoResearch	Cat# 703545155; RRID: AB_2340375
Anti-rabbit IgG, Cy3	Jackson ImmunoResearch	Cat# 711165152; RRID: AB_2307443
Anti-mouse Caspase 1	Adipogen	Cat# AG-20B-0042
Anti-mouse IL-1β	R&D (Fisher)	Cat# AF-401-SP
Anti-mouse β-actin	Abcan	Cat# P30002
anti-rabbit secondary antibody	Amersham Pharmacia	Cat# NA934V
Anti-mouse IgG, Cy3	Jackson ImmunoResearch	Cat# 715165151; RRID: AB_2315777
Chemicals, Peptides and Recombinant Proteins		
GSK1016790A	Sigma	Cat# G0798; RRID: IMSR_JAX: 000664
GSK2193874	Sigma	Cat# SML0942
HC067047	Sigma	Cat# SML0143
Tamoxifen	Sigma	Cat# T5648
Adenosine 5'-triphosphate	Fisher Scientific	Cat# BP413
Lipopolysaccharide	Sigma	Cat# L2637
MSU crystals	Custom made	N/A
N-acetyl-L-cysteine	Sigma	Cat# 616-91-1
Glutathione Monoethyl Ester	Merk	Cat# 118421-50-4
Ac-YVAD-AMC	Bachem	Cat# I-1630
SiO_2	US SILICA	Cat# 06122818
Alum	Fisher Scientific	Cat# 77161
Gramicidin A	Santa Cruz Biotech, Inc	Cat# sc-203061A
Nigericin. sodium salt	Adipogen	Cat# G-CN2-0020-M005
BAPTA-AM	Sigma	Cat# 126150-97-8
Cytochalasin D	Thermo Fisher Scientific	Cat# PHZ1063
Ca074Me	Sigma	Cat# 147859-80-1
CPPD	InvivoGen	Cat# tlrl-cppd
Uric acid	Sigma	Cat# U2625
FITC–Dextran	Sigma	Cat# 46945
Uricase	Sigma	Cat# U0880
Clostridium histolyticum null	Sigma	Cat# C5138
DNase	Sigma	Cat# 9003-98-9
M-CSF	PeproTech	Cat# 315-02
Resiniferatoxin	Sigma-Aldrich	Cat# 138977-28-3
Red blood cell lysis buffer	Roche	Cat# 11814389001
Bovine serum albumin	Sigma	Cat# B4287
Critical Commercial Assays		
TNF-a ELISA Kit	R&D System	Cat# MTA00B

IL-1β ELISA Kit	R&D System	Cat# MLB00C
MPO ELISA Kit	R&D System	Cat# DY3667
RNeasy Mini Kit	QIAGEN	Cat# 74104
ThermoScript RT-PCR First-Strand cDNA Synthesis	Life Technology	Cat# 11146024
FastStart Universal SYBR Green mix	Roche	Cat# 04913850001
Zombie UV™ Fixable Viability Kit	Biolegend	Cat# 423108 (77474)
Experimental Models: Organisms/strains		
Mouse: *c57bl/6j*	The Jackson Laboratory	00664
Mouse: *Trpv4^{eGFP}*	MMRRC	032771-UCD
Mouse: *Trpv4$^{-/-}$*	Suzuki et al., 2003	N/A
Mouse: *Cx3cr1^{CreER}*	The Jackson Laboratory	021160
Mouse: *Col2A^{CreER}*	The Jackson Laboratory	006774
Mouse: *Trpv1^{Cre}*	From Dr. Qin Liu	N/A
Mouse: *S100A8^{Cre}*	The Jackson Laboratory	021614
Mouse: *Trpv4$^{f/f}$*	Luo et al., 2018	N/A
Mouse: *Pirt^{GCaMP3}*	From Dr. Qin Liu	N/A
Mouse: *Nlrp3$^{-/-}$*	SMOC	N/A
Mouse: *Caspase-1$^{-/-}$*	SMOC	N/A
Mouse: *Trpv1^{Cre}; Trpv4$^{f/f}$*	This paper	N/A
Mouse: *Col2A^{CreER}; Trpv4$^{f/f}$*	This paper	N/A
Mouse: *S100A8^{Cre}; Trpv4$^{f/f}$*	This paper	N/A
Mouse: *Cx3cr1^{CreER}; Trpv4$^{f/f}$*	This paper	N/A
Oligonucleotides		
Primer: mTRPV4 Forward: CCTGCTGGTCACCTACATCA	This paper	N/A
Primer: mTRPV4 Reverse: CTCAGGAACACAGGGAAGGA	This paper	N/A
Primer: mTRPV4 Forward: TGCTTCTCAAGTGTTCACGCCTCTT	This paper	N/A
Primer: mTRPV4 Reverse: ATGTGCTGAAAGACCCCGATCTT	This paper	N/A
Primer: mGAPDH Forward: GCACAGTCAAGGCCGAGAAT	This paper	N/A
Primer: mGAPDH Reverse: GCCTTCTCCATGGTGGTGAA	This paper	N/A
Primer: hGAPDH Forward: ACGAATTTGGCTACAGCAACAG	This paper	N/A
Primer: hGAPDH Reverse:	This paper	N/A

GTCTACATGGCAACTGTGAGGA		
Primer: hTRPV4 Forward: ACAACAGCAAGATTGAGAACCG	This paper	N/A
Primer: hTRPV4 Reverse: TAGTAGGCGGTGAGAGTGAAGA	This paper	N/A
Primer: hTRPV1 Forward: AGATCGGGGTCTTGGCCTAT	This paper	N/A
Primer: hTRPV1 Reverse: AGTTCTTCTCGCAGGTGTCG	This paper	N/A
Primer: hTRPM8 Forward: CTCAGCATGAGGAACAGAAGGA	This paper	N/A
Primer: hTRPM8 Reverse: TTCACCAAGTCGCTTTCACTGT	This paper	N/A
Primer: hTRPA1 Forward: TTTCTCAGTGACCACAATGGCT	This paper	N/A
Primer: hTRPA1 Reverse: TTGTGAAGTGCAAGGTGCAAAA	This paper	N/A
Software and Algorithms		
Flow Jo	Tree Star	Version 10
GraphPad Prism	GraphPad Software	Version 6

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