

Title: KCNN4 links PIEZO-dependent mechanotransduction to NLRP3 inflammasome activation

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Abstract: Immune cells sense the microenvironment to fine-tune their inflammatory responses. Patients with cryopyrin associated periodic syndrome (CAPS), caused by mutations in the *NLRP3* gene, develop auto-inflammation triggered by non-antigenic, e.g. environmental cues. However, the underlying mechanisms are poorly understood. Here, we uncover that KCNN4, a calcium-activated potassium channel, links PIEZO-mediated mechanotransduction to NLRP3 inflammasome activation. Yoda1, a PIEZO1 agonist, lowers the threshold for NLRP3 inflammasome activation. PIEZO-mediated sensing of stiffness and shear stress increases NLRP3-dependent inflammation. Myeloid-specific deletion of *PIEZO1/2* protects mice from gouty arthritis. Activation of PIEZO1 triggers calcium influx, which activates KCNN4 to evoke potassium efflux promoting NLRP3 inflammasome activation. Activation of PIEZO signaling is sufficient to activate the inflammasome in cells expressing CAPS-causing NLRP3 mutants via KCNN4. Finally, pharmacologic inhibition of KCNN4 alleviates auto-inflammation in CAPS patient cells and in CAPS-mimicking mice. Thus, PIEZO-dependent mechanical inputs augment inflammation in NLRP3-dependent diseases including CAPS.

37 **One Sentence Summary:** PIEZO-mediated mechanotransduction stimulates KCNN4-dependent
38 potassium efflux to potentiate NLRP3 inflammasome activation.

39

40 INTRODUCTION

41 Immune cells primarily respond to pathogen exposure and tissue injury using pattern recognition
42 receptors (PRRs) to elicit an inflammatory response (1, 2). However, it is increasingly recognized
43 that other parameters in the microenvironment alter during inflammation and that immune cells
44 are capable to adapt their responses to those variations. More recently, specific changes in immune
45 cell activation and effector function in response to mechanical cues and forces have been put
46 forward (3). The process of mechanotransduction governs conversion of mechanical inputs into
47 biochemical or electrical signals activating signaling pathways that ultimately affect cellular
48 function.

49 Macrophages are particularly sensitive to changes in tissue stiffness or tension as they are adherent
50 and contact-dependent. Canonical macrophage functions such as phagocytosis (4, 5), generation
51 of reactive oxygen species (ROS) (6), and secretion of cytokines (4, 7–9) have been demonstrated
52 to change in response to sensing of substrate rigidity. On the other hand, blood monocytes can
53 respond to high shear stress promoting adhesion, phagocytosis and cytokine secretion (10).

54 Macrophages and monocytes, as other cells, employ conserved mechanisms by which they can
55 sense and respond to multiple types of mechanical forces or to surrounding physical properties of
56 different tissues. Sensing through mechano-gated ion channels recently gained a lot of attention.
57 Among them, PIEZO1 and TRPV4 have been mostly characterized in immune cells. PIEZO1 is a
58 member of the PIEZO family of mechanically activated cation channels (11), while TRPV4
59 represents a member of the TRP superfamily of non-selective cation channels (12). In response to
60 mechanical inputs, physical distortion of the plasma membrane directly leads to opening of these
61 channels, triggering ion influx, including Ca^{2+} , to elicit signal transduction.

62 PIEZO1 was shown to co-localize with Toll-like receptor 4 (TLR4) to promote ROS production,
63 thereby enhancing pathogen engulfment and killing upon macrophage activation (6). Moreover, a
64 pro-inflammatory macrophage phenotype was shown to be promoted by stiff substrates through
65 PIEZO1-dependent Ca^{2+} influx and NF- κ B activation (7). PIEZO1 is also activated by cyclical
66 hydrostatic pressure resulting in endothelin 1 (EDN1)-dependent hypoxia-inducible factor 1 α
67 (HIF1 α) stabilization ensuring efficient clearance of intranasal *Pseudomonas aeruginosa* (13). In
68 analogy, the role of TRPV4 has been predominantly investigated in macrophages (4, 14, 15).

69 The inflammasome is a cellular complex that provides a first line of response against pathogens
70 and sterile insults. In most cases, the inflammasome complex consists of a sensing receptor, the
71 adaptor ASC and the effector proteinase Caspase-1. Upon activation, its assembly leads to self-
72 activation of Caspase-1, which cleaves pro-IL-1 β and pro-IL-18 into their mature forms promoting
73 their secretion and also cleaves Gasdermin D triggering pro-inflammatory lytic cell death, called
74 pyroptosis (16–19). NLRP3 inflammasome activation occurs in two principal steps, (1) priming
75 through Toll-like or cytokine receptor signaling resulting in robust expression of the
76 inflammasome components and (2) activation leading to inflammasome complex assembly, which
77 can be triggered by various stimuli, including a remarkable variety of environmental cues (16–19).
78 Gain of function mutations in the *NLRP3* gene lead to the development of Cryopyrin- associated
79 periodic syndromes (CAPS), auto-inflammatory conditions that often occur at joints and eyes,

80 highly mechanical sites, and that can be triggered via cold exposure (20). However, whether
81 mechanical inputs are directly linked to NLRP3 inflammasome activation has not been
82 systematically addressed.

83 In light of a potential role of mechanotransduction in NLRP3-dependent inflammation, we set out
84 to explore whether and how mechanical stress can impact NLRP3 inflammasome activation. We
85 find that activation of PIEZO signaling, either through treatment with agonists, via enhancing of
86 substrate stiffness or via inducing of shear stress, amplifies NLRP3 inflammasome activation.
87 Mechanistically, we provide evidence for PIEZO1 activation to evoke calcium influx to induce
88 KCNN4-dependent potassium efflux, thereby potentiating NLRP3 inflammasome activation. In
89 search for disease relevance of our findings, we also demonstrate this mechanism to contribute to
90 NLRP3-dependent inflammation such as monosodium urate-induced arthritis and CAPS. Hence,
91 our results may pave the way for so far unanticipated therapeutic strategies with the goal to
92 alleviate inflammation in these inflammatory conditions.

93

94 RESULTS

95

96 **Activation of PIEZO1 by its agonists potentiates NLRP3 inflammasome activation**

97 To address whether PIEZO-mediated mechanotransduction affects NLRP3 inflammasome
98 activation, we first used Yoda1, a PIEZO1 agonist (21), and THP-1 cells, a human acute leukemia
99 monocytic cell line widely used for assessing NLRP3 inflammasome activation. THP-1 cells in
100 suspension were primed with LPS and exposed to nigericin to activate the NLRP3 inflammasome
101 in absence or presence of Yoda1. Yoda1 significantly enhanced susceptibility of THP-1 cells to
102 nigericin-induced inflammasome activation as evidenced by increased amounts of cleaved and
103 secreted IL-1 β and Caspase-1 in supernatants measured by ELISA and/or Western blotting (Fig.
104 1A and 1B) and enhanced pyroptosis determined by Sytox Green uptake (Fig. 1C). Nigericin
105 induces NLRP3 inflammasome activation in a potassium efflux-dependent manner (22), we thus
106 wondered if it was the same with potassium efflux-independent activators, imiquimod R837 and
107 its derivative CL097 (23). Yoda1 also dramatically lowered the threshold of inflammasome
108 activation in response to R837 or its derivative CL097 (fig. S1A-S1F). No induction of pyroptosis
109 was observed in *NLRP3* knockout (KO) THP-1 cells (Fig. 1C and fig. S1C and S1F) confirming
110 that Yoda1-induced potentiation of inflammasome activation was NLRP3-dependent. The release
111 of cleaved IL-1 β and Caspase-1 in response to LPS plus nigericin, R837 or CL097 were also
112 markedly enhanced in Yoda1-stimulated murine bone marrow-derived macrophages (BMDMs) as
113 compared to control-treated cells (fig. S1G-S1L). Nigericin-induced pyroptosis was also
114 significantly amplified by Yoda1 in human peripheral blood mononuclear cells (PBMCs) (Fig.
115 1D). The PIEZO1 agonists Jedi1 and Jedi2 also enhanced R837-dependent NLRP3 inflammasome
116 activation, although to a lesser extent as compared to Yoda1 (fig. S1M). Both Jedi1 and Jedi2
117 have been shown to exert weaker and shorter effects on PIEZO1 activation as compared to Yoda1
118 (24). In line with a previous study (7), Yoda1 treatment also slightly enhanced the production and
119 release of TNF α (fig. S1G, S1I and S1K). Enhanced TNF production has been primarily linked to
120 PIEZO1-induced NF- κ B-dependent transcription of this cytokine (7). However, Yoda1 treatment
121 did not affect the expression of NLRP3, pro-Caspase-1, pro-IL1 β , ASC, NEK7 and Gasdermin D
122 (Fig. 1B). Formation of ASC specks is a hallmark of cells in which the NLRP3 inflammasome is
123 activated (25). LPS-primed BMDMs stimulated with nigericin (Fig. 1E and 1F), R837 (fig. S1N,

124 S1O) or CL097 (fig. S1N and S1O) showed significantly enhanced cell numbers containing ASC
125 specks when cotreated with Yoda1 as compared to cells without Yoda1 treatment. Yoda 1,
126 independent of NLRP3, had no potentiating but, if anything, rather an inhibitory effect on
127 activation of the Pyrin and AIM2 inflammasomes (fig. S2A and S2B).

128 To confirm that the effect of Yoda1 on NLRP3 inflammasome activation was PIEZO-dependent,
129 we next generated *PIEZO1* KO, *PIEZO2* KO and *PIEZO1/2* double knockout (dKO) THP-1 cells
130 using CRISPR/Cas9-mediated gene editing. Deletion of either gene in THP1 cells was confirmed
131 using Sanger sequencing (fig. S3A-S3C). Yoda1-induced potentiation of inflammasome activation
132 in response to nigericin (fig. S4A-S4C), R837 (fig. S4D-S4F) or CL097 (fig. S4G-S4I) was
133 abolished in *PIEZO1* KO and *PIEZO1/2* dKO cells but not in *PIEZO2* KO cells, confirming that
134 effects of Yoda1 occur through PIEZO1 activation. Neither *PIEZO1* nor *PIEZO2* was required to
135 activate the NLRP3 inflammasome in response to nigericin (fig. S4A-S4C), R837 (fig. S4D-S4F)
136 or CL097 (fig. S4G-S4I) in the absence of Yoda1. Re-expression of PIEZO1 in *PIEZO1* KO THP-
137 1 cells restored increased sensitivity to inflammasome activation in response to nigericin (Fig. 1G-
138 1I), R837 (fig. S5A-S5C) or CL097 (fig. S5D-S5F). To address whether enhancing expression of
139 PIEZO1 potentiates effects of Yoda1 stimulation, we next generated wild-type (WT) and *NLRP3*
140 KO THP-1 cells ectopically expressing PIEZO1. In WT cells ectopically expressing PIEZO1,
141 Yoda1 was sufficient to trigger cleavage and secretion of IL-1 β and Caspase-1 as well as
142 pyroptosis in LPS-primed cells, while Yoda1 didn't show such effects in *NLRP3* KO cells
143 ectopically expressing PIEZO1 (fig. S6A-S6C). Taken together, PIEZO1 activation constitutes a
144 potent mechanism to amplify NLRP3 inflammasome activation in macrophages.

145

146 **PIEZO-dependent mechanotransduction modulates NLRP3-dependent inflammation**

147 We next wondered whether physiologic PIEZO activation had an impact on NLRP3
148 inflammasome activation. To this end, we first subjected THP-1 cells to different cell culture
149 substrate rigidities. THP-1 cells cultured on stiff substrates secreted more IL-1 β than cells cultured
150 on soft substrates in response to nigericin and R837 (Fig. 2A). Deletion of both *PIEZO1* and
151 *PIEZO2* attenuated the secretion of IL-1 β triggered by nigericin and R837 in cells cultured on stiff
152 as well as on soft substrates (Fig. 2A). We next applied shear stress to cultured THP-1 cells.
153 Likewise, enhanced shear stress amplified IL-1 β release in a PIEZO-dependent manner (Fig. 2B).
154 To address whether PIEZO-dependent mechanotransduction was important in NLRP3-dependent
155 inflammation *in vivo*, we next used monosodium urate (MSU) crystal-induced acute arthritis in
156 mice. To this end, intra-articular injections of MSU crystals into ankles of back paws were
157 performed. Ankle swelling over time, and ankle temperature were quantified to evaluate the
158 severity of joint inflammation. To confirm dependence of this model on NLRP3 inflammasome
159 activation, experiments were performed in wild-type (*Nlrp3*^{+/+} mice) and *Nlrp3* knockout mice
160 (*Nlrp3*^{-/-} mice). As expected, ankle swelling, and ankle temperature were significantly attenuated
161 in *Nlrp3*^{-/-} mice as compared to *Nlrp3*^{+/+} mice (Fig. 2C and 2D). We then generated myeloid-
162 specific *Piezo1/2* double knockout mice (*Piezo1*^{fl/fl};*Piezo2*^{fl/fl};*LysM-Cre* mice) by crossing
163 *Piezo1/2* floxed mice (26, 27) with lysozyme M (*LysM*) promoter-dependent Cre recombinase
164 expressing mice (*LysM-Cre* mice) (28) and compared them with corresponding *Piezo1/2* floxed
165 control mice (*Piezo1*^{fl/fl};*Piezo2*^{fl/fl}). Strikingly, arthritis in mice lacking *Piezo1/2* in the myeloid
166 compartment was significantly attenuated as compared to floxed control mice (Fig. 2E and 2F).
167 These results thus indicate that PIEZO-dependent mechanical inputs are important to modulate
168 NLRP3-dependent inflammation in macrophages *in vitro* and *in vivo*.

169

170 **PIEZO-mediated Ca²⁺ influx triggers KCNN4-dependent K⁺ efflux to promote NLRP3**
171 **inflammasome activation**

172 Activation of PIEZO channels evoke cellular Ca²⁺ influx (11). We thus monitored cytosolic Ca²⁺
173 level using a genetically-encoded fluorescent Ca²⁺ indicator, jGCaMP7S (29), in WT and
174 *PIEZO1/2* dKO THP-1 cells treated with nigericin or R837 in absence or presence of Yoda1.
175 Ionomycin, a potent and selective Ca²⁺ ionophore, was used as a positive control (Fig. 3A). As
176 previously shown, Yoda1 treatment increased cytosolic Ca²⁺ levels in WT cells. The increase of
177 cytosolic Ca²⁺ levels was further enhanced in response to nigericin (Fig. 3A) or R837 (fig. S7A)
178 in presence of Yoda1. In contrast, cells lacking *PIEZO1* and *PIEZO2* did not show an obvious
179 increase in cytosolic Ca²⁺ in response to Yoda1 or Yoda1 plus nigericin (Fig. 3A) or R837 (fig.
180 S7A). We then asked whether potentiation of NLRP3 inflammasome activation by Yoda1 was
181 Ca²⁺ influx-dependent. To this end, we compared the effect of Yoda1 in THP-1 cells treated in
182 culture medium with or without Ca²⁺. Indeed, enhanced IL-1 β and Caspase-1 release as well as
183 pyroptosis in presence of Yoda1 in nigericin- (Fig. 3B-3D), R837- (fig. S7B-S7D) and CL097-
184 (fig. S7E-S7G) treated cells were significantly decreased under Ca²⁺-free medium conditions as
185 compared to cells treated in Ca²⁺-containing medium. Thus, Ca²⁺ influx contributes to PIEZO1-
186 mediated potentiation of NLRP3 inflammasome activation.

187 We next asked how PIEZO1-mediated Ca²⁺ influx amplifies NLRP3 inflammasome activation. To
188 address this question, we performed a genome-wide CRISPR/Cas9-mediated knockout screen in
189 THP-1 cells using the human CRISPR Brunello lentiviral pooled sgRNA library (30). R837 was
190 used to induce NLRP3 inflammasome activation to set up this screen. To obtain hits specifically
191 related to PIEZO1-dependent mechanisms of inflammasome activation, we have chosen a R837
192 concentration in which cotreatment with R837 and Yoda1 led to approximately 85% cell death,
193 while stimulation with R837 alone resulted in basic levels (10 to 15%) of cell death. R837 plus
194 Yoda1-induced cell death was applied twice to ensure maximal elimination of Yoda1-sensitive
195 cells. For the assessment of enrichment of sgRNAs, co-treated cells were compared to vehicle
196 control-treated cells (Fig. 4A). In this screen, NLRP3, ASC (PYCARD) and PIEZO1 were
197 identified as top hits as expected. In addition, ATP11A and CDC50A (TMEM30A), which were
198 reported to directly mediate PIEZO1 activation on membranes (31), were also identified.
199 Intriguingly, KCNN4, a Ca²⁺-activated potassium channel, which was reported to act downstream
200 of PIEZO1 in red blood cell (32), was the first-ranked hit. We further validated the involvement
201 of KCNN4 in this context using both genetic and pharmacological approaches. Deletion of *KCNN4*
202 using CRISPR/Cas9-mediated gene editing in THP-1 cells (fig. S8A) attenuated the enhancing
203 effect of Yoda1 on R837-induced pyroptosis, cleavage and secretion of IL-1 β and Caspase-1 (Fig.
204 4B-4D and fig. S8B-S8D). Re-expression of EGFP-tagged KCNN4 restored the enhancing effect
205 of Yoda1 on NLRP3 inflammasome activation in *KCNN4* KO cells (Fig. 4B-4D). Deletion of
206 *KCNN4* didn't affect NLRP3 inflammasome activation induced by R837 alone (Fig. 4B-4D)
207 indicating that KCNN4, as PIEZO proteins, is not required for NLRP3 inflammasome activation
208 in the absence of Yoda1. To further consolidate the role of potassium efflux mediating the effect
209 of Yoda1 on NLRP3 inflammasome activation, we blocked potassium efflux by increasing
210 extracellular potassium concentrations and assessed their effect on NLRP3 inflammasome
211 activation. In line with previous studies (22, 23, 33), increasing extracellular potassium
212 concentrations completely blocked nigericin-induced (Fig. 4E-4G), but not R837-induced (fig.
213 S9A and S9B) NLRP3 inflammasome activation. However, increasing extracellular potassium
214 concentrations completely blocked enhancing effects of Yoda1 on R837-induced NLRP3
215 inflammasome activation (Fig. 4E-4G and fig. S9C-S9G). Consistently, pharmacological

216 inhibition of KCNN4 by TRAM-34 (34) similarly inhibited pyroptosis, cleavage and secretion of
217 IL-1 β and Caspase-1 induced by R837 plus Yoda1 in THP-1 cells (fig. S9C-S9E). Similar results
218 were also obtained in BMDMs (fig. S9F-S9G). These results suggest that potassium efflux via
219 KCNN4 mediates the enhancing effect of PIEZO1 activation on NLRP3 inflammasome activation.

220

221 **PIEZO-KCNN4-dependent K⁺ efflux promotes NLRP3-dependent auto-inflammation in** 222 **CAPS cells**

223 In CAPS patients, auto-activation of NLRP3 inflammasome occurs in absence of infection or
224 tissue injury (20). However, inflammation frequently occurs at sites of mechanical impact and in
225 response to cold exposure (20). We thus asked whether PIEZO-dependent mechanotransduction
226 was important in triggering auto-activation of the NLRP3 inflammasome in CAPS. To this end,
227 we expressed three CAPS-causing NLRP3 (mouse) mutants, D301N, T346M and R258W, in
228 *NLRP3*-deficient THP-1 cells. *NLRP3*-deficient cells expressing WT NLRP3 or GFP and WT cells
229 expressing GFP were used as controls. Expression of NLRP3 and GFP was confirmed by Western
230 blotting (fig. S10A). Pyroptotic cell death was determined by propidium iodide incorporation. In
231 line with previous studies (35–38), cells expressing CAPS-causing NLRP3 mutants displayed
232 significantly enhanced pyroptosis in response to LPS priming alone as compared to control-treated
233 cells (fig. S10B). Importantly, Yoda1 stimulation, similar to LPS treatment, was sufficient to
234 induce pyroptosis in CAPS-causing NLRP3 mutant expressing cells (fig. S10B). Like for LPS
235 treatment, Yoda1 had only minor effects on induction of pyroptosis in control cells (fig. S10B).
236 To verify that Yoda1-induced pyroptosis in CAPS mutant expressing cells was PIEZO-dependent,
237 we next expressed GFP, WT NLRP3 and CAPS-causing NLRP3 mutants in WT, *PIEZO1* KO,
238 *PIEZO2* KO and *PIEZO1/2* dKO THP-1 cells. The expression of NLRP3 and GFP was confirmed
239 by Western blotting (fig. S10C). LPS-induced pyroptosis in cells expressing CAPS-causing
240 NLRP3 mutants was not dependent on PIEZO1 or PIEZO2 as the response was unaltered in
241 *PIEZO1* KO, *PIEZO2* KO and *PIEZO1/2* dKO cells (fig. S10D). However, Yoda1-induced
242 pyroptosis in cells expressing CAPS-causing NLRP3 mutants depended on PIEZO1 expression
243 (Fig. 5A). We further tested whether PIEZO1-dependent activity was KCNN4-dependent. Indeed,
244 inhibition of KCNN4 dramatically inhibited Yoda1-induced inflammasome activation in these
245 cells (Fig. 5B and 5C). To test whether mechanostimulation alone, in the absence of Yoda1, could
246 activate NLRP3 in cells expressing CAPS-causing NLRP3 mutants, we next subjected WT and
247 *PIEZO1/2* dKO THP-1 cells expressing GFP, WT NLRP3 and CAPS-causing NLRP3 mutants to
248 changes in substrate stiffness. Increasing substrate stiffness significantly enhanced IL-1 β release
249 in WT cells expressing GFP, WT NLRP3 and mutant NLRP3 expressing THP-1 cells, while this
250 response was significantly reduced in corresponding *PIEZO1/2* dKO cells (Fig. 5D). The increase
251 was more pronounced in mutant NLRP3 expressing cells indicating that CAPS mutations render
252 cells more susceptible to changes in substrate stiffness. To corroborate a role of PIEZO-KCNN4-
253 dependent NLRP3 inflammasome activation in cells endogenously expressing a CAPS-causing
254 NLRP3 mutant, we next used mice carrying an intronic floxed neomycin-resistance gene (NeoR
255 that prevents the expression of *Nlrp3*^{A350V}, a mutation genetically similar to the one observed in
256 human Muckle-Wells syndrome, a distinct subgroup of CAPS (36). These mice were crossed with
257 *LysM* promoter-dependent Cre recombinase expressing mice to obtain myeloid-specific
258 expression of mutant NLRP3 (*Nlrp3*^{A350V/+}; *LysM-Cre*⁺ mice) as compared to mice lacking Cre
259 recombinase and mutant NLRP3 expression (*Nlrp3*^{A350V/+}; *LysM-Cre*⁻ mice). Liver macrophages
260 were isolated from these mice and primed with low dose of LPS to ensure the expression of NLRP3
261 and IL-1 β and further treated with Yoda1. Low dose of LPS induced moderate secretion of IL-1 β

262 as expected (Fig. 6A). Treatment of Yoda1 potentiated secretion of IL-1 β and Caspase-1, while no
263 IL-1 β was detected in cells isolated from control mice under tested conditions (Fig. 6A and 6B).
264 Importantly, inhibition of KCNN4 attenuated Yoda1-induced inflammasome activation in these
265 cells (Fig. 6A and 6B).

266 To corroborate these findings in primary human cells, we next isolated PBMCs of a CAPS patient
267 carrying a heterozygous germline *NLRP3 E313K* mutation. Even though less pronounced as
268 compared to LPS stimulation, Yoda1 treatment alone was sufficient to enhance secretion of IL-1 β
269 from patient PBMCs, which was significantly inhibited upon KCNN4 inhibition (Fig. 6C). Yoda1
270 also amplified IL-1 β in cells co-treated with LPS as compared to cells treated with LPS alone.
271 Inhibition of KCNN4 also inhibited this effect of Yoda1 but had no effect on IL-1 β release induced
272 by LPS alone.

273 Thus, activation of PIEZO1 is sufficient to activate the inflammasome in a KCNN4-dependent
274 manner in cells expressing CAPS-causing NLRP3 mutants.

275

276 **Pharmacologic inhibition of KCNN4 activity attenuates CAPS in mice**

277 To open a therapeutic perspective from above findings, we finally asked the question whether
278 pharmacologic KCNN4 inhibition had a beneficial effect on auto-inflammation in CAPS. It was
279 recently reported that macrophage-specific expression of *Nlrp3^{A350V}* suffices to drive CAPS in
280 mice (39). The chemokine receptor *Cx3cr1* is widely expressed in the mononuclear phagocyte
281 system. Cre-driven recombination in *Cx3cr1-CreER* mice after tamoxifen induction was reported
282 to occur in tissue-resident macrophages (40), likely to be exposed to mechanical stress. Therefore,
283 we crossed *Nlrp3^{A350V/A350V}* mutant mice with *Cx3cr1-CreER* mice to obtain *NLRP3^{A350V/+};Cx3cr1-*
284 *CreER⁺* mice with tamoxifen-inducible expression of NLRP3^{A350V}. After tamoxifen induction,
285 *NLRP3^{A350V/+};Cx3cr1-CreER⁻* control mice and *NLRP3^{A350V/+};Cx3cr1-CreER⁺* mice were treated
286 with vehicle or the KCNN4 inhibitor TRAM34 (Fig. 7A). In line with previous studies showing
287 that tamoxifen-induced global *Nlrp3^{A350V}* expression triggered mild nonlethal autoinflammation
288 associated with body weight loss (41, 42), vehicle-treated *NLRP3^{A350V/+};Cx3cr1-CreER⁺* mice
289 showed gradual body weight loss during treatment; while vehicle-treated *NLRP3^{A350V/+};Cx3cr1-*
290 *CreER⁻* control mice didn't lose their weight (Fig. 7B). Importantly however, TRAM34-treated
291 *Nlrp3^{A350V/+};Cx3cr1-CreER⁺* mice did not experience a significant body weight loss (Fig. 7B).
292 Body weight loss inversely correlated with changes in body temperature (Fig. 7C) and serum levels
293 of IL-1 β (Fig. 7D) in mice with corresponding genotypes, indicating that autoinflammation in adult
294 CAPS-mimicking mice is alleviated in response to inhibition of KCNN4.

295 Altogether, our results suggest that KCNN4 acts downstream of PIEZO signaling-induced Ca²⁺
296 influx to promote NLRP3 inflammasome activation, a mechanism that may contribute to
297 microenvironmental effects on severity of CAPS and other NLRP3-dependent inflammatory
298 conditions.

299

300 **DISCUSSION**

301

302 Immune cells including macrophages are constantly exposed to microenvironmental changes, such
303 as shear stress in the circulation during extravasation, membrane reorganization during
304 transmigration, hydrostatic pressure in blood vessels, lung and heart (27). These changes redirect
305 their responses to physiological and pathological cues (27). The NLRP3 inflammasome plays
306 critical roles in innate immune responses detecting various endogenous and exogenous danger
307 signals. In this study, we have provided a direct link between PIEZO protein-mediated

308 mechanosensing and the NLRP3 inflammasome-dependent immune response. Based on our data,
309 we suggest a model, whereby mechanical cues act in parallel to trigger KCNN4-dependent K^+
310 efflux (Fig. 7E). In CAPS, a condition of sterile auto-inflammation, no specific NLRP3 activator
311 is present. Therefore, KCNN4-dependent K^+ efflux might represent a key step to induce NLRP3
312 inflammasome activation. This is supported by our data showing that PIEZO activation is
313 sufficient to trigger NLRP3 inflammasome activation in a KCNN4-dependent manner.
314 Furthermore, KCNN4 inhibition alleviates auto-inflammation in a CAPS-mimicking mouse
315 model. In other NLRP3-dependent inflammatory conditions that are primarily caused by specific
316 NLRP3 activators that may or may not trigger K^+ efflux, KCNN4 activation may have rather
317 synergistic effects on NLRP3 inflammasome activation. In this study, we showed that MSU-
318 induced arthritis in mice is attenuated in the absence of PIEZO-dependent mechanotransduction,
319 clearly demonstrating the importance of mechanical stress in other NLRP3-dependent
320 inflammatory conditions. Whether inhibition of KCNN4 is indeed beneficial in such a context
321 remains to be explored in the future.

322 In this study, we found that PIEZO-dependent effects on NLRP3 inflammasome activation
323 depended on extracellular Ca^{2+} . The role of Ca^{2+} in the activation cascade of the NLRP3
324 inflammasome in response to several NLRP3 activators including nigericin and ATP is
325 controversially discussed (43). Importantly however, we emphasize here that Ca^{2+} influx or PIEZO
326 activation is not required per se for NLRP3 inflammasome activation in response to such stimuli
327 when used at higher concentrations. Yet, we provide evidence for a mechanism in which enhancing
328 K^+ efflux through Ca^{2+} -dependent activation of KCNN4 lowers the threshold of NLRP3
329 inflammasome activation. Activation of PIEZO1 is therefore efficient at concentrations of
330 nigericin that are not sufficient for robust NLRP3 inflammasome activation on its own. Our data
331 thus do not contradict a generally accepted model in which K^+ is the primary trigger of NLRP3
332 inflammasome activation. Most importantly, we demonstrate that our findings are of high
333 relevance in the context of NLRP3-dependent inflammatory disorders.

334 So far, mechanisms underlying Ca^{2+} -dependent NLRP3 activation remained ill-defined. One study
335 suggested that Ca^{2+} promotes interaction of ASC with NLRP3 (44). It was also proposed that
336 elevated Ca^{2+} levels in the cytoplasm resulted in mitochondrial Ca^{2+} overload that impaired
337 mitochondrial function, thereby activating the NLRP3 inflammasome (45). We propose here that
338 Ca^{2+} primarily amplifies K^+ efflux in a KCNN4-dependent manner, which could be of relevance
339 for other Ca^{2+} mobilizing mechanisms reported during NLRP3 activation.

340 While our data provide evidence for PIEZO-mediated mechanotransduction to be important in
341 NLRP3 inflammasome activation, a recent study addressed the role of TRPV4, another important
342 mechano-gated ion channel in this context. They find that deletion of *TRPV4* affected crystal- but
343 not non-crystal-induced NLRP3 activation. Mechanistically, they propose that TRPV4 is
344 important for efficient phagocytosis of crystals and phagocytosis-dependent ROS production.
345 Crystal-containing phagosomes are destined for degradation to lysosomes. However, inefficient
346 digestion of crystals in lysosomes results in their accumulation, which causes lysosomal leakage.
347 Deletion or inhibition of TRPV4 limited phagocytosis, thereby attenuating ROS production and
348 lysosomal leakage leading to reduced NLRP3 inflammasome activation (46). In line with this
349 study, PIEZO1 activity has also been shown to enhance phagocytosis in macrophages but so far
350 not in the context of crystal uptake and NLRP3 inflammasome activation (6). In our study, we put
351 forward a phagocytosis-independent PIEZO function in NLRP3 inflammasome activation. Yet,
352 we cannot exclude that crystal-induced phagocytosis might be also limited in MSU-induced
353 arthritis in mice lacking PIEZO proteins in the myeloid compartment.

354 In CAPS patients, inflammation is often more prominent in eyes, joints and bones, sites favoring
355 movement-associated mechanical stress. Moreover, urticaria seen in CAPS patients is frequently
356 cold-induced. In this study, we discovered that PIEZO-mediated signaling is sufficient to activate
357 the NLRP3 inflammasome in cells expressing CAPS-causing NLRP3 mutants and patient CAPS
358 cells. Furthermore, we showed that KCNN4 inhibition can efficiently alleviate auto-inflammation
359 in a tamoxifen-induced CAPS-mimicking mouse model, which is a model for human CAPS caused
360 by somatic *NLRP3* mutations (47). Gouty arthritis is yet another condition where mechanical
361 impact may play an important role. Indeed, we demonstrated that genetic ablation of PIEZO
362 function in mice reduces MSU-induced joint inflammation to a level seen for NLRP3 deletion.
363 Thus, targeting of this pathway may provide a novel therapeutic strategy for treatment of CAPS
364 patients and potentially other NLRP3-related inflammatory diseases.

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366 **Materials and Methods**

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368 **Mice**

369 *Piezo1^{fl/fl}* (Strain #:029213), *Piezo2^{fl/fl}* (Strain #:027720) and *Nlrp3^{A350VneoR}* (Strain #:017969) mice
370 were obtained from The Jackson Laboratory. *Piezo1^{fl/fl}* and *Piezo2^{fl/fl}* mice were crossed with
371 *LysM-Cre* mice to obtain mice with myeloid-specific deletion of *Piezo1* and *Piezo2*. *Cx3cr1-*
372 *CreER* mice were from Prof. Marco Prinz at University of Freiburg, Germany. *Nlrp3^{A350VneoR}* mice
373 were crossed with *Cx3cr1-CreER* mice to obtain *Nlrp3^{A350VneoR};Cx3cr1-CreER* mice with
374 tamoxifen-inducible expression of NLRP3^{A350V} in Cx3cr1-positive cells. Mice were housed under
375 specific pathogen-free conditions with controlled temperature (19-23°C) and humidity (50-60%)
376 on a 12-h light/dark cycle with unrestricted access to water and standard laboratory chow.
377 Maintenance and animal experimentation were in accordance with the local ethical committee
378 (Com'Eth) in compliance with the European legislation on care and use of laboratory animals (La
379 cellule AFiS (Animaux utilisés à des Fins Scientifiques): APAFIS#36729-2022041911158105 v2
380 and APAFIS #43633-2023042814302902 v6). No exclusion of animals used for experiments was
381 performed. Littermates were chosen randomly according to their genotypes.

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383 **Reagents**

384 Imiquimod (R837) (tlrl-imq), MSU crystals (tlrl-msu-25), CL097 (tlrl-c97) and MCC950 (inh-
385 mcc) were purchased from InvivoGen. Nigericin sodium salt (N7143), Propidium Iodide (P4170),
386 Lipopolysaccharides from *Escherichia coli* 055: B5 (L2880) and Phorbol 12-myristate 13-acetate
387 (PMA) (P1585) were obtained from Sigma-Aldrich. Yoda1 (5586) and TRAM34 (2946) were
388 purchased from Bio-technie. Sytox™ Green Nucleic Acid Stain (S7020) was purchased from
389 Thermo Fisher Scientific. Human macrophage-colony stimulating factor (hM-CSF) (11343117)
390 was obtained from Immunotools. Anti-Caspase1 (p20) (human) antibody (AG-20B-0048-C100),
391 anti-Caspase1 (p20) (mouse) antibody (AG-20B-0042), anti-NLRP3 antibody (AG-20B-0014-
392 C100) were purchased from AdipoGen. Anti-human IL-1β antibody (AF-201-NA) was from R&D
393 Systems. Anti-murine IL-1β antibody (5129-100) was from BioVision. Anti-tubulin antibody
394 (T5168) was from Sigma-Aldrich. Anti-GFP antibody (ab13970) and anti-NEK7 antibody
395 (ab133514) were obtained from Abcam. Anti-ASC antibody (sc-22514) was obtained from Santa
396 Cruz Biotechnology. Anti-Gasdermin D antibody (NBP2-33422) was obtained from Novus
397 Biologicals.

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Plasmids

The pX330-P2A-EGFP and pX330-P2A-RFP plasmid was previously generated by inserting P2A-GFP and P2A-RFP sequence into pX330-U6-Chimeric_BB-CBh-hSpCas9 (26). KCNN4 CDS was amplified from THP-1 cDNA by PCR. KCNN4 fused with EGFP was cloned into pBOB plasmid using ligation-independent cloning (LIC). Calcium reporter jGCaMP7s sequence was amplified from pGP-CMV-jGCaMP7s (104463, Addgene) and cloned into pBOB plasmid by LIC. C-terminal EGFP-tagged CAPS-causing mouse Nlrp3 mutants R258W, D301N and T346M were cloned into pBOB plasmid by LIC. Mouse Piezo1 CDS was amplified from a gift plasmid from Prof. Ardem Patapoutian and cloned into pBOB plasmid by LIC. The primers used are listed below:

Name	Sequences
pBOB mPiezo1 CDS-F	5'ATGGAGCCGCACGTGCTGGGCGCCGGGC3'
pBOB mPiezo1 CDS-R	5'CTACTCCCTCTCACGTGTCCACTTA 3'
PBOB KCNN4-EGFP F1	5'ATGGGCGGGGATCTGGTGCTTGGC 3'
PBOB KCNN4-EGFP R1	5'TCCTCGCCCTTGCTCACCATCTTGGACTGCTGGC TGGGTCTGGA 3'
PBOB KCNN4-EGFP F2	5'AACCCAGCCAGCAGTCCAAGATGGTGAGCAAGG GCGAGGAGCTGT 3'
PBOB KCNN4-EGFP R2	5'CTACTTGTACAGCTCGTCCATGCCG 3'
pBOB-jGCaMP7s-F	5'ATGGGTTCTCATCATCATCATC 3'
pBOB-jGCaMP7s-R	5'TTACTTCGCTGTCATCATTTGTACA 3'
mNlrp3 R258W-F	5'TATCCACTGCTGGGAGGTGAGCCTC 3'
mNlrp3 R258W-R	5'GAGGCTCACCTCCCAGCAGTGGATA 3'
mNlrp3 D301N-F	5'TGGATGGCTTTAATGAGCTACAAGG 3'
mNlrp3 D301N-R	5'CCTTGTAGCTCATTAAAGCCATCCA 3'
mNlrp3 T346M-F	5'CTGCTCATAACGATGAGGCCGGTAG 3'
mNlrp3 T346M-R	5'CTACCGGCCTCATCGTTATGAGCAG 3'

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Generation of knockout cell lines using CRISPR/Cas9-mediated gene editing

For the generation of *PIEZO1* KO, *PIEZO2* KO, *PIEZO1/2* double knockout (dKO) and *KCNN4* KO THP-1 cell lines, two sgRNAs (sgRNA 1 and sgRNA 2) for each gene were designed and cloned into pX330-P2A-EGFP or pX330-P2A-RFP, respectively, using T4 ligation. THP-1 cells were transfected with mixture of two sgRNAs-expressing plasmids (0.5 µg each) using XtremeGENE 9 according to the manufacturer's manual. 24 h after transfection, GFP and RFP double-positive cells were sorted and collected using BD FACS Aria™ III Cell Sorter. Single cell colonies were obtained by seeding into 96-well plates via series of dilution. The sequences of sgRNAs are listed below:

Name	Sequences
<i>hPIEZO1</i> sgRNA 1	5' CACCAAGATGCCCAGGTCAG 3'
<i>hPIEZO1</i> sgRNA 2	5' CCCGGCAGAGCCCACATCCA 3'
<i>hPIEZO2</i> sgRNA 1	5' TATGAAGAGGATTTCAATGG 3'
<i>hPIEZO2</i> sgRNA 2	5' AACAACTGACTTTCCCAGCAG 3'
<i>hKCNN4</i> sgRNA 1	5' TTCGGCGTCTCAAGGCCCCC 3'

<i>hKCNN4</i> sgRNA 2	5' CAGAGATGCTGTGGTTTCGGG 3'
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Validation of obtained THP-1 knockout cell lines by Sanger sequencing

For the validation of *PIEZO1* KO, *PIEZO2* KO, *PIEZO1/2* dKO, *KCNN4* KO THP-1 clones, genomic fragments containing sgRNA targeting sites were amplified by PCR and cloned into pUC57 plasmid using LIC. After transformation of *E. coli* competent cells, plasmids were purified from at least 6 bacteria colonies and subjected to Sanger sequencing. Obtained sequences were aligned to reference sequence to determine the insertion/deletion. The primers used are listed below:

Name	Sequences
PIEZO1 KO GT-F	5' CCTGTCCTCTTGGTCTGACCGCGGC 3'
PIEZO1 KO GT-R	5' CCATGTCGGGGCGTGGAGGTTTCTG 3'
PIEZO2 KO GT-F	5' CTGGTTGTCTTCCCTTTTTTCCTGA 3'
PIEZO2 KO GT-R	5' AACCTAGAAAATGGAGTCGCTTAGC 3'
KCNN4 KO GT-F	5' TGCCCCAAGACACACCCTAGCCCCCT 3'
KCNN4 KO GT-R	5' CAAGTCCCCAGTCCCTCCTCCCTCA 3'

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Cell culture

THP-1, mouse primary bone marrow-derived macrophages (BMDMs) and HEK293t cell lines were cultured at 37°C with 5% CO₂. THP-1 cells were grown in RPMI 1640 supplement with 10 mM HEPES, 10% fetal calf serum (FCS), 2.5 g/l glucose, 1 mM sodium pyruvate, gentamycin and 50 μM β-mercaptoethanol. HEK293t cells were grown in DMEM (1 g/ml glucose) supplement with 10% fetal calf serum, penicillin and streptomycin. BMDMs were differentiated from bone marrow progenitors isolated from the tibia and femur in DMEM (4,5g/l glucose) supplement with 50 ng/ml recombinant hM-CSF, 10% heat-inactivated fetal calf serum, penicillin and streptomycin for 7 days. Human bloods from healthy control and CAPS patient were collected after the patient gave their signed informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS (GE Healthcare) and cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, penicillin and streptomycin. For treatment of cells, BMDMs and THP-1 cells were primed with 1 μg/mL LPS for 3 h, followed by the treatment of nigericin, Imiquimod and CL097 in presence or absence of 25 μM Yoda1. For treatment of inhibitor, cells were pretreated with inhibitor for 45 min and followed by stimulation in presence of inhibitor. To assess the involvement of K⁺ efflux, LPS-primed cells were treated with NLRP3 inflammasome activator in the culture medium supplemented with 5 mM, 15 mM, 25 mM, or 35 mM KCl. For the experiments with calcium-free medium, BMDMs and THP-1 cells were primed with 1 μg/mL LPS for 3h in DMEM (4,5g/l glucose) containing 10% FCS, cells were washed once with PBS before treatment with nigericin, R837 or CL097 in DMEM (4,5g/l glucose) without calcium containing 10% dialyzed FCS.

For the stiffness-related experiments using wild type THP-1 cells, cells were seeded on silicone gels of 0.2 KPa and 64 KPa (5165-5EA and 5145-5EA, Sigma-Aldrich) with culture medium containing 100 nM PMA for 3 h, followed by replacement of fresh medium and overnight incubation at 37 °C. On the next day, cells were treated with nigericin or R837. For the stiffness-related experiments using THP-1 cells expressing CAPS-causing *Nlrp3* mutants, cells were seeded

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457 on normal tissue culture plates, 0.2 KPa or 64 KPa silicone gels with culture medium containing
458 100 nM PMA for 3 h, followed by replacement of fresh medium and supernatants were collected
459 after 4 hours incubation at 37 °C.

460 For shear stress related experiments, shear stress was generated by a rotating unit device as
461 described previously (48). Briefly, each rotating unit is made of one circular rotating plate in
462 Plexiglas (3.2 cm diameter) fixed to a rotating motor. Rotating plate is replaced in contact with the
463 culture medium which generates a shear flow over cells when rotates. The shear stress τ exposed
464 to cells depends on the circular plate rotation speed Ω , the distance from the axis r and the height
465 of the plate h and is given by: $\tau = \eta \Omega r/h$, η being the medium viscosity. In these experiments,
466 the rotating plates were set up at height $h = 4$ mm and at speed $\Omega = 8$ and 16 rounds *per* min, which
467 generates maximal shear stress at the edge of rotating plate $\tau \approx 1.8 \times 10^{-3}$ Pa and 3.6×10^{-3} Pa,
468 respectively. WT and *PIEZO1/2* dKO cells were seeded on 6-well tissue culture plates with culture
469 medium containing 100 nM PMA for 3h, followed by replacement of fresh medium and overnight
470 incubation at 37°C. On the next day, cells were treated with nigericin or R837 in presence or
471 absence of indicated shear stress.

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473 **Immunoblotting**

474 Both cell lysates and culture supernatants were subjected to immunoblotting. For cell lysates, cells
475 were lysed on ice with 1× RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1
476 mM EGTA, 1% Triton X-100, 1 mM NaVO₄, 1.5 mM Sodium pyrophosphate, 1 mM NaF, 1 mM
477 β-Glycerophosphate) supplemented with protease inhibitor cocktail. The immunoblot was
478 prepared using Glycine SDS-PAGE gels. For the supernatants, the proteins were extracted using
479 methanol-chloroform precipitation protocol as previously described (26), separated by Tricine
480 SDS-PAGE and analyzed by immunoblotting. The PVDF membranes were incubated with
481 primary antibody at 4 °C overnight. After 3 times washes with TBS-T, membranes were incubated
482 with HRP-conjugated secondary antibody for 1 hour at RT. Membranes were again washed with
483 TBS-T for 3 times and were incubated with Immobilon Forte Western HRP substrate
484 (WBLUF0500, EMD Millipore Corporation). Images were captured using AI600 Imager from GE
485 Healthcare Life Science.

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487 **Flow cytometry**

488 For analysis of lytic cell death, after treatments, THP-1 cells were spin down and resuspended in
489 cold PBS with 1 μM of Sytox Green or propidium iodide (PI). Sytox Green- or PI- positive cells
490 were analyzed by BD FACS Celesta™ Flow Cytometer. For cell sorting, THP-1 cells were spin
491 down and resuspended in cold PBS supplemented with 1% of fetal calf serum, followed by sorting
492 at sterile condition using BD FACS Aria™ III Cell Sorter in fresh medium for further culture.

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494 **Measurement cytokines using ELISA**

495 IL-1β and TNFα levels in collected culture supernatants were determined by ELISA according to
496 the user's manual. Human IL-1 beta/IL-1F2 DuoSet ELISA (DY201), mouse IL-1 beta/IL-1F2
497 DuoSet ELISA (DY401), human TNF-alpha DuoSet ELISA (DY210), mouse TNF-alpha DuoSet
498 ELISA (DY410) and DuoSet ELISA Ancillary Reagent Kit 2 (DY008) were purchased from R&D
499 system.

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501 **Immunofluorescence and live-video imaging**

502 For immunofluorescence, cells plated on coverslips (12-mm) were fixed with 4%
503 paraformaldehyde for 15 min at room temperature after treatments. Coverslips were washed three
504 times with PBS for 5 min each time and permeabilized with 0.1% saponin in PBS for 10 min,
505 followed by blocking in PBST (PBS plus 0.05% Tween-20) containing 0.5% BSA for 1 h. The
506 coverslips were further incubated with anti-ASC antibody (1/100 dilution) for 1 h at room
507 temperature, followed by incubation with the secondary for 1h at room temperature (avoid the
508 coverslips in direct light). After three times washing with PBST, cells were stained with DAPI and
509 mounted for further imaging. For live-video imaging, THP-1 WT and *PIEZO1/2* dKO cells stably
510 expressing jGCaMP7S were seeded on μ -dish 35 mm high glass bottom (81158, Ibbidi) in culture
511 medium containing 100 nM PMA for 3 h, followed by replacement of fresh medium and
512 incubation overnight. On the next day, cells were treated with R837, Yoda1 or R837 plus Yoda1.
513 The images were acquired using a Nikon spinning disk with an interval of 20s. Ionomycin was
514 added at ~16 min after treatment. Fluorescence intensities of individual cells over time were
515 quantified with Image J.

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517 **Lentivirus packaging and infection**

518 The 3rd generation lentiviral vector system with three packaging helper plasmids (pVSVG, pMDL
519 and pREV) was used in this study. The lentivirus packaging was performed in HEK293t cells.
520 HEK293t cells were transfected with Lipofectamine 2000 according to the user's manual. THP-1
521 cells (4.0×10^5 cells per well in 6-well plate) were infected in 1.5 mL lentivirus-contained culture
522 medium plus 1 mL fresh medium containing final concentration of 10 μ g/mL polybrene. 48 hours
523 after viral infection, cells were used for further experiments.

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525 **Genome-wide CRISPR/Cas9-mediated knockout screen**

526 The Brunello human genome-wide CRISPR/Cas9 knockout lentiviral library containing 76,441
527 sgRNAs systemically targeting 19,114 genes on human genome was used in this study. Briefly,
528 THP-1 cells stably expressing Cas9 protein were infected with the pooled Brunello lentivirus
529 library at ROI of 0.2. After puromycin selection for 3 days, cells were further expanded. To ensure
530 a representation of minimal 500 cells per sgRNA, at least 4.0×10^7 cells were used in each group.
531 An adequate number of cells were primed with LPS in suspension for 3 hours and treated with
532 vehicle (control group) or R837 plus Yoda1 (treated group). When pyroptotic cell death reached
533 ~85% in the treated group, R837 and Yoda1 were washed out and the survivals were expanded.
534 When cell number reached 4.0×10^7 cells, these cells were repeatedly treated with R837 plus
535 Yoda1 to reach reached ~85% of pyroptotic cell death. R837 and Yoda1 were washed out and the
536 survivals were further expanded to reach 4.0×10^7 cells. Genomic DNA from both control- and
537 treated groups was isolated using the phenol/chloroform extraction method. Fragments containing
538 sgRNA were amplified from genomic DNA by PCR referring to the protocol from broad institute
539 GPP Web Portal (<https://portals.broadinstitute.org/gpp/public/resources/protocols>). PCR
540 fragments were cleaned up using NucleoSpin gel and PCR clean-up kit (740609.50, MACHEREY-
541 NAGEL) according to the users' manual and subjected to next generation sequencing (NGS).
542 Sequencing was performed on an Illumina HiSeq 4000 sequencer as 50 bp single end reads by the
543 GenomEast platform, a member of the 'France Genomique' consortium (ANR-10-INBS-0009).
544 Image analysis and base calling were performed using RTA version 2.7.7 and bcl2fastq version
545 2.20.0.422. Sequencing reads were trimmed, aligned and counted using PinAPL-Py and guide
546 RNAs (Brunello library). The Matching Threshold for bowtie2 alignment was defined as 35. Data
547 were statistically analyzed using Model-based Analysis of Genome-wide CRISPR/Cas9 Knockout

548 (MAGeCK v0.5.9.3). The method ranks sgRNAs based on p -values calculated from the negative
549 binomial model, and uses a modified robust ranking aggregation (RRA) algorithm to identify
550 positively or negatively selected genes. Read counts were normalized by total number of reads.
551 Enrichment testing of paired samples was performed using the test function in MAGeCK with --
552 paired option. Volcano plot was generated using ggplot package in R and significance was defined
553 as $FDR < 0.05$.

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555 **MSU-induced acute arthritis mouse model**

556 MSU crystal (0.5 mg in 10 μ l PBS) was injected intra-articularly into one ankle of each adult
557 mouse. The thickness of injected ankles was monitored using a caliper at 0, 2, 6, 12 and 24 hours
558 after MSU injection. Ankle temperature was measured using an infrared thermometer at 12 hours
559 after MSU injection.

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561 **CAPS-mimicking mouse model with nonlethal autoinflammation**

562 Adult mice were daily treated with tamoxifen 50 mg/kg via intraperitoneal injection for five
563 consecutive days. Then, mice were further daily treated with vehicle (10% DMSO:90% corn oil)
564 or TRAM34 dissolved in 10% DMSO:90% corn oil (15 mg/kg) via intraperitoneal injection for
565 eight days. Body weight loss was monitored every day starting from the first day of TRAM34
566 treatment. Rectal temperature was measured and blood was collected on the day after the last day
567 of TRAM34 treatment. The timeline of this experimental procedure is illustrated in Fig.7A.

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569 **Statistical analysis**

570 Preliminary experiments were performed and sample size was determined based on generally
571 accepted rules to test preliminary conclusions reaching statistical significance, where applicable.
572 For FACS experiments, the percentage of Sytox green-positive and PI-positive cells was provided.
573 For immunofluorescence experiments, a minimum of 150 cells were counted per condition, and
574 the percentage containing ASC specks cells was calculated. A non-parametric *Mann-Whitney* test
575 (two-tailed) was used for statistical analysis of ankle temperature, rectal temperature, and serum
576 IL-1 β levels. Differences between groups were assessed for statistical significance by ANOVA or
577 using an unpaired Student t test (when only two sets of data were compared). Statistical
578 significance was indicated using the following symbols: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

579

580 **Supplementary Materials**

581

582 Figures S1-S10

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759

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763

764

765

766 **Figure legends:**

767
768 **Figure 1. Activation of PIEZO1 by Yoda1 potentiates nigericin-induced NLRP3**
769 **inflammasome activation.** (A) ELISA measurements of IL-1 β in culture supernatants from THP-
770 1 cells primed with 1 μ g/ml LPS for 3 h and followed by treatment with 5 μ M nigericin (Ni) in
771 presence or absence of 25 μ M Yoda1. (B) Immunoblotting of culture supernatants (Sup) and
772 lysates (Lys) from LPS-primed THP-1 cells treated as in panel A. Antibodies against IL-1 β and
773 Caspase-1 (CASP1) were used. An antibody against Tubulin was used as a loading control. “s.e.”
774 short exposure, “l.e.” long exposure. (C) Uptake of Sytox Green in LPS-primed wild type (WT)
775 and *NLRP3* KO THP-1 cells treated as described in panel A. (D) Uptake of Sytox Green from
776 isolated PBMCs primed with 1 μ g/ml LPS for 3 h and followed by treatment with 5 μ M nigericin
777 (Ni) in presence or absence of 25 μ M Yoda1. (E) Representative immunofluorescence images of
778 ASC speck formation in LPS-primed BMDMs stimulated with 2.5 μ M nigericin in the presence
779 or absence of 25 μ M Yoda1. White arrows indicate ASC specks (green). Scale bars: 10 μ m. (F)
780 Quantification of macrophages containing ASC specks in experiments as described in panel D.
781 (G) ELISA measurements of IL-1 β in culture supernatants from WT and *PIEZO1* KO THP-1 cells
782 expressing empty vector (e.v.) or mouse Piezo1. Cells primed with 1 μ g/ml LPS for 3 h and
783 followed by treatment with 5 μ M nigericin (Ni) in presence or absence of 25 μ M Yoda1 for 40
784 min. (H) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed cells
785 treated as described for panel G. Antibodies against IL-1 β and Caspase-1 (CASP1) were used. An
786 antibody against Tubulin was used as a loading control. (I) Uptake of Sytox Green from LPS-
787 primed cells treated as described in panel F. Sytox Green uptake was analyzed by FACS after
788 staining. “ND” not detected. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data shown are representative
789 of at least three independent experiments, except for data shown in panel D which are
790 representative of two independent experiments.

791
792 **Figure 2. PIEZO-mediated mechanosensing potentiates NLRP3 inflammasome activation.**
793 (A) ELISA measurements of IL-1 β in culture supernatants from WT and *PIEZO1/2* double KO
794 (dKO) THP-1 cells cultured on soft (0.2 KPa) or stiff (64 KPa) silicone. Cells were treated with 5
795 μ M nigericin (Ni) for 40 min or 100 μ M R837 for 2 h. (B) ELISA measurements of IL-1 β in
796 culture supernatants from PMA-differentiated WT and *PIEZO1/2* double KO (dKO) THP-1 cells
797 treated with 5 μ M nigericin (Ni) for 40 min or 100 μ M R837 for 2 h in absence or presence of
798 shear stress of 1.8×10^{-3} Pa or 3.6×10^{-3} Pa. (C) Measurement of the percentage of ankle swelling
799 in *Nlrp3*^{+/+} (n=6) and *Nlrp3*^{-/-} (n=4) mice at 0, 2, 6 12 and 24 hours after intra-articular injection
800 of MSU (0.5 mg in 10 μ l PBS). (D) Ankle temperature of *Nlrp3*^{+/+} (n=6) and *Nlrp3*^{-/-} (n=4) mice
801 at 12 hours after intra-articular injection of MSU. (E) Measurement of the percentage of ankle
802 swelling in *Piezo1*^{fl/fl}; *Piezo2*^{fl/fl} (n=5) and *Piezo1*^{fl/fl}; *Piezo2*^{fl/fl}; *LysM-Cre* (n=5) mice at 0, 2, 6 12
803 and 24 hours after intra-articular injection of MSU (0.5 mg in 10 μ l PBS). (F) Ankle temperature
804 of *Piezo1*^{fl/fl}; *Piezo2*^{fl/fl} (n=5) and *Piezo1*^{fl/fl}; *Piezo2*^{fl/fl}; *LysM-Cre* (n=5) mice at 12 hours after intra-
805 articular injection of MSU. “ns” not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data
806 shown in panel A are representative of three experiments. Data shown in panel B are representative
807 of two experiments.

808
809 **Figure 3. Ca²⁺ influx is required for PIEZO-mediated mechanosensing and potentiation of**
810 **NLRP3 inflammasome activation.** (A) Measurement of cytosolic Ca²⁺ levels in WT and
811 *PIEZO1/2* dKO THP-1 cells using a Ca²⁺ reporter jGCaMP7s. Cells were treated with 5 μ M

812 Nigericin (Ni), 25 μ M Yoda1 or 5 μ M Nigericin plus 25 μ M Yoda1 (Ni+Yoda1). The images were
813 acquired using a Nikon spinning-disk microscope with an interval of 60s. Stimuli were added into
814 the culture medium at 3 min as indicated by a red arrow, and ionomycin was added at 15 min as
815 indicated by a blue arrow. Fluorescence intensities of individual cells over time were quantified.
816 $n=127$ for “WT Ni+Yoda1” group; $n=110$ for “WT Yoda1” group; $n=89$ for “WT Ni” group;
817 $n=105$ for “*PIEZO1/2* dKO Ni+Yoda1” group; $n=96$ for “*PIEZO1/2* dKO Yoda1” and $n=83$ for
818 “*PIEZO1/2* dKO Ni” group. **(B)** ELISA measurements of IL-1 β in culture supernatants from LPS-
819 primed THP-1 cells treated with 5 μ M Nigericin (Ni) in presence or absence of 25 μ M Yoda1 in
820 medium with or without (w/o) Ca²⁺. **(C)** Immunoblotting of culture supernatants (Sup) and lysates
821 (Lys) from LPS-primed THP-1 cells in experiments as described for panel B. Antibodies against
822 IL-1 β and Caspase-1 (CASP1) were used. An antibody against Tubulin was used as a loading
823 control. **(D)** Uptake of Sytox Green in LPS-primed THP-1 cells in experiments as described for
824 panel B. Sytox Green uptake was analyzed by FACS after staining. ** $p < 0.01$, *** $p < 0.001$.
825 Data are representative of at least three independent experiments.

826
827 **Figure 4. KCNN4 acts downstream of PIEZO-mediated calcium influx to promote NLRP3**
828 **inflammasome activation.** **(A)** Genome-wide CRISPR/Cas9 screening identifies genes involved
829 in Yoda1-mediated pyroptosis. RRA scores for the comparison of R837 plus Yoda1 vs vehicle.
830 **(B)** ELISA measurements of IL-1 β in culture supernatants from WT and *KCNN4* KO THP-1 cells
831 expressing GFP or GFP-tagged KCNN4 (*KCNN4*). Cells primed with 1 μ g/ml LPS for 3 h and
832 followed by treatment with 100 μ M R837 in presence or absence of 25 μ M Yoda1 for 1 h. **(C)**
833 Immunoblotting of culture supernatants (Sup) and lysates (Lys) from cells in experiments as
834 described in panel B. Antibodies against IL-1 β and Caspase-1 (CASP1) were used. An antibody
835 against Tubulin was used as a loading control. **(D)** Uptake of Propidium Iodide (PI) in LPS-primed
836 cells in experiments as described for panel B. **(E)** ELISA measurements of IL-1 β in culture
837 supernatants from LPS-primed BMDMs treated with 50 μ M R837 or 15 μ M nigericin with or
838 without 25 μ M Yoda1 in medium containing indicated concentrations of extracellular KCl. **(F)**
839 Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed BMDMs in
840 experiments as described for panel E. **(G)** Uptake of Sytox Green in LPS-primed THP-1 cells
841 followed by treatment with 100 μ M R837 or 15 μ M nigericin with or without 25 μ M Yoda1 in
842 medium containing indicated concentrations of extracellular KCl. “ns” not significant, * $p < 0.05$,
843 ** $p < 0.01$, *** $p < 0.001$. Data are representative of at least three independent experiments.

844
845 **Figure 5. PIEZO stimulation is sufficient to trigger NLRP3 inflammasome activation in**
846 **cultured cells expressing CAPS-causing NLRP3 mutants.** **(A)** Uptake of Propidium Iodide (PI)
847 in WT, *PIEZO1* KO, *PIEZO2* KO and *PIEZO1/2* dKO THP-1 cells expressing GFP, WT or
848 D301N-, T346M-, R258W-mutated mouse *Nlrp3*. Cells were treated with 25 μ M Yoda1 for 6 h.
849 **(B)** Uptake of Propidium Iodide (PI) in WT THP-1 cells expressing GFP, WT or D301N-, T346M-
850 , R258W-mutated mouse *Nlrp3*. Cells were treated with vehicle, 1 μ g/ml LPS, 25 μ M Yoda1 or
851 25 μ M Yoda1 plus 5 μ M TRAM34 for 6h. **(C)** Immunoblotting of culture supernatants (Sup) and
852 lysates (Lys) from WT THP-1 cells expressing GFP, WT or D301N-, T346M-, R258W- mutated
853 mouse *Nlrp3*. Cells were treated with 25 μ M Yoda1 in presence or absence of 5 μ M TRAM34 for
854 6h. Antibodies against Caspase-1 (CASP1) and GFP were used. An antibody against Tubulin was
855 used as a loading control. **(D)** ELISA measurements of IL-1 β in culture supernatants from WT and
856 *PIEZO1/2* dKO (dKO) THP-1 cells expressing GFP, WT or R258W-, D301N-, T346M-mutated
857 mouse *Nlrp3* cultured on normal tissue culture plates (T.C.), soft (0.2 KPa) or stiff (64 KPa)

858 silicone. Cells were treated with 100 nM PMA for 3 hours and supernatants were collected at 4
859 hours after replacement of fresh medium. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are
860 representative of at least three independent experiments.

861

862 **Figure 6. PIEZO1 stimulation is sufficient to trigger NLRP3 inflammasome activation in**
863 **primary cells isolated from CAPS-mimicking mice and a CAPS patient.** (A) ELISA
864 measurements of IL-1 β in culture supernatants in fetal liver macrophages from *Nlrp3*^{A350V/+};*LysM-*
865 *Cre*⁺ (*LysM-Cre*⁺) and *Nlrp3*^{A350V/+};*LysM-Cre*⁻ (*LysM-Cre*⁻) mice (n=7 for each genotype) as
866 indicated. Cells were primed with 1 ng/ml LPS for 1 h, followed by treatment with vehicle or 25
867 μ M Yoda1 in presence or absence of 5 μ M TRAM34 for 3 h. (B) Immunoblotting of culture
868 supernatants (Sup) and lysates (Lys) from cells in experiments as described in panel B. Antibodies
869 against NLRP3, IL-1 β and Caspase-1 (CASP1) were used. An antibody against Tubulin was used
870 as a loading control. (C) ELISA measurements of IL-1 β in culture supernatants from isolated
871 PBMCs of a CAPS patient (*NLRP3 E313K* mutation). Cells were treated with or without 1 μ g/ml
872 LPS in presence of vehicle, 25 μ M Yoda1, 5 μ M TRAM34 or 25 μ M Yoda1 plus 5 μ M TRAM34
873 for 4 h. “ND” not detected, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are representative of at
874 least three independent experiments.

875

876 **Figure 7. Inhibition of KCNN4 attenuates autoinflammation in CAPS-mimicking mice.** (A)
877 Experimental timeline. Adult mice were injected intra-peritoneally with 50 mg/kg Tamoxifen
878 (TAM) for five consecutive days and followed by daily intra-peritoneal injections of vehicle or 15
879 mg/kg TRAM34 for eight consecutive days. On day 8 (D8), rectal temperature was measured and
880 blood was collected. (B) Percentage of body weight loss of *Nlrp3*^{A350V/+};*Cx3cr1-CreER*⁻ (*Cx3cr1-*
881 *CreER*⁻) and *Nlrp3*^{A350V/+};*Cx3cr1-CreER*⁺ (*Cx3cr1-CreER*⁺) mice injected intra-peritoneally with
882 vehicle or 15 mg/kg TRAM34 after tamoxifen induction. (C) Rectal temperature at D8 of
883 *Nlrp3*^{A350V/+};*Cx3cr1-CreER*⁻ (*Cx3cr1-CreER*⁻) and *Nlrp3*^{A350V/+};*Cx3cr1-CreER*⁺ (*Cx3cr1-CreER*⁺)
884 mice intra-peritoneally injected with vehicle or 15 mg/kg TRAM34 after tamoxifen induction. (D)
885 Serum IL-1 β at D8 of *Nlrp3*^{A350V/+};*Cx3cr1-CreER*⁻ (*Cx3cr1-CreER*⁻) and *Nlrp3*^{A350V/+};*Cx3cr1-*
886 *CreER*⁺ (*Cx3cr1-CreER*⁺) mice intra-peritoneally injected with vehicle or 15 mg/kg TRAM34
887 after tamoxifen induction. n=8 for *Nlrp3*^{A350V/+};*Cx3cr1-CreER*⁻ treated with vehicle (*Cx3cr1-*
888 *CreER*⁻_Vehicle); n=10 for *Nlrp3*^{A350V/+};*Cx3cr1-CreER*⁺ mice treated with vehicle (*Cx3cr1-*
889 *CreER*⁺_Vehicle); n=11 for *Nlrp3*^{A350V/+};*Cx3cr1-CreER*⁺ mice treated with TRAM34 (*Cx3cr1-*
890 *CreER*⁺_TRAM34). * $p < 0.05$, ** $p < 0.01$. (E) Proposed model of the mechanism linking
891 mechanostimulation to NLRP3 inflammasome activation *in vivo*.

892

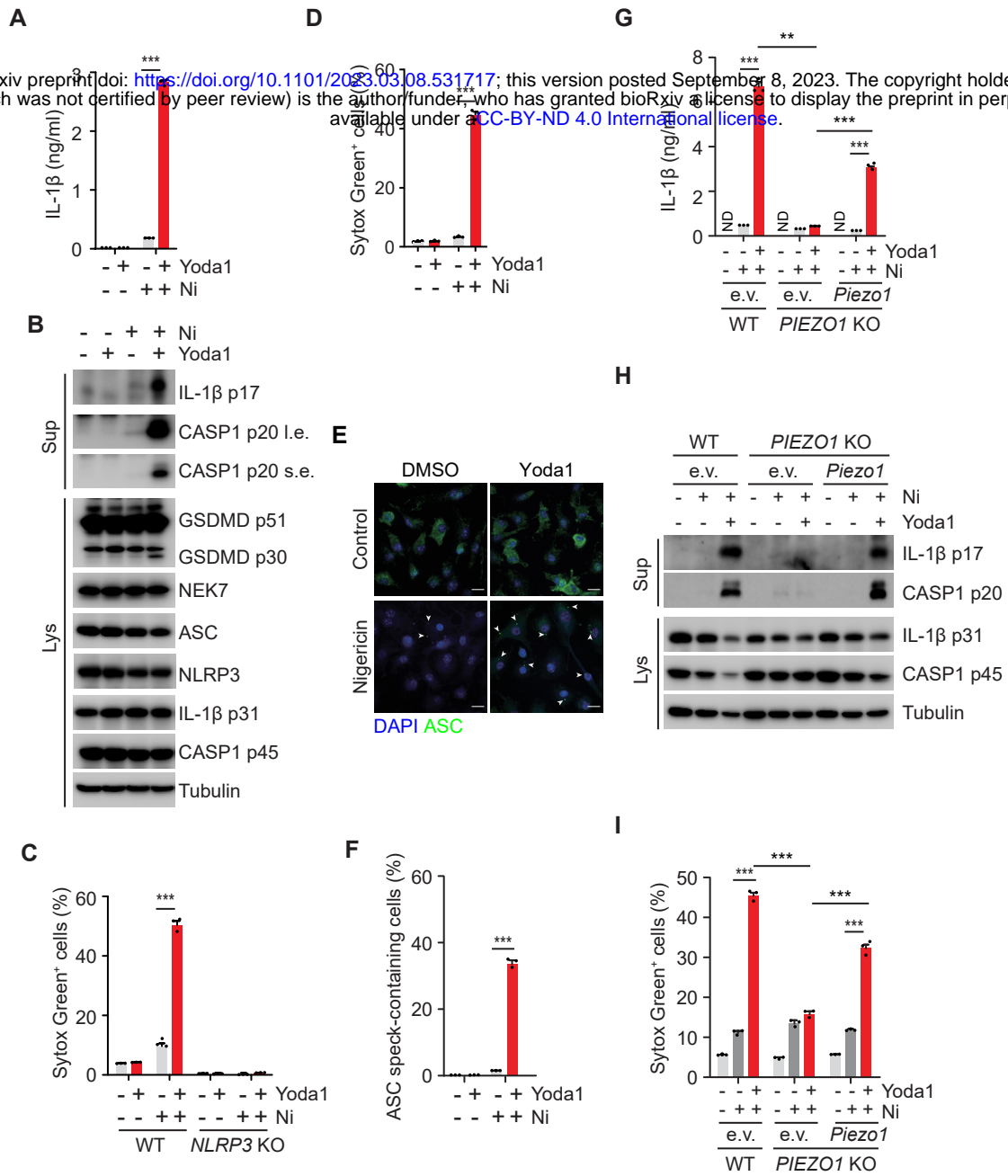


Figure 1

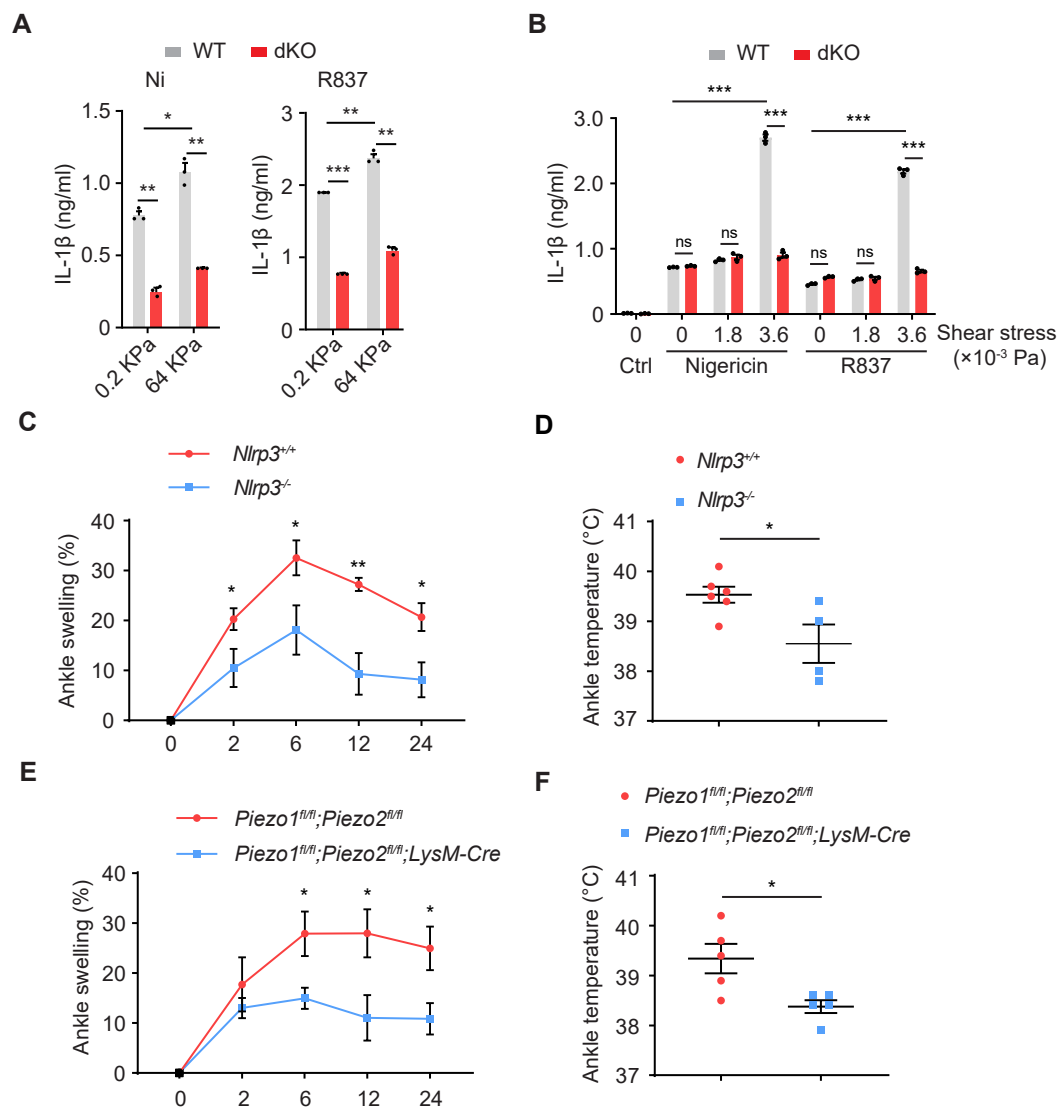


Figure 2

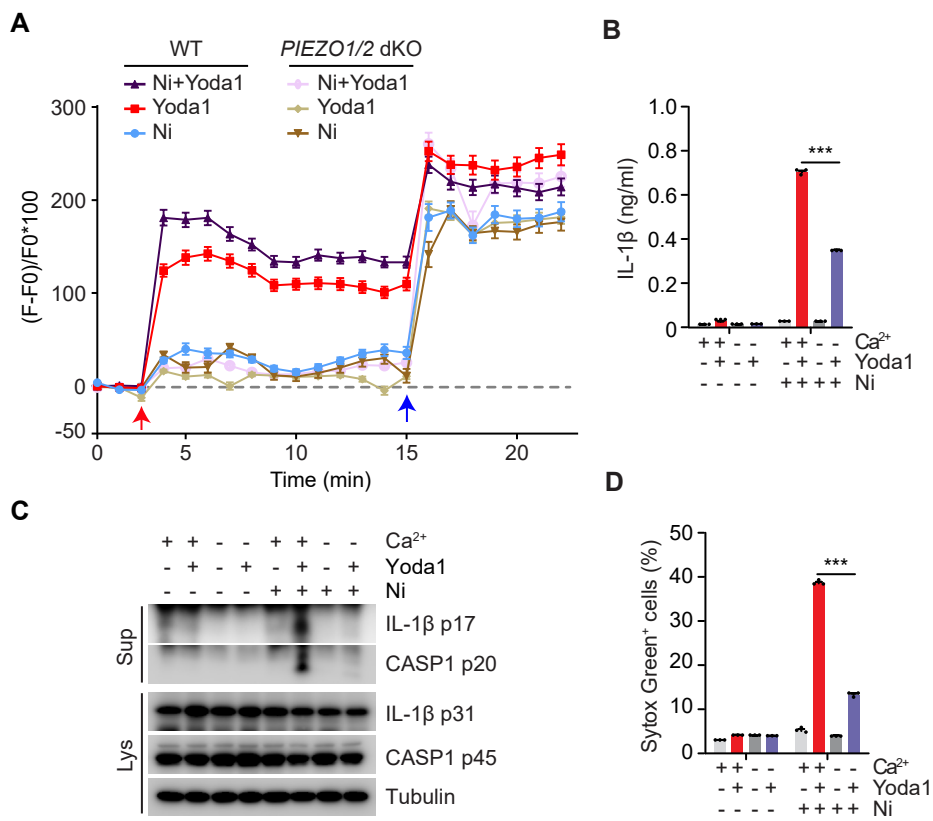


Figure 3

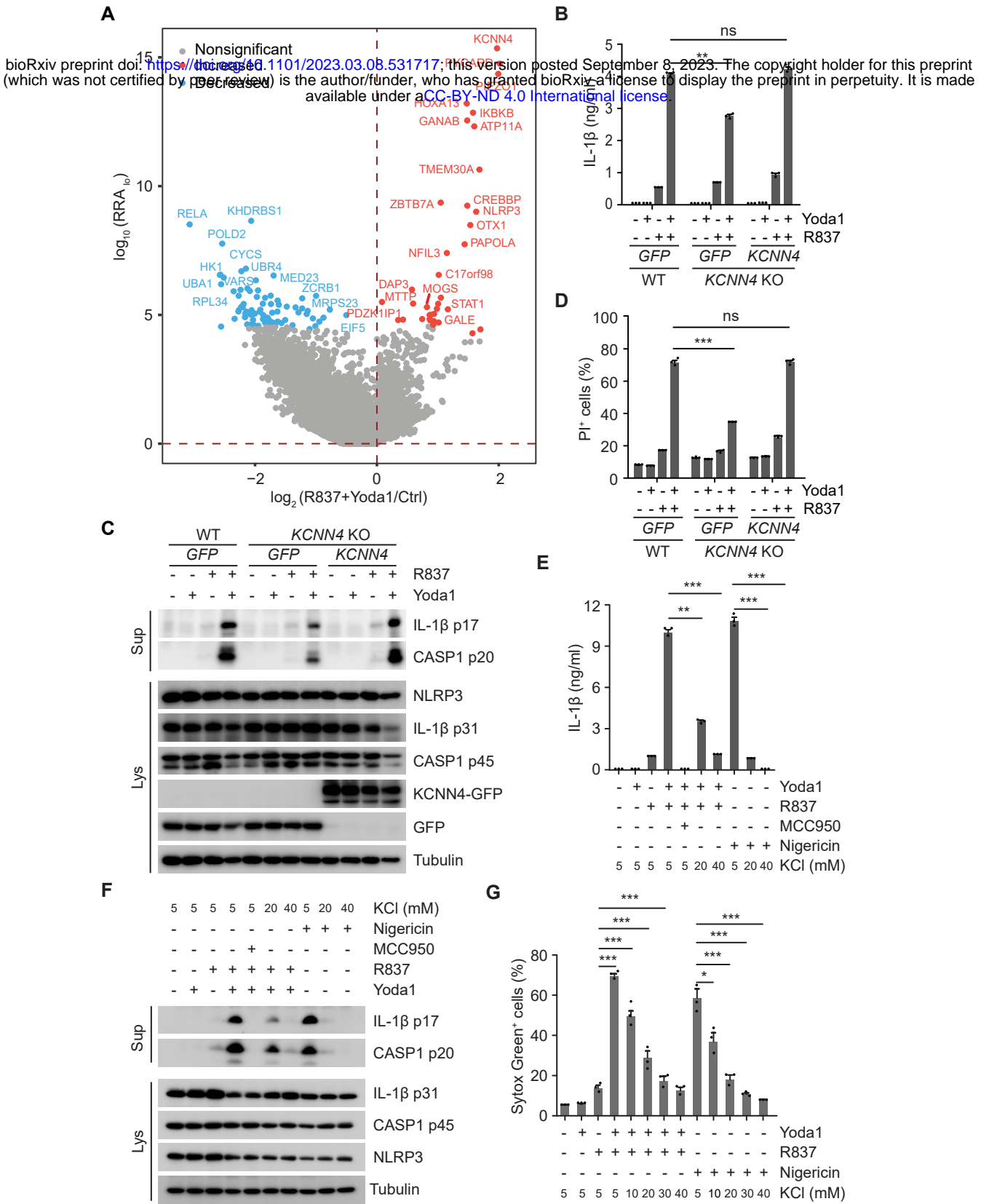


Figure 4

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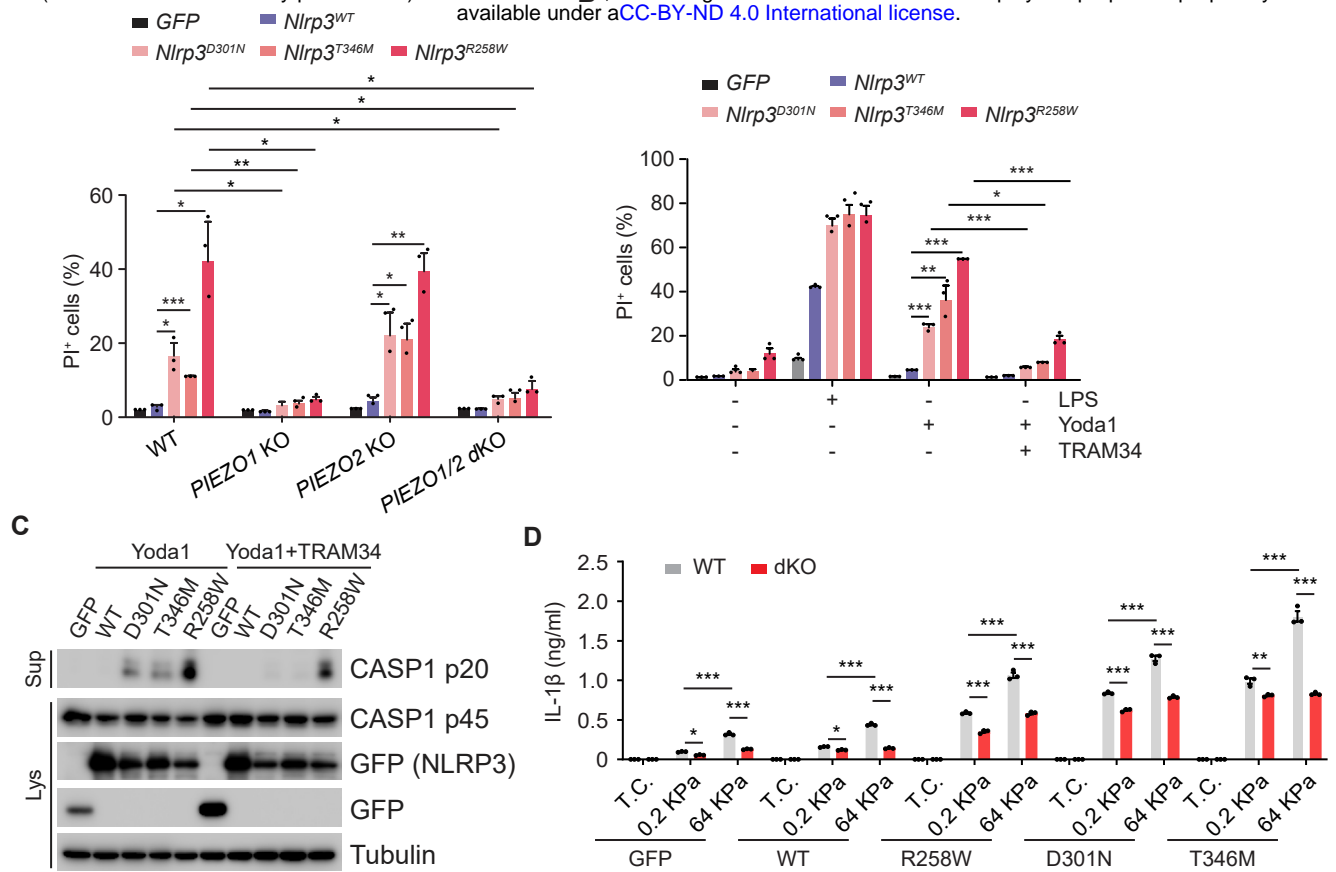


Figure 5

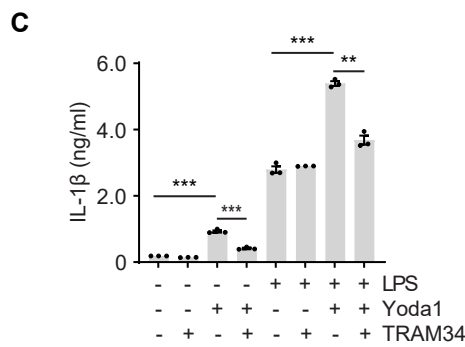
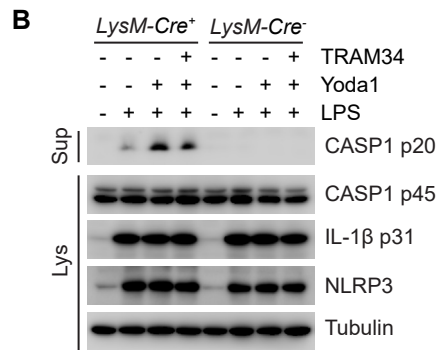
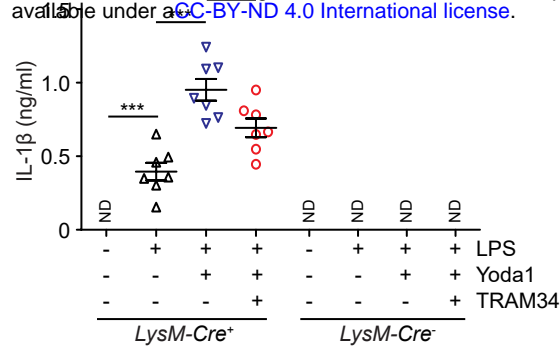
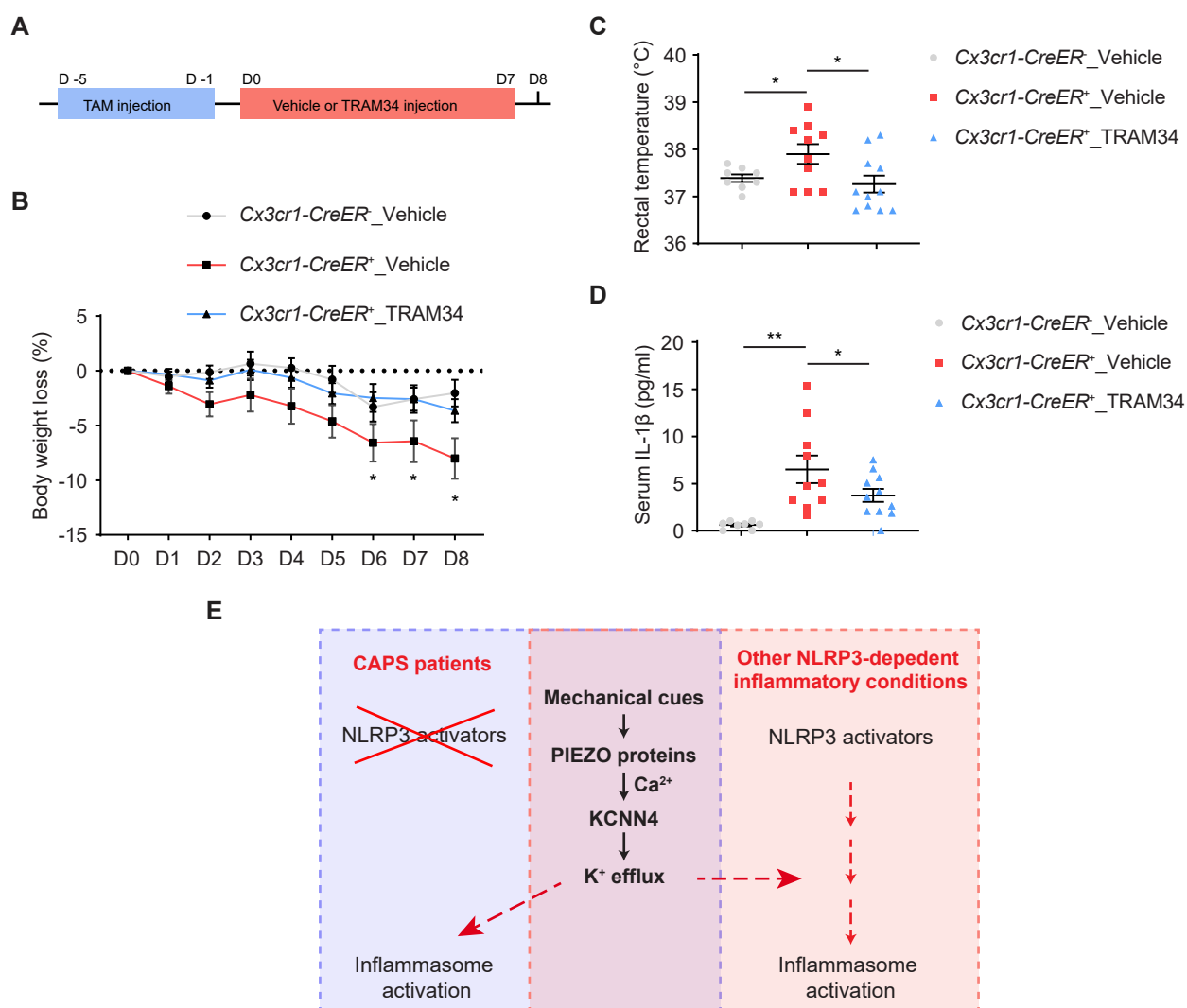


Figure 6



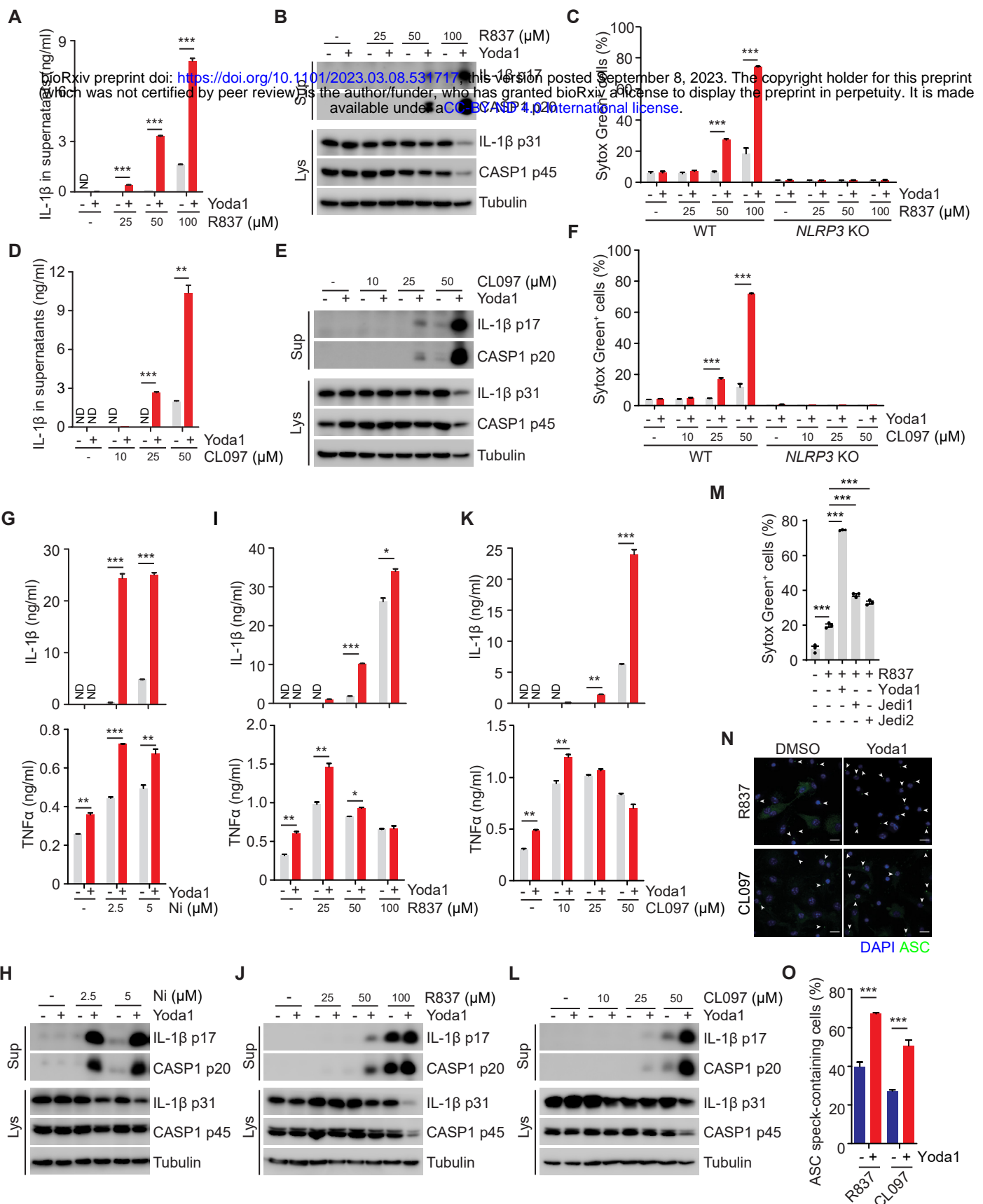


Fig. S1. Activation of PIEZO1 by Yoda1 potentiates R837- and CL097-induced NLRP3 inflammasome activation. (A, D) ELISA measurements of IL-1 β in culture supernatants from THP-1 cells primed with 1 μ g/ml LPS for 3 h and followed by treatment with R837 (A) or CL097 (D). (B, E) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed THP-1 treated as described for panel A and D. Antibodies against IL-1 β and Caspase-1 (CASP1) were used. Antibody against Tubulin was used as a loading control. (C, F) Uptake of Sytox Green in LPS-primed WT and NLRP3 KO THP-1 cells treated as described for panel A and D. (G, I, K) ELISA measurements of IL-1 β and TNF α in culture supernatants from LPS-primed BMDMs treated with nigericin (Ni) (G), R837 (I), CL097 (K) as indicated in presence or absence of 25 μ M Yoda1. (H, J, L) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed BMDMs treated as in panel G, I, K. Antibodies against IL-1 β and Caspase-1 (CASP1) were used. Antibody against Tubulin was used as a loading control. (M) Uptake of Sytox Green in LPS-primed WT THP-1 cells treatment with 100 μ M R837 in presence or absence of 25 μ M Yoda1, 100 μ M Jedi1 or 100 μ M Jedi2. (N) Representative immunofluorescence images of ASC speck formation in LPS-primed BMDMs stimulated with 100 μ M R837 or 50 μ M CL097 in the presence or absence of 25 μ M Yoda1. White arrows indicate ASC specks. Scale bars: 10 μ m. (O) Quantification of macrophages containing ASC specks in panel N. "ND" not detected. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are representative of at least three independent experiments.

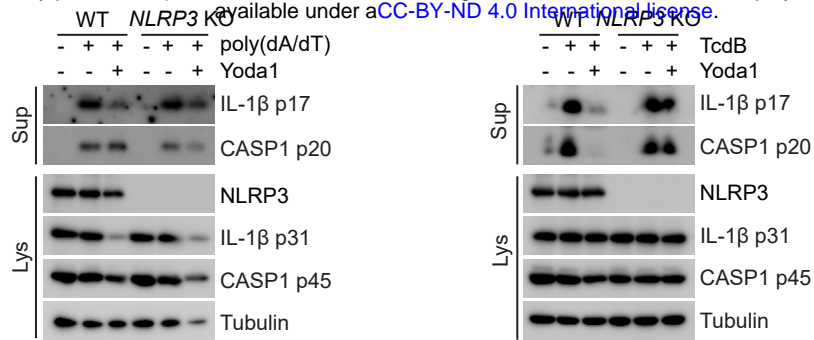


Fig. S2. Yoda1 does not enhance the activity of AIM2- and Pylrin inflammasome. (A, B) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed WT and *NLRP3* KO BMDMs transfected with 1 μ g/ml poly(dA:dT) for 4 h (**A**) or treated with 1 nM TcdB for 2 h (**B**) in presence or absence of 25 μ M Yoda1. Antibodies against IL-1 β and Caspase-1 (CASP1) were used. Antibody against Tubulin was used as a loading control. Data are representative of three independent experiments.

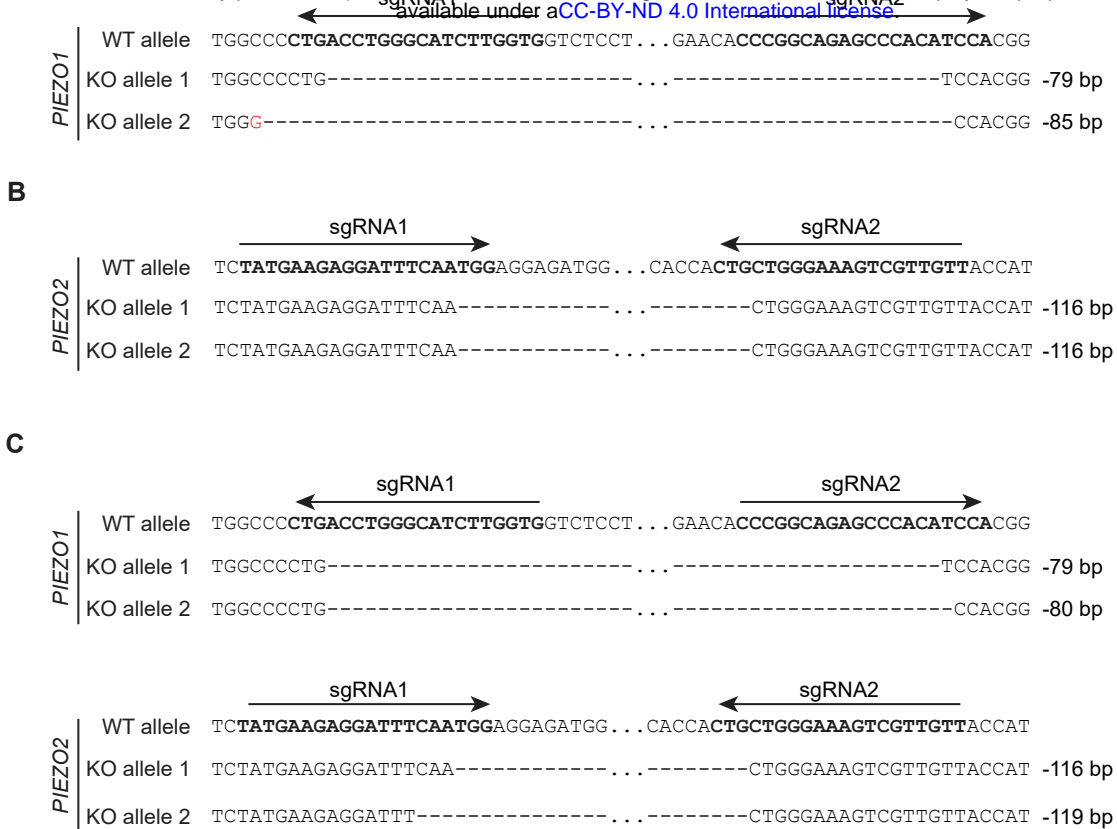
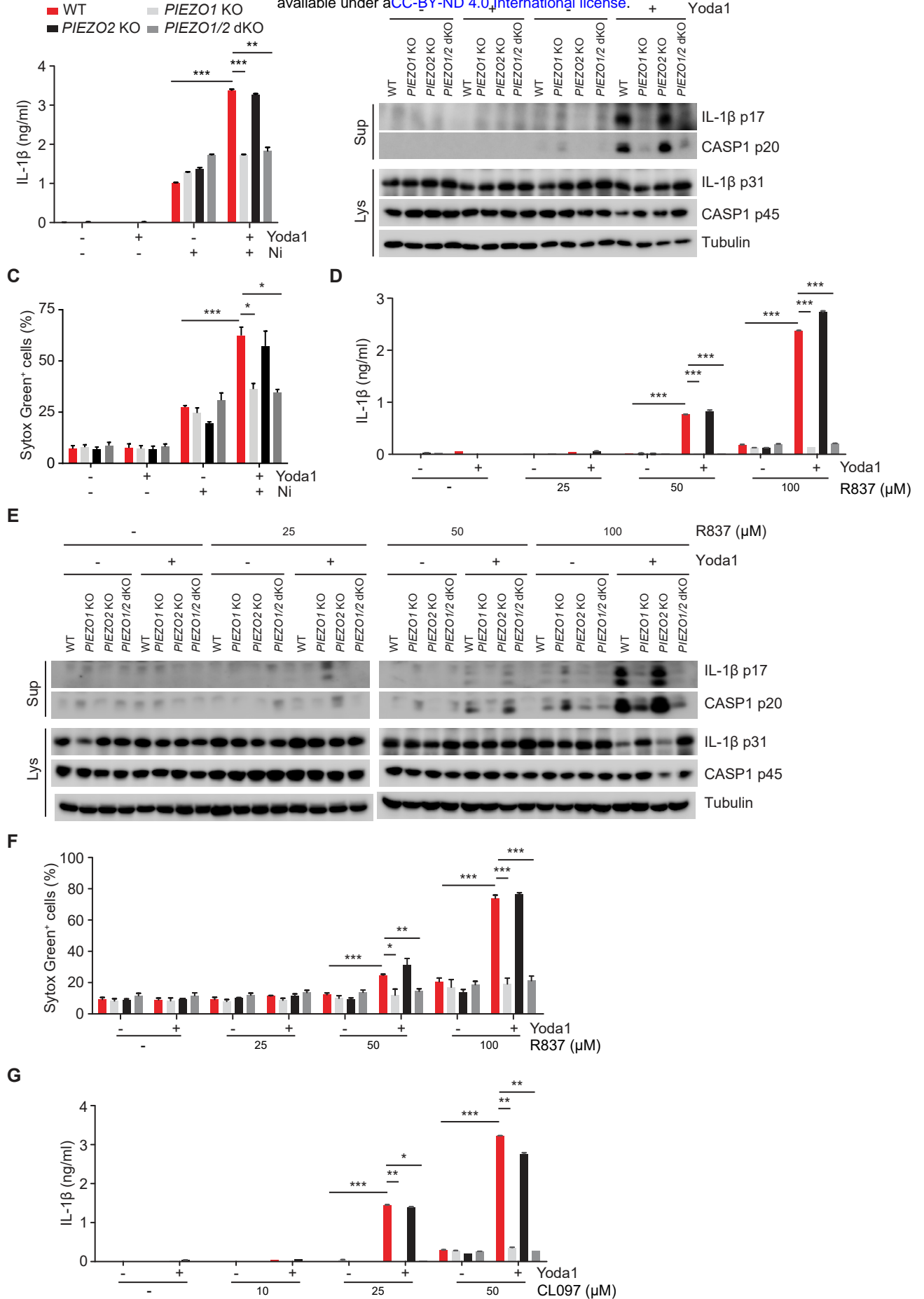


Fig. S3. Validation of *PIEZO1* KO, *PIEZO2* KO and *PIEZO1/2* dKO THP-1 cells. (A-C) Sequences of *PIEZO1* and/or *PIEZO2* alleles validated by Sanger sequencing in *PIEZO1* KO (A), *PIEZO2* KO (B), *PIEZO1/2* dKO (C). The sequences of sgRNAs are indicated by arrows. A mutation is highlighted in red, "-" indicates a deleted nucleotide, "..." in *PIEZO1* alleles indicates a fragment of 34 bps; "..." in *PIEZO2* alleles indicates a fragment of 96 bps. The sequencing was performed once.



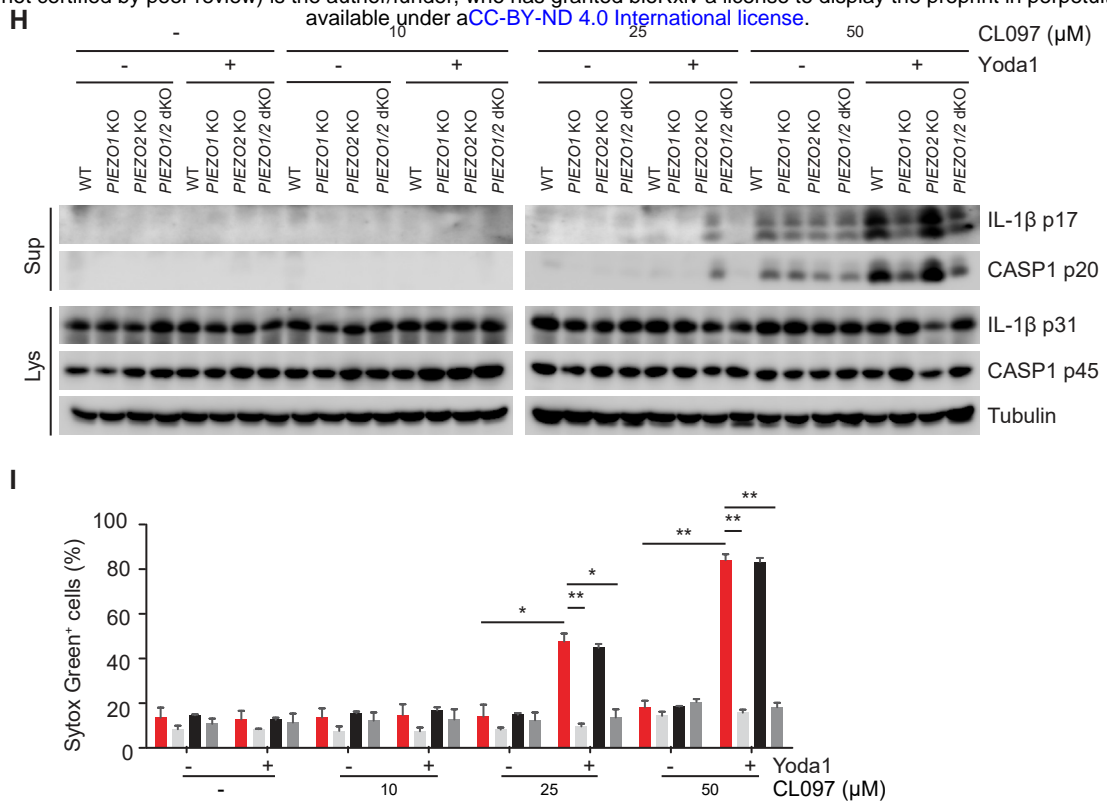


Fig. S4. Deletion of *PIEZO1* abolishes Yoda1-dependent NLRP3 inflammasome activation. (A, D, G) ELISA measurements of IL-1β in culture supernatants from WT, *PIEZO1* KO, *PIEZO2* KO and *PIEZO1/2* dKO THP-1 cells primed with 1 µg/ml LPS for 3 h and followed by treatment with nigericin (A), R837 (D) and CL097 (G) as indicated in presence or absence of 25 µM Yoda1. (B, E, H) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed THP-1 cells in experiments as described for panel A, D and G. Antibodies against IL-1β and Caspase-1 (CASP1) were used. An antibody against Tubulin was used as a loading control. (C, F, I) Uptake of Sytox Green from LPS-primed cells in experiments as described in panel A, D and G. Sytox Green uptake was analyzed by FACS after staining. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are representative of at least three independent experiments.

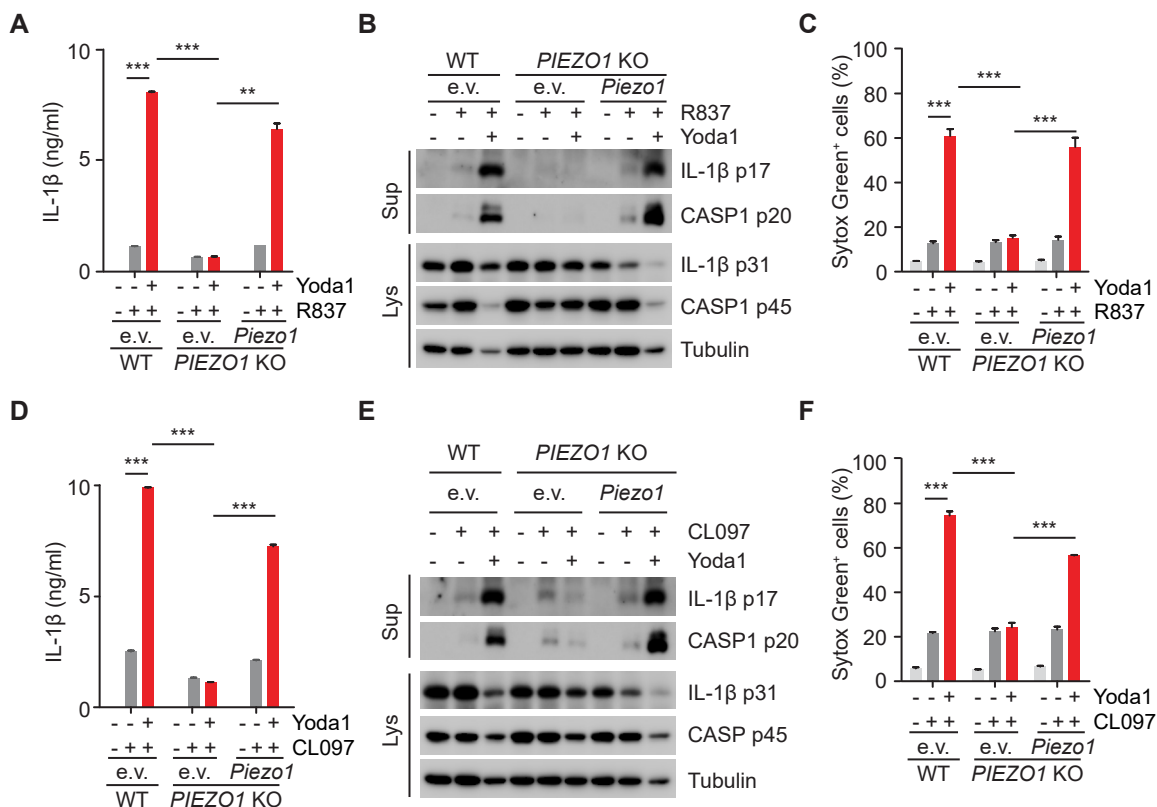


Fig. S5. Re-expression of Piezo1 restores Yoda1-dependent NLRP3 inflammasome activation in *PIEZO1* KO cells. (A, D) ELISA measurements of IL-1 β in culture supernatants in WT and *PIEZO1* KO THP-1 cells expressing empty vector (e.v.) or mouse Piezo1. Cells were primed with 1 μ g/ml LPS for 3 h, followed by treatment with 100 μ M R837 (A), 50 μ M CL097 (D) in presence or absence of 25 μ M Yoda1. (B, E) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed THP-1 cells in experiments as described in panel A and D. Antibodies against IL-1 β and Caspase-1 (CASP1) were used. Antibody against Tubulin was used as a loading control. (C, F) Uptake of Sytox Green from LPS-primed cells in experiments as described for panel A and D. Sytox Green uptake was analyzed by FACS after staining. ** $p < 0.01$, *** $p < 0.001$. Data are representative of at least three independent experiments.

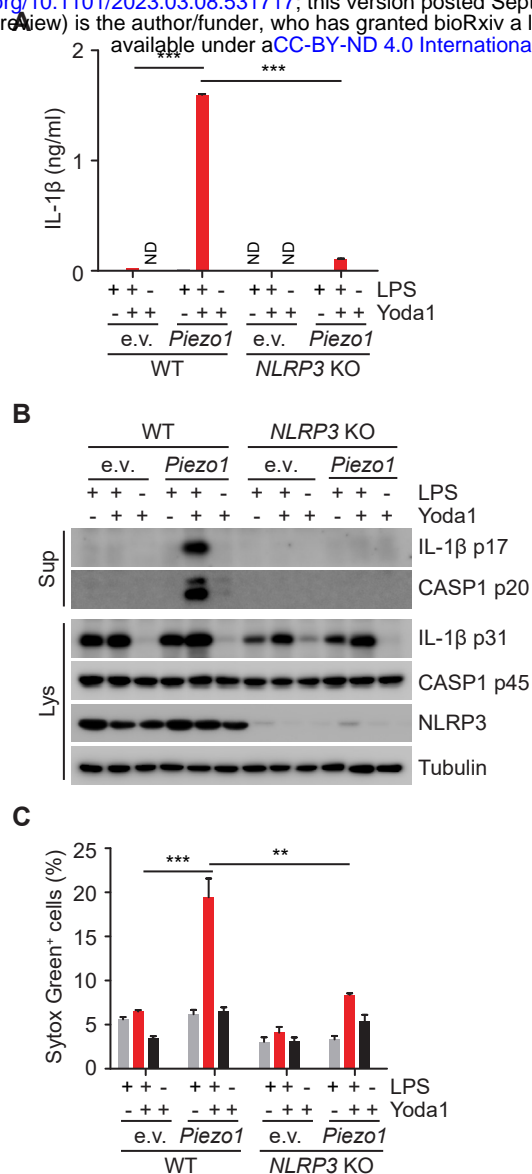


Fig. S6. Yoda1 is sufficient to activate NLRP3 inflammasome in cells ectopically-expressing Piezo1. (A) ELISA measurements of IL-1 β in culture supernatants in WT and *NLRP3* KO THP-1 cells expressing empty vector (e.v.) or mouse Piezo1. Cells were primed with or without 1 μ g/ml LPS for 3 h, followed by treatment with 25 μ M Yoda1. (B) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from cells in experiments as described in panel A. Antibodies against IL-1 β and Caspase-1 (CASP1) were used. An antibody against Tubulin was used as a loading control. (C) Uptake of Sytox Green in cells in experiments as described in panel A. Sytox Green uptake was analyzed by FACS after staining. “ND” not detected. ** $p < 0.01$, *** $p < 0.001$. Data are representative of at least three independent experiments.

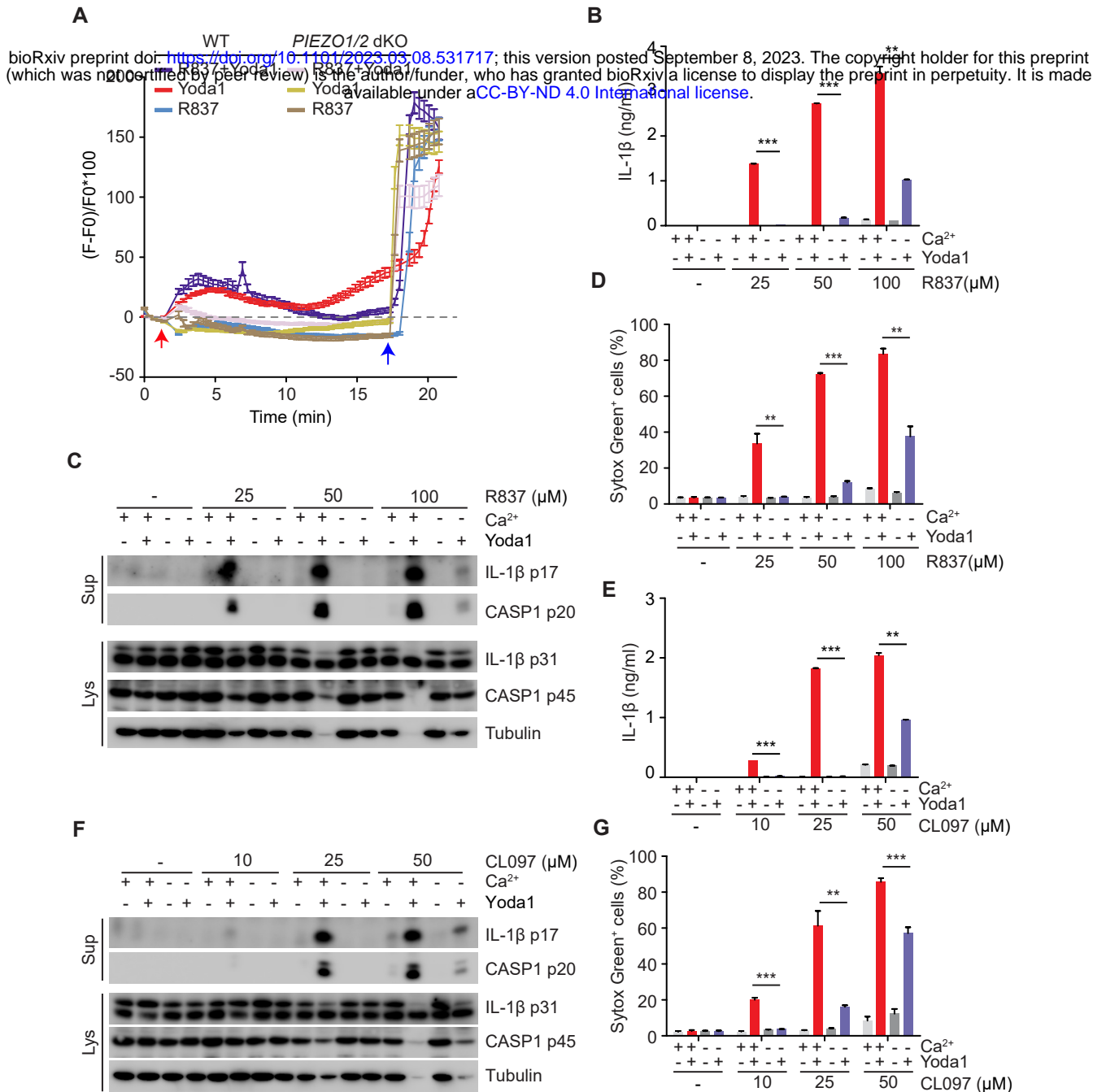


Fig. S7. Ca²⁺ influx is required for PIEZO-dependent NLRP3 inflammasome activation. (A) Measurement of cytosolic Ca²⁺ levels in WT and *PIEZO1/2* dKO THP-1 cells using the Ca²⁺ reporter jGCaMP7s. Cells were treated with 100 μ M R837, 25 μ M Yoda1 or 100 μ M R837 plus 25 μ M Yoda1. The images were acquired using a Nikon spinning-disk microscope with an interval of 20s. Stimuli were added into the culture medium at 1 min as indicated by a red arrow, and ionomycin was added at the end of the experiments as indicated by a blue arrow. Fluorescence intensities of individual cells over time were quantified. n=79 for “WT R837+Yoda1” group; n=96 for “WT Yoda1” group; n=90 for “WT R837” group; n=113 for “*PIEZO1/2* dKO R837+Yoda1” group; n=83 for “*PIEZO1/2* dKO Yoda1” and n=91 for “*PIEZO1/2* dKO R837” group. (B, E) ELISA measurements of IL-1 β in culture supernatants from LPS-primed THP-1 treated with R837 (B), CL097 (E) as indicated in presence or absence of 25 μ M Yoda1 in medium with Ca²⁺ or without (w/o) Ca²⁺. (C, F) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed THP-1 cells in experiments as described for panel B and E. Antibodies against IL-1 β and Caspase-1 (CASP1) were used. An antibody against Tubulin was used as a loading control. (D, G) Uptake of Sytox Green in LPS-primed THP-1 cells in experiments as described in panel B and E. Sytox Green uptake was analyzed by FACS after staining. ***p* < 0.01, ****p* < 0.001. Data are representative of at least three independent experiments.

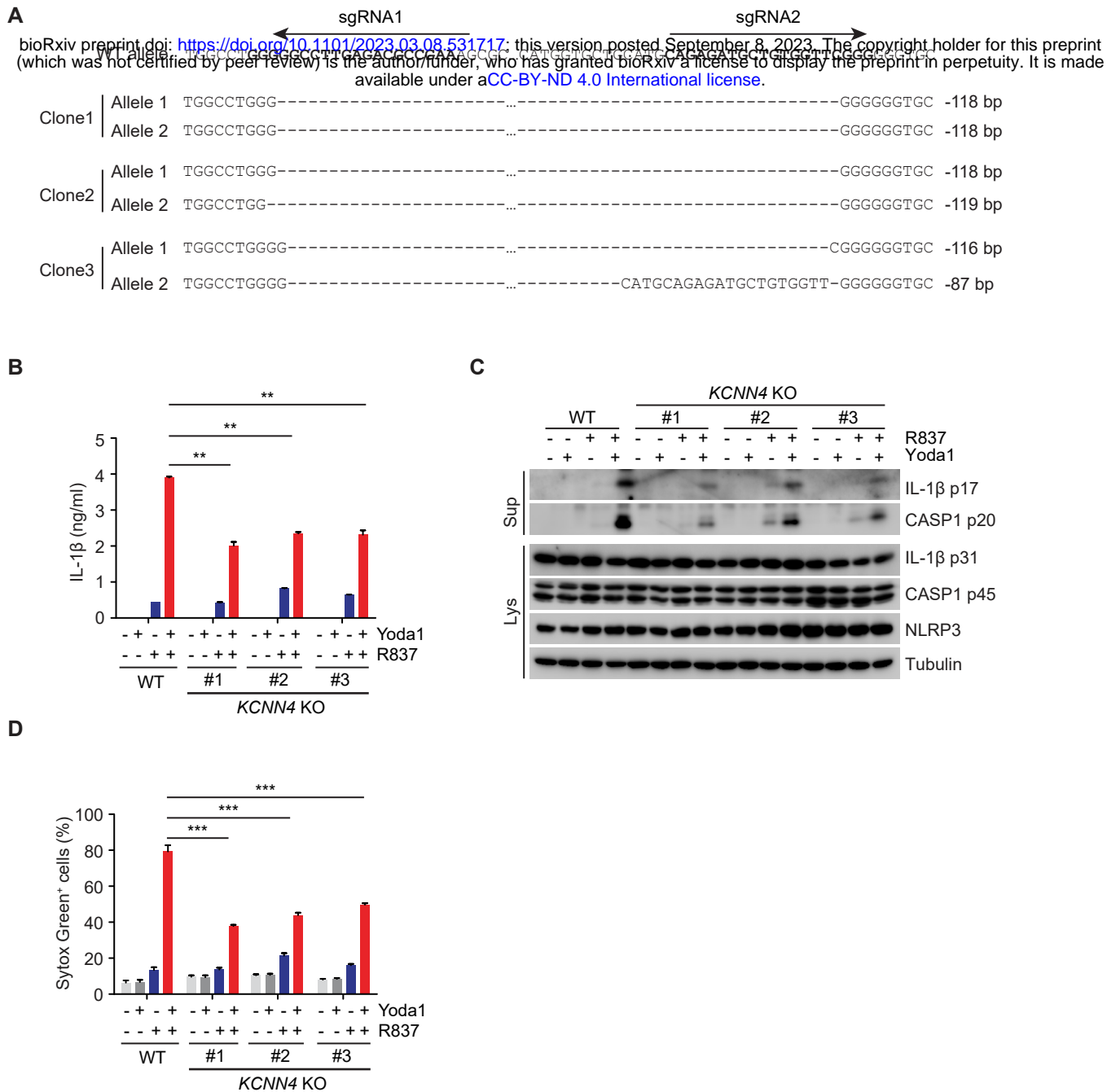


Fig. S8. Deletion of KCNN4 abolishes Yoda1-dependent NLRP3 inflammasome activation. (A) Generation of THP-1 *KCNN4* KO cells using CRISPR/Cas9-mediated gene editing. Validation of *KCNN4* KO THP-1 cells by Sanger Sequencing. sgRNAs were indicated by arrows. “-” means a deleted nucleotide, “...” means a fragment of 65 bps. (B) ELISA measurements of IL-1 β in culture supernatants from WT and *KCNN4* KO THP-1 cells primed with 1 μ g/ml LPS for 3 h and followed by treatment with 100 μ M R837 in presence or absence of 25 μ M Yoda1 for 1 h. (C) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed THP-1 cells in experiments as described for panel B. Antibodies against IL-1 β , Caspase-1 (CASP1) and NLRP3 were used. An antibody against Tubulin was used as a loading control. (D) Uptake of Sytox Green in LPS-primed cells in experiments as described for panel B. ** $p < 0.01$, *** $p < 0.001$. Data are representative of three independent experiments.

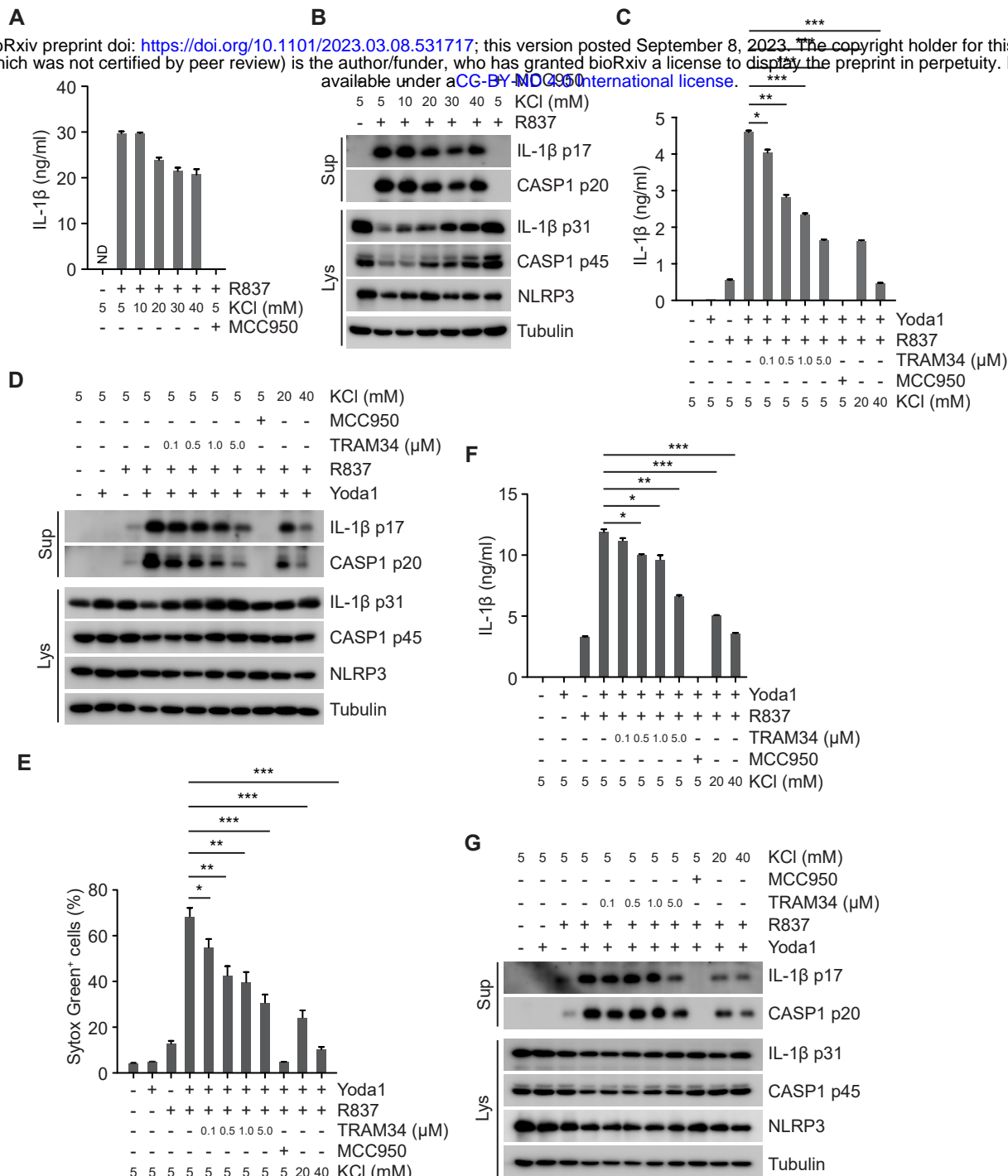


Fig. S9. Inhibition of KCNN4 blocks the effect of Yoda1 on NLRP3 inflammasome activation. (A) ELISA measurements of IL-1β in culture supernatants from LPS-primed BMDMs treated with 100 μM R837 in medium containing indicated concentrations of extracellular KCl for 3 h. (B) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed BMDMs in experiments as described for panel A. Antibodies against IL-1β, Caspase-1 (CASP1) and NLRP3 were used. An antibody against Tubulin was used as a loading control. (C) ELISA measurements of IL-1β in culture supernatants from LPS-primed THP-1 cells treated as indicated with 100 μM R837, 25 μM Yoda1 or 100 μM R837 plus 25 μM Yoda1 in presence of TRAM34, 10 μM MCC950 or extracellular KCl for 1 h. (D) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from THP-1 cells in experiments as described for panel C. (E) Uptake of Sytox Green in THP-1 cells in experiments as described for panel C. (F) ELISA measurements of IL-1β in culture supernatants from LPS-primed BMDMs treated as indicated with 50 μM R837, 25 μM Yoda1 or 50 μM R837 plus 25 μM Yoda1 in presence of TRAM34, 10 μM MCC950 or extracellular KCl for 1 h. (G) Immunoblotting of culture supernatants (Sup) and lysates (Lys) from LPS-primed BMDMs treated as for panel F. Antibodies against IL-1β, Caspase-1 (CASP1) and NLRP3 were used. An antibody against Tubulin was used as a loading control. "ND" not detected. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Data are representative of at least three independent experiments.

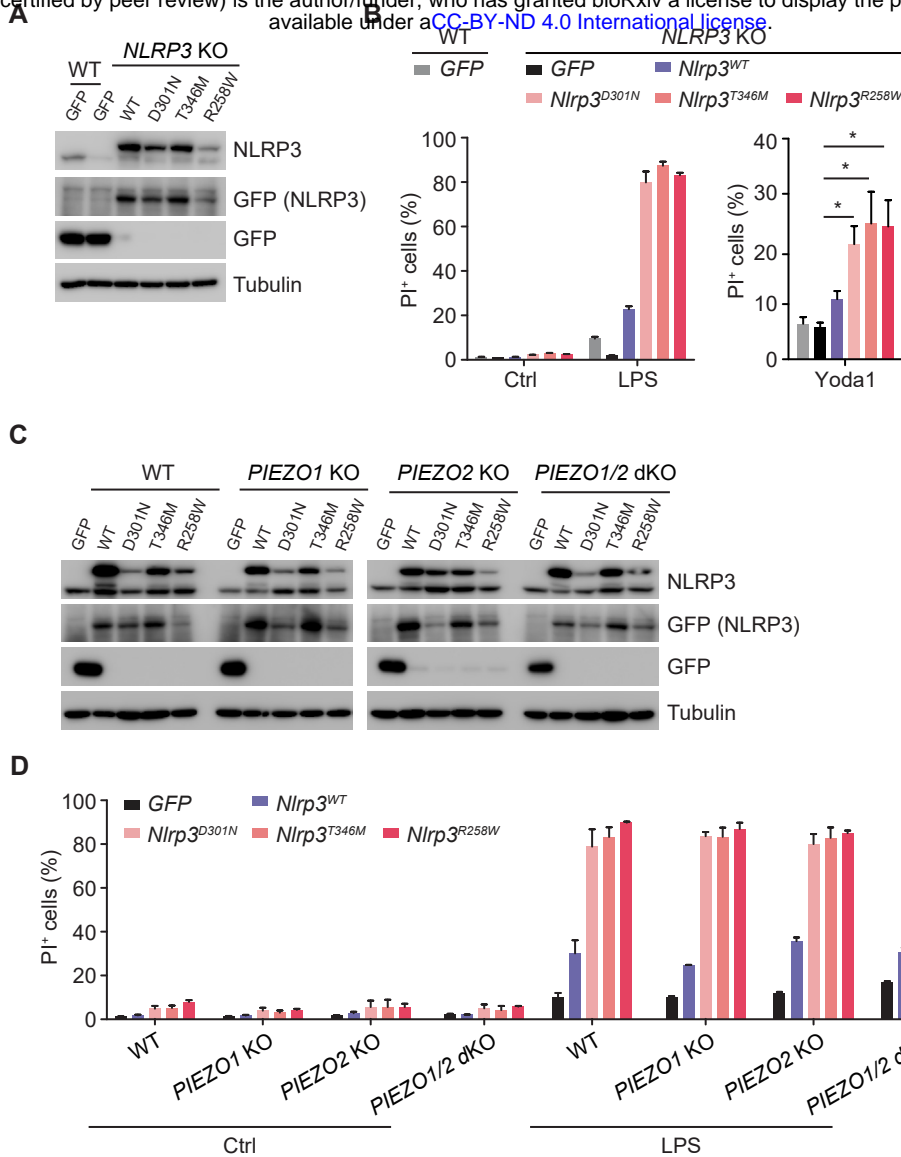


Fig. S10. Yoda1 is sufficient to activate the NLRP3 inflammasome in cells expressing CAPS-causing NLRP3 mutants. (A) Immunoblotting of lysates from WT and *NLRP3* KO THP-1 cells expressing GFP, WT- or D301N-, T346M-, R258W mutated *Nlrp3*. Antibodies against NLRP3 and GFP were used. An antibody against Tubulin was used as a loading control. (B) Uptake of Propidium Iodide (PI) in WT and *NLRP3* KO THP-1 cells expressing GFP, WT or D301N-, T346M-, R258W-mutated mouse *Nlrp3*. Cells were treated with vehicle, 1 μ g/ml LPS or 25 μ M Yoda1. Propidium Iodide uptake was analyzed by FACS after staining. (C) Immunoblotting of lysates from WT, *PIEZO1* KO, *PIEZO2* KO and *PIEZO1/2* dKO THP-1 cells expressing GFP, WT or D301N-, T346M-, R258W-mutated *Nlrp3*. Antibodies against NLRP3 and GFP were used. An antibody against Tubulin was used as a loading control. (D) Uptake of Propidium Iodide (PI) in WT, *PIEZO1* KO, *PIEZO2* KO and *PIEZO1/2* dKO THP-1 cells expressing GFP, WT or D301N-, T346M-, R258W-mutated mouse *Nlrp3*. Cells were treated with vehicle or 1 μ g/ml LPS for 6 h. Propidium Iodide uptake was analyzed by FACS after staining. * $p < 0.05$. Data are representative of at least three independent experiments.