Human non-canonical inflammasomes activate CASP3 to limit intracellular *Salmonella* replication in macrophages.

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Abstract

Inflammasomes are multiprotein signaling platforms that activate inflammatory caspases to induce pyroptosis. In humans, canonical inflammasomes activate CASP1, which cleaves the pore-forming protein gasdermin D (GSDMD) and the cytokines IL-1β and IL-18. In contrast, the non-canonical inflammasome detects bacterial lipopolysaccharide (LPS) through CASP4/5, which cleave GSDMD to drive pyroptosis. While CASP1 substrates are well characterized, CASP4/5 substrates remain less defined. Here, we show that in response to intracellular LPS and gram-negative bacterial infection, CASP4/5 directly cleave and activate the executioner caspases CASP3/7. CASP3 in turn cleaves and activates gasdermin E (GSDME). Surprisingly, CASP3, but not GSDME, was required for restricting intracellular *Salmonella* replication, suggesting that CASP4/5-induced apoptosis contributes to host defense. We further show that most GSDMD cleavage during non-canonical inflammasome activation is mediated by CASP1, and that GSDMD is the primary driver of pyroptosis. Finally, we confirm that CASP4/5 activate CASP3/7 and GSDME in human primary macrophages. These findings establish CASP4/5 as dual apoptotic initiator and inflammatory caspases and reveal a central role for the apoptotic signaling cascade in non-canonical inflammasome-mediated immunity.

32 **Introduction**

33 The detection and elimination of invading pathogens by the immune system is essential for host
34 survival. It is critical that the immune response to invading pathogens be tightly regulated such
35 that an appropriate response can be mounted without excessive inflammation, a condition known
36 as sepsis. Sepsis is a life threatening condition which mainly occurs in children under the age of
37 five and is one of the leading causes of death worldwide[1,2].

38 A key component of the host response to invading pathogens is the assembly of multi-protein
39 signaling complexes called inflammasomes[3]. Inflammasome assembly activates cysteine
40 proteases called caspases that induce inflammation and lytic cell death known as pyroptosis,
41 which is critical for infection control[4,5]. The canonical inflammasome pathway activates caspase-
42 1 (CASP1), while the non-canonical pathway activates caspase-4 and caspase-5 (CASP4/5) in
43 humans and caspase-11 (CASP11) in mice, collectively referred to as inflammatory caspases[6,7].
44 CASP1 activation can be triggered by diverse stimuli, such as host or pathogen proteases, and
45 potassium efflux[8-11]. In contrast, CASP4/5 and -11 sense and are activated by intracellular
46 lipopolysaccharide (LPS) from Gram-negative bacteria like *Salmonella enterica* serovar
47 Typhimurium (*Stm*), an intracellular pathogen that is a major cause of food-borne disease
48 globally[6,12-14]. A recent study demonstrated that while CASP1 is important during early infection,
49 CASP4 is essential in preventing intracellular *Salmonella* replication at late stages of infection in
50 human macrophages[15]. However, the mechanism by which CASP4 restricts replication at late
51 stages remains unclear.

52 The substrates of inflammatory caspases are crucial mediators of immunity yet their full repertoire
53 and functional significance remain incompletely defined[16]. Human inflammatory caspases are
54 known to cleave the interleukin family of cytokines, IL-1β and IL-18, and the pore-forming
55 protein gasdermin D (GSDMD), leading to pyroptosis[17-23]. In this manner, pyroptosis protects the
56 host from infection[24-28]. In the absence of GSDMD, CASP1 can process the apoptotic executioner
57 caspases-3 and -7 (CASP3/7) to induce apoptosis[29]. A proteomic screen also identified CASP7
58 as a substrate of inflammasome-activated CASP1 in mice, but in the same study, CASP3
59 activation was found to be independent of CASP1[30]. It was later shown that, like human cells,
60 CASP1 could mediate CASP3 processing in mice to induce apoptosis in the absence of GSDMD
61 when infected with *Salmonella enterica* serovar Typhimurium or poly(dA:dT)[31,32]. This process
62 was dependent on CASP1 cleaving Bid (BH3-interacting domain death agonist), which
63 subsequently induced caspase-9 (CASP9)-dependent activation of CASP3[31,32]. These findings

64 suggest that CASP1 activation can lead to either direct or indirect activation of executioner CASP3
65 and -7 and that inflammatory caspases can initiate apoptosis.

66 Apoptosis is a regulated form of cell death that maintains organismal development and
67 homeostasis and is generally thought to be immunologically silent. Emerging evidence suggests
68 that the induction of apoptosis during infections may have important consequences for host
69 defense. For example, dendritic cells activate both pyroptosis and apoptosis during late stages of
70 infection and this is essential for restricting *Legionella pneumophila* replication[33,34]. Similarly,
71 activation of the apoptotic signaling cascade during viral infection led to CASP3-dependent
72 cleavage of gasdermin E (GSDME), which induced pyroptosis and protected human skin
73 organoids from viral replication[35,36]. Whether the induction of apoptosis or CASP3 and GSDME-
74 mediated pyroptosis contributes broadly to host defense remains unknown. Moreover, while
75 CASP1 is well-established to activate the apoptotic signaling cascade, whether human non-
76 canonical inflammasomes (CASP4/5) similarly cleave apoptotic caspases remains less clear.

77 Substrates of the non-canonical inflammasome pathway are generally not well-defined. A recent
78 proteomics screen identified CASP7 as a substrate of CASP4 in humans, though its functional
79 significance remained unknown[37]. We also recently reported that IL-1β and IL-18 are substrates
80 of CASP4/5[21]. Notably, in CASP4/5-deficient cells, we observed a loss of CASP3 activation in
81 response to intracellular LPS, suggesting that the human non-canonical inflammasomes engage
82 the apoptotic pathway. However, whether this is a direct processing event and its functional
83 significance regarding pathogen defense remained to be elucidated.

84 In this study, we identify CASP3 and CASP7 as direct substrates of human inflammatory
85 caspases. We found that CASP4/5 directly cleave CASP3/7, and active CASP3 subsequently
86 cleaves and activates GSDME during intracellular LPS stimulation and *Salmonella* infection.
87 CASP3-induced GSDME-mediated pyroptosis has been described in the context of
88 chemotherapeutics activating apoptotic signaling or during cytotoxic lymphocyte-mediated cell
89 death[38], but this pathway has not been known to be activated during non-canonical inflammasome
90 activation. We demonstrate that CASP3/7 and GSDME processing occurs in primary human cells
91 in response to intracellular LPS and *Salmonella* infection. Interestingly, we discovered that
92 GSDME was dispensable, but CASP3 was required for inhibiting
93 intracellular *Salmonella* replication, demonstrating that non-canonical inflammasomes mediate
94 host defense in part by activating the apoptotic signaling cascade.

Results

Human inflammatory caspases directly cleave and activate executioner CASP3 and CASP7

Because previous work has shown that CASP1 can cleave CASP3/7, and CASP4 has been implicated in CASP7 processing[37], we first investigated whether inflammatory caspases directly target CASP3 and -7. We expressed human GSDMD (as a control), CASP3, or CASP7 in HEK 293T cells and incubated lysates with recombinant active CASP1, CASP4, CASP5, CASP8, or mouse CASP11 for 1 or 3 hours. As expected, inflammatory caspases efficiently processed GSDMD at both time points (Fig. 1A). CASP1, CASP4, CASP5 and CASP8 processed human CASP3 (Fig. 1B). Mouse CASP11 treated lysates exhibited very minimal processing of human CASP3 at prolonged incubation, suggesting a divergence between human and murine non-canonical inflammasomes. This aligns with prior reports that CASP4/5 can cleave human IL-18, whereas CASP11 cannot cleave mouse IL-18[20-22]. Surprisingly, only CASP1 and CASP4 processed CASP7 in this assay (Fig. 1C), despite the well-established role of CASP8 in cleaving both CASP7 and CASP3[39]. (Fig. 1B,C), indicating that complementary methods are needed to confirm processing.

We next sought to determine whether active inflammatory caspases would cleave CASP3/7 in cells. We generated *CASP3/7* double knockout (KO) HEK 293T cells (Fig. S1A) and co-transfected these cells with either active or catalytically inactive human inflammatory caspases along with catalytically inactive CASP3 or CASP7. Consistent with our *in vitro* assays, all inflammatory caspases processed catalytically inactive CASP3 and CASP7 (Fig. S1B,C), and this processing was dependent on their catalytic activity and not on the catalytic activity of CASP3 or CASP7.

We also confirmed that inflammatory caspases cleave CASP3/7 using an orthogonal activation mechanism. We utilized our previously established dimerizable inflammatory caspase cell system in which cells express a modified caspase where the caspase activation and recruitment domains (CARD) are swapped with a dimerizable domain (DmrB), allowing selective activation of CASP1, CASP4, or CASP5 by the small molecule drug AP20187[21]. HEK 293T cells expressing DmrB-caspases and IL-18, a known substrate of inflammatory caspases that serves as a positive control, were transfected with either CASP3 or CASP7. Following AP20187 treatment, we observed activation of the DmrB-CASP1, -4, and -5, as indicated by IL-18 cleavage (Fig. S1D-I). Consistent with our *in vitro* biochemical assays, activated CASP1, -4, and -5 all processed CASP3

126 (Fig. S1D-F) and CASP7 (Fig. S1G-I). These findings suggest that upon direct activation in cells,
127 inflammatory caspases cleave the executioner caspases CASP3 and CASP7.

128 Caspases cleave peptide bonds at aspartic (Asp) acid residues[40,41]. During apoptosis, the initiator
129 caspases (CASP8/9) activate executioner caspases by cleaving CASP3 at Asp175 and CASP7
130 at Asp198, generating catalytically active large and small subunits[30,42]. Because inflammatory
131 caspase processing of CASP3/7 produced fragments that were detected by antibodies specific
132 for their active forms, we hypothesized that this cleavage activates the executioner caspases. To
133 test this, we co-expressed catalytically competent CASP3 (Fig. 1D) or CASP7 (Fig. 1E) with
134 GSDME, a known substrate of CASP3[43], along with either catalytically active or inactive human
135 inflammatory caspases in HEK 293T *CASP3/7* KO cells. Consistent with their activation, CASP3,
136 and to a lesser extent CASP7, induced GSDME cleavage, indicating that inflammatory caspases
137 cleave and activate CASP3/7. We ruled out that inflammatory caspases do not directly process
138 GSDME through recombinant protein assays. Lysates from GSDME-expressing HEK293T cells
139 were incubated with apoptotic (CASP3, CASP6, CASP7, CASP8) or inflammatory (CASP1,
140 CASP4, CASP5, CASP11) caspases (Fig. 1C). As expected, CASP3 robustly cleaved GSDME,
141 while CASP7 produced only minimal processing after prolonged incubation, suggesting CASP7-
142 mediated GSDME cleavage is possible but likely not physiologically relevant. Collectively, these
143 findings demonstrate that inflammatory caspases directly cleave and activate CASP3/7, and that
144 CASP3 is the primary mediator of GSDME cleavage.

145 **CASP1, CASP8, and CASP9 are dispensable for CASP3/7 and GSDME processing during**
146 **non-canonical inflammasome activation by intracellular LPS.**

147 Our in vitro and cellular assays indicated that inflammatory caspases cleave and activate
148 CASP3/7, and that CASP3 can subsequently cleave GSDME to drive pyroptosis[38]. Although
149 CASP3-mediated GSDME cleavage has been shown to induce pyroptosis, whether GSDME
150 contributes to non-canonical inflammasome mediated cell death is unknown. To address this, we
151 transfected LPS into PMA-differentiated THP-1 macrophages to activate CASP4/5 (Fig. 2A).
152 CASP1 can cleave CASP3/7 and can be activated via the NLRP3 inflammasome downstream of
153 CASP4/5 due to K+ efflux through GSDMD pores[8,9,44]. Prior work indicated that during CASP1
154 activation, both GSDME and GSDMD contribute to cell death and cytokine release[45]. To rule out
155 a contribution from downstream CASP1 activation, we pretreated THP1 cells with the NLRP3
156 inhibitor MCC950[46] prior to LPS transfection (Fig. 2A). LPS treatment resulted in CASP3, CASP7,
157 and GSDME cleavage. As expected, LPS also induced robust GSDMD cleavage and CASP1

158 activation, as evidenced by the appearance of the cleaved 30 kDa fragment of GSDMD and p10
159 species of CASP1 by immunoblotting (Fig. 2A). MCC950 abolished CASP1 activation, which led
160 to a slight decrease in GSDMD processing (Fig. 2A). Interestingly, we observed increased
161 CASP3, CASP7 and GSDME cleavage in MCC950-treated cells compared to LPS-only treated
162 cells (Fig. 2A), suggesting that in the absence of CASP1, CASP4/5 activation induces robust
163 processing and activation of CASP3/7, and GSDME. Consistent with this notion and the increased
164 CASP3/7 processing, we observed increased CASP3/7 activity compared to control and LPS-
165 only treated cells (Fig. 2C) using an intracellular CASP3/7 activity reporter dye. Despite loss of
166 CASP1 activation, the kinetics of pyroptosis remained unchanged in MCC950-treated cells
167 compared to LPS-only treated cells, as assessed by Sytox Green uptake, likely due to elevated
168 GSDME processing (Fig. 2B).

169 Because GSDME can be processed by CASP3 downstream of the extrinsic and intrinsic
170 apoptotic pathways mediated by CASP8 and CASP9 respectively[43], we next sought to genetically
171 dissect the contribution of CASP1, CASP8, and CASP9 to CASP3 activation and GSDME
172 processing. In *CASP1* KO THP-1 cells, LPS still triggered robust cleavage of CASP3, CASP7,
173 and GSDME, accompanied by increased CASP3/7 activity (Fig. 2D-F). In contrast, GSDMD
174 processing was nearly abolished, confirming that most GSDMD cleavage downstream of non-
175 canonical inflammasome activation is driven by CASP1. This aligns with our previous
176 observations that in cells, GSDMD is a poor substrate for CASP4 and -5 compared to CASP1[21].
177 Importantly, these results indicate that CASP1 is not required for CASP3/7, and GSDME
178 processing.

179 Both CASP8 and CASP9 are known to activate CASP3 and CASP7 to induce apoptosis, a
180 process constantly ongoing to maintain cellular homeostasis[47,48]. Like in *CASP1* KO cells, deletion
181 of CASP8 (Fig. 2G-I) or CASP9 (Fig. 2J-L) did not block CASP3/7 or GSDME processing, nor
182 alter GSDMD cleavage or pyroptosis. In fact, CASP3/7 activity was modestly increased,
183 suggesting that CASP8 or -9 may normally compete for CASP3/7 engagement.

184 Finally, because CASP1 is known to cleave Bid, a member of the Bcl-2 family of proteins that
185 activates CASP9 to induce CASP3/7 cleavage[31,32,49], we sought to determine whether CASP4/5
186 activate CASP3/7 indirectly through activation of Bid. We incubated lysates from THP-1
187 macrophages with equivalent activity units of recombinant caspases and assessed processing of
188 substrates at different time points by immunoblotting. As expected, CASP1 and CASP8
189 processed Bid into the truncated form (Fig. 2M). However, CASP4 and CASP5 failed to cleave

190 Bid, but were active and able to process IL-18 and GSDMD (Fig. 4M). In this assay, where multiple
191 substrates are present simultaneously, IL-18 processing appeared to be favored by CASP4
192 compared to other caspases while GSDMD processing was favored by CASP1 (Fig. 2M). Taken
193 together, these data indicate that CASP1, CASP8, and CASP9 are dispensable for CASP3/7
194 activation during noncanonical inflammasome activation and support a model in which CASP4/5
195 directly cleave CASP3/7 to promote CASP3/GSDME-mediated pyroptosis.
196
197 **CASP3, and CASP4/5 are required to induce GSDME processing during non-canonical**
198 **inflammasome activation by intracellular LPS.**

199 Our findings above suggest that CASP4/5 activate the executioner CASP3/7, leading to
200 CASP3/GSDME-mediated pyroptosis. To directly test this, we transfected WT, *CASP4/5* KO,
201 *CASP3* KO, or *CASP7* KO cells with LPS (Fig. 3A-I). We then assessed CASP3/7 and GSDME
202 processing by immunoblotting, cell death by Sytox Green uptake, and CASP3/7 activity using a
203 cell permeable reporter dye. Loss of CASP4/5 significantly impaired CASP3/7 and GSDME
204 cleavage, cell death, and CASP3/7 activity compared to control cells in response to intracellular
205 LPS (Fig 3A-C). *CASP3* KO cells lacked GSDME cleavage but exhibited increased GSDMD
206 processing in LPS transfected cells (Fig. 3D). Despite loss of GSDME processing, pyroptosis was
207 not significantly diminished (Fig. 3E), suggesting functional compensation through enhanced
208 GSDMD activation. CASP3 deficiency slightly reduced overall CASP3/7 activity, likely due to
209 preserved CASP7 activity (Fig. 3F). Unexpectedly, *CASP7* KO cells retained CASP3 activation
210 and GSDME processing (albeit less than control cells), but CASP1 processing into the p10
211 species and GSDMD processing were nearly abolished, resulting in delayed cell death (Fig.
212 3G,H). *CASP7* KO cells also had attenuated CASP3/7 activity, as expected (Fig. 3I). Taken
213 together, these findings demonstrate that CASP4/5 activate CASP3/7, with CASP3 serving as the
214 primary protease that cleaves GSDME. While CASP3/GSDME activation can drive pyroptosis
215 downstream of the non-canonical inflammasome in part, loss of CASP3 is buffered by increased
216 GSDMD activity, highlighting plasticity in the execution of pyroptosis.
217
218 Mammals encode six gasdermins: gasdermin A, B, C, D, E, and PJVK (also known as
219 DFNB59)[36,50,51]. Typically, their N-terminal pore-forming domains are autoinhibited by the C-
220 termini, but upon cleavage, the N-termini are liberated and form pores to induce pyroptosis.
221 Inflammatory caspases cleave GSDMD to induce pyroptosis[17-19]. While CASP3 has been reported
222 to cleave GSDME to induce pyroptosis, this has primarily been described in the context of

223 chemotherapy-induced apoptosis or cytotoxic lymphocyte-mediated cell death[38,52,53]. Whether
224 CASP3/GSDME contributes to pyroptosis during non-canonical inflammasome activation has not
225 been established.

226 To address this, we transfected WT, *GSDMD* KO, *GSDME* KO, or *GSDMD/E* double KO THP-1
227 macrophages with LPS and assessed gasdermin processing, cell death, CASP3/7 activation, and
228 IL-1α/β release (Fig. 3J-M). Immunoblots revealed increased CASP3/7 processing, but decreased
229 CASP1 processing in *GSDMD*, *GSDME*, or *GSDMD/E* KO cells compared to control cells with
230 LPS transfection (Fig. 3J), suggesting that both GSDMD and GSDME may contribute to
231 downstream CASP1 activation via the NLRP3 inflammasome. Loss of either GSDMD or GSDME
232 led to increased CASP3 and CASP7 activation compared to WT cells. However, the CASP3/7
233 activity was only significantly increased in *GSDME* KO cells (Fig. 3L). GSDME processing was
234 increased in *GSDMD* KO cells, but GSDMD processing was attenuated in *GSDME* KO cells
235 compared to WT cells (Fig. 3J). Interestingly, GSDME loss did not affect cell death, whereas
236 GSDMD deficiency markedly reduced lysis, and *GSDMD/E* double KO cells phenocopied
237 *GSDMD* KO cells (Fig. 3K), implying that GSDMD is the principal driver of lytic cell death in
238 response to intracellular LPS.

239 In contrast, IL-1α/β release required both gasdermins, as assessed by the HEK Blue assay which
240 reports on the activity of active IL-1α/β[21] (Fig. 3M). Either GSDMD or GSDME was sufficient to
241 release similar amounts of active IL-1α/β as WT cells, but loss of both GSDMD/E resulted in
242 significantly attenuated cytokine release (Fig. 3M), consistent with reports that both contribute to
243 cytokine release during canonical inflammasome signaling[45]. Altogether these findings suggest
244 that both GSDMD and GSDME contribute to cytokine release during non-canonical
245 inflammasome activation but GSDMD is the primary driver of pyroptosis.

246 **CASP3, and CASP4/5 are required for GSDME processing during non-canonical**
247 **inflammasome activation by intracellular *Salmonella*.**

248 The non-canonical inflammasomes detect gram-negative bacteria. We next asked if CASP4/5
249 cleave and activate CASP3/7 during bacterial infections. We infected WT, *CASP4/5* KO, and
250 *CASP3* KO cells with *Salmonella enterica* serovar Typhimurium (*Stm*) then assessed CASP3/7
251 and GSDME processing by immunoblotting, cell death by Sytox Green uptake, and CASP3/7
252 activity using a cell permeable reporter dye. Log-phase *Salmonella* induces expression of the type
253 3 secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) that activates

254 both canonical and non-canonical inflammasome signaling [54-57]. Thus, we first infected WT and
255 *CASP4/5* KO (Fig. S2A,B) or *CASP3* KO THP1-1 macrophages with *Salmonella* grown to
256 stationary phase (*Stm*$^{Stat.}$) to minimize the contribution of the canonical caspase-1 signaling
257 pathway then measured cell death and processing of caspases and gasdermins by
258 immunoblotting. Since CASP4 is thought to contribute to resolving infection at later time in
259 infection, we tested different multiplicity of infections (MOIs) to mimic different infection burdens.
260 In control cells, we observed dose-dependent cleavage of GSDME and GSDMD (Fig.
261 S2A,C). Importantly, CASP3/7 and GSDME processing was largely abrogated in *CASP4/5* KO
262 cells while GSDMD processing was retained, likely due to the activation of the NLRP3
263 inflammasomes (Fig. S2A)[57-59]. GSDME, but not GSDMD processing, was also abrogated in
264 *CASP3* KO cells (Fig. S2C). Interestingly, despite cleavage of the pore-forming proteins, we
265 observed little to no cell death by Sytox Green (Fig. S2B,D). This indicates that CASP3/7
266 activation and subsequent GSDME cleavage is CASP4/5-dependent, but processing does not
267 generate enough pores to induce cell lysis with stationary phase *Salmonella*.

268 Given that there was no cell death observed with (*Stm*$^{Stat.}$) and the fact that SPI-1 is important for
269 *Salmonella* to invade and replicate in host cells[60,61], we focused on SPI-1-induced infection
270 models for further studies. We infected WT, *CASP4/5* KO and *CASP3* KO cells with SPI-1-
271 induced *Salmonella* (*Stm*$^{SPI-1}$) (Fig. 4A-D). Like infections with stationary phase *Salmonella*, we
272 observed CASP4/5-dependent activation of CASP3/7, and CASP3-dependent processing of
273 GSDME (Fig. 4A,C). However, consistent with the notion that CASP1 is the dominant protease
274 that processes GSDMD in cells, SPI-1-induced *Stm* infection triggered more cell death than
275 stationary-phase *Stm* infections, which was attenuated in *CASP4/5* KO cells compared to control
276 cells, but unchanged between control and *CASP3* KO cells (Fig. 4B,D).

277 To determine the contribution of the gasdermin proteins to cell death, we infected WT, *GSDMD*,
278 *GSDME*, or *GSDMD/E* KO THP-1 macrophages with *Stm*$^{SPI-1}$ (Fig. 4E,F). We observed similar
279 results in activation of CASP3/7 with *Salmonella* infection as with LPS transfections, but there
280 were some notable differences in cell death (Fig. 4E,F). GSDME processing was increased in
281 *GSDMD* KO cells and GSDMD processing was increased in *GSDME* KO cells unlike with LPS
282 treatment where GSDMD processing was attenuated in *GSDME* KO cells, likely due to the
283 activation of CASP1 by SPI-1-induced *Salmonella* (Fig. 4E). Indeed, CASP1 processing was
284 similar in WT and *GSDME* KO cells, but was attenuated in *GSDMD* and *GSDMD/E* KO cells
285 compared to WT cells. Strikingly, cell death, as assessed by Sytox Green uptake, was not

286 attenuated in *GSDMD*, *GSDME*, or *GSDMD/E* KO cells at 24h post infection compared to WT
287 cells and although not significant in all cases, trended higher (Fig. 4F).

288 Despite the loss of both pore-forming proteins, *GSDMD/E* KO cells displayed pyroptotic
289 morphology (Fig. 4G). This implies that enhanced CASP3/7 activity may drive rapid cell lysis,
290 potentially through alternative pore-forming mechanisms such as Ninjurin-1 (NINJ1)-mediated
291 membrane rupture[60,62] or pannexin-1 (PANX1), a substrate of CASP3/7 that can form pores
292 independently of GSDMD and GSDME[63]. To test this, WT, *GSDMD* KO and *GSDMD/E* KO cells
293 were infected with *Stm*$^{SPI-1}$ at MOI 50 after pretreatment with spironolactone, a pannexin-1
294 inhibitor, or glycine, an osmoprotectant that inhibits NINJ1-mediated lysis, or a combination of
295 both[62,63]. Pretreatment with PANX1 and NINJ1 inhibitors significantly reduced LDH release in all
296 groups (Fig. 4H), demonstrating that CASP3-mediated PANX1 activation and NINJ1 lysis
297 occurring downstream of apoptosis contribute to cell death during bacterial infection, like recently
298 described[15,64]. However, inhibiting PANX1 and NINJ1 had no impact on pore formation in WT
299 cells, as assessed by Sytox Green uptake, but significantly inhibited Sytox Green uptake in
300 *GSDMD/E* KO cells (Fig. 4I). Collectively, these findings suggest that during intracellular LPS
301 sensing, GSDMD is the dominant pore-forming protein mediating cell lysis, while both GSDMD
302 and GSDME contribute to cytokine release. In contrast, during *Salmonella* infection, gasdermin-
303 independent pathways can drive cell rupture downstream of non-canonical inflammasome
304 mediated CASP3/7 activation.

305 **GSDMD and CASP3 are essential, but GSDME is dispensable for limiting *Salmonella***
306 **replication in human macrophages.**

307 Recent work revealed that CASP4 plays a role in limiting intracellular *Salmonella* replication,
308 particularly at late stages of infection, though the mechanism remains unclear[15]. Activation of
309 pyroptosis in response to infection is generally thought to be host-protective by limiting cellular
310 replicative niches and activating cytokines that potentiate innate and adaptive immune responses.
311 We hypothesized that CASP4 prevents replication by activating CASP3 to induce GSDME-
312 mediated pyroptosis once bacterial burdens become high. To test the contribution of GSDME in
313 preventing *Salmonella* replication, we infected WT, *GSDMD*, *GSDME,* and *GSDMD/E* KO THP-
314 1 macrophages with GFP-expressing *Salmonella (GFP-Salmonella*) at increasing MOIs (MOI =
315 20, 50, or 100) and measured the change in GFP intensity during infection as a proxy for bacterial
316 replication (Fig. 5). We collected images and quantified the fold change in *GFP-Salmonella*
317 intensity at 24 h post infection relative to the start of imaging (t = 2 h) infection (Fig. 5A,).

318 Unexpectedly, GSDME was dispensable for limiting bacterial replication (Fig. 5B). Only *GSDMD*
319 KO cells had significantly higher GFP-*Salmonella* burdens relative to WT infected cells (Fig. 5B),
320 consistent with a recent study demonstrating that GSDMD is essential for limiting intracellular
321 *Salmonella* replication[60]. *GSDME* KO cells had decreased burdens compared to WT cells,
322 suggesting that GSDME may promote, instead of limit infection and replication. Consistent with
323 this idea, *GSDMD/E* KO cells had similar bacterial burdens to WT cells and had decreased
324 burdens compared to *GSDMD* KO cells. Cell death was assessed by LDH release and observed
325 to be largely independent of both GSDMD and GSDME (Fig. 5C), consistent with contributions
326 from PANX1 and NINJ1 activation.

327 We next examined the role of CASP3 in restricting bacterial replication, but not CASP7 as *CASP7*
328 KO cells had a defect in GSDMD processing. WT and *CASP3* KO cells were infected with GFP-
329 *Salmonella* and images of infected cells were collected to quantify the fold change in GFP
330 intensity. In WT cells, GFP intensity remained stable, indicating replication control at all MOIs (Fig.
331 5D,E). In contrast, *CASP3* KO cells showed a significant increase in bacterial burden across all
332 MOIs tested compared to WT cells (Fig. 5D,E). We tested two independent *CASP3* KO clones
333 and LDH release at MOI = 20 was decreased in one clone compared to WT cells but unaffected
334 at MOI = 50 and 100 (Fig. 5F). Together, these data indicate that in human macrophages,
335 GSDMD and CASP3 are essential for restricting intracellular *Salmonella*, whereas GSDME is
336 dispensable and may even facilitate replication.

337 **CASP3/7 and GSDME are cleaved in primary human macrophages during non-canonical**
338 **inflammasome activation.**

339 To evaluate the physiological relevance of CASP4/5-induced CASP3/GSDME activation, we
340 transfected primary human monocyte-derived macrophages (differentiated with macrophage
341 colony stimulating factor (M-CSF)) with LPS [65]. Consistent with observations in THP-1 cells, LPS
342 transfection induced robust processing of CASP3/7 and GSDME in primary macrophages (Fig.
343 6A). Representative immunoblots from three independent donors are shown (Fig. 6A).
344 Pretreatment with the NLRP3 inhibitor MCC950 blocked CASP1 activation and reduced GSDMD
345 processing but did not alter CASP3/7 or GSDME cleavage, indicating that CASP3/7 activation is
346 primarily mediated by CASP4/5, whereas GSDMD processing is largely downstream of CASP1
347 (Fig. 6A). Intracellular LPS-induced cell death and CASP3/7 activity were comparable between
348 untreated and MCC950-treated cells (Fig. 6B-D), consistent with non-canonical inflammasome
349 activation. Representative images from Donor 7 illustrate pyroptotic morphology and Sytox Green

350 uptake used to monitor cell death kinetics (Fig. 6B). Aggregate analysis across all donors
351 confirmed that cell death was unchanged by MCC950 treatment (Fig. 6E). Importantly, *Salmonella*
352 infection induced CASP3/7, GSDME, and GSDMD processing in primary cells (Fig. 6F). Together,
353 these findings demonstrate that intracellular LPS and bacterial infections trigger CASP4/5-
354 dependent activation of CASP3, leading to GSDME cleavage and pyroptosis in primary human
355 macrophages.

356 **Discussion**

357 Cell death is central to maintaining cellular homeostasis and resolving infections, with
358 inflammasome-mediated pyroptosis serving as a critical defense mechanism against intracellular
359 pathogens[66]. Previous studies have revealed complex crosstalk between apoptotic and pyroptotic
360 caspases, particularly in canonical inflammasome signaling, where CASP1 can indirectly or
361 directly activate executioner CASP3 and -7 [23,29,31,32,67-70]. These signaling cascades appears to be
362 both species- and stimulus-dependent. Direct cleavage of CASP3/7 by CASP1 has been reported
363 in human cells under certain conditions, such as dipeptidyl peptidase 8/9 inhibitor treatment[29],
364 while in mouse bone marrow–derived macrophages, CASP1-mediated CASP3/7 activation
365 requires upstream CASP8 and CASP9 activation induced by CASP1-dependent Bid cleavage in
366 response to inflammasome agonists like LPS plus nigericin (NLRP3 agonist), *Salmonella* (NLRC4
367 agonist), or poly(dA:dT) (AIM2 agonist) [31,32]. Whether human non-canonical inflammasomes
368 (CASP4/5) similarly activate apoptotic executioner caspases remained unclear.

369 In the present study, we demonstrate that human CASP4/5 directly cleave CASP3 and CASP7,
370 and CASP3 subsequently cleaves GSDME, but our data suggests that most of the cell death is
371 mediated by GSDMD. Unlike CASP1, CASP4/5 did not cleave Bid, and neither CASP8 nor
372 CASP9 contributed to CASP3 activation during non-canonical inflammasome signaling. These
373 data identify CASP4/5 as direct upstream activators of apoptotic executioner caspases in human
374 cells. Although previous *in vitro* studies suggested that murine CASP11 (the ortholog of human
375 CASP4) can cleave mouse CASP3/7, we did not observe significant CASP11 processing of
376 human CASP3[69,71]. Other work indicates that in mouse macrophages, CASP11 triggers CASP1
377 activation, that then cleaves CASP3[70]. This points to species-specific differences in non-canonical
378 inflammasome signaling, consistent with other observations that human CASP4/5, but not mouse
379 CASP11, can cleave IL-18[20-22].

380 Our data also reveal that pharmacological or genetic inhibition of CASP1 attenuated GSDMD
381 processing while increasing GSDME cleavage, indicating that CASP4/5 preferentially drive

382 CASP3/GSDME-mediated pyroptosis. Both GSDMD and GSDME contributed to IL-1β release as
383 measured by immunoblotting, in agreement with prior observations[45]. However, IL-1β levels
384 measured using an IL-1 receptor activation assay were not significantly reduced by single
385 GSDMD or GSDME deletion, likely reflecting contributions from IL-1α release downstream of pore
386 formation, which activates IL-1α via calpains[72]. Consistent with this, *GSDMD/E* double knockout
387 cells transfected with LPS showed a reduction in released IL-1α/β compared to wild-type or single
388 knockout cells, highlighting the collective role of these gasdermins in coordinating cytokine
389 release.

390 The CASP4/5–CASP3/GSDME axis may function as a backup pathway when pathogens inhibit
391 canonical CASP1-GSDMD signaling. For example, SARS-CoV-2 NSP5 protease cleaves
392 GSDMD N-term to inactivate the pore forming ability, but the protease also activates CASP1 and
393 CASP8, that subsequently activate CASP3 to induce GSDME-mediated pyroptosis[73]. Similarly,
394 Enterovirus 3C protease cleaves and inactivates GSDMD N-term[74], and gram-negative bacterial
395 pathogen *Shigella flexneri* can degrade GSDMD, thereby impairing canonical pyroptosis and
396 promoting infection[75]. Consistently, we observed increased CASP3/7 and GSDME cleavage in
397 GSDMD-deficient cells, highlighting the compensatory nature of this pathway, similar to
398 observations made by others[32,45].

399 While GSDMD has long been recognized as the primary effector of LPS-induced pyroptosis via
400 inflammatory caspases[17-19], our data suggest that in human macrophages, most GSDMD
401 processing is mediated by CASP1, which is activated downstream of the non-canonical pathway.
402 In contrast, CASP4/5 preferentially induce GSDME-mediated pyroptosis via CASP3 activation,
403 but the GSDME pores are sublytic. As CASP4/5 can cleave and inactivate IL-1β[21] and CASP3 is
404 known to inactivate GSDMD[29], IL-1β[76,77], and IL-18[78], it is possible that this pathway may have
405 evolved to induce pyroptosis while limiting inflammation that could result in sepsis. GSDME-
406 mediated pyroptosis may also have a distinct role in promoting a sublytic phase that enables rapid
407 IL-1β release, which could have important ramifications for pathogen defense[45].

408 Our data suggest reciprocal regulation between CASP1 and CASP3-induced pyroptosis. In
409 CASP1-deficient cells, GSDME processing was increased, while GSDMD processing was
410 increased in CASP3-deficient cells, suggesting that these pathways can compensate for one
411 another to maintain overall cell death. This crosstalk may ensure robust inflammatory responses
412 even when pathogens attempt to inhibit one arm of the pathway. Notably, GSDMD appears to
413 play a dominant role in the kinetics of pyroptotic cell death, consistent with previous reports that

414 GSDMD pore formation precedes apoptotic caspase activation during canonical inflammasome
415 signaling[29,79]. However, this only applied in the context of when CASP4/5 were activated by LPS.
416 In this study, neither GSDMD nor GSDME were required for *Salmonella* induced cell lysis at 24h
417 post infection, contrasting with a recent study demonstrating that GSDMD and NINJ1 were
418 essential for LDH release in THP-1 macrophages infected with *Salmonella*[15]. Differences in
419 infection timepoint (6 h vs 24 h here), cell priming (e.g., Pam3CSK4 vs. unprimed in our study)
420 and contributions of alternative pore-forming proteins such as NINJ1 or PANX1 may explain these
421 discrepancies. Notably, recent work in mice found that LPS or *Escherichia coli* infection plus heat
422 shock induced cell lysis that was independent of the typical pore-forming proteins such as,
423 GSDMD, GSDME, and MLKL (mixed lineage kinase domain-like protein), but was critically
424 dependent on NINJ1, which mediates plasma membrane rupture downstream of several cell
425 death pathways[62,80]. Furthermore, this death only occurred with certain priming agents, such as
426 poly(I:C) and LPS, but not Pam3CSK4[62]. Hence, it's possible that under certain conditions,
427 *Salmonella* can induce rapid cell lysis that is independent of GSDMD and GSDME. More studies
428 using human cells are needed to understand the precise contributions of the different pore-
429 forming proteins in pathogen defense.

430 Much of our understanding of the innate immune response to *Salmonella* has stemmed from work
431 using mice, but emerging studies using human cells have begun to yield critical insights. Recent
432 work in human macrophages demonstrated that CASP1-dependent pyroptosis is important for
433 limiting intracellular *Salmonella* replication early during infection but CASP4 was important for
434 restricting replication at later stages of infection[15]. Our data suggests that CASP4 inhibits
435 replication by activating CASP3/7 to induce apoptosis and cell lysis that is likely dependent on
436 NINJ1 or PANX1. Indeed, NINJ was reported to be important for cell death in THP1 macrophages
437 infected with *Salmonella*[15]. Recent work also revealed that CASP3 can activate PANX1 to induce
438 pyroptosis independently of CASP1, CASP8, GSDMD, and GSDME[63]. Consistent with this notion,
439 we found that *GSDMD/E* KO cells exhibited pyroptotic morphology and GSDME was dispensable
440 for inhibiting bacterial replication. Also in agreement, a recent report also noted that GSDME was
441 dispensable, and that GSDMD is the only gasdermin that confers protection against acute
442 *Salmonella* infection in the gut of mice[81]. Additional support for this mechanism stems from
443 observations that apoptosis is critical for restricting *Legionella* infection and replication in dendritic
444 cells[34], and apoptosis was reported to be essential for limiting *Salmonella* replication in mice[64,82].
445 Collectively, this implies that apoptosis may play a broader role in preventing intracellular

446 pathogen replication and future studies should focus on delineating the contributions of apoptosis
447 and pyroptosis in restricting infection.

448 Controlled activation of pyroptosis protects the host from infection, and therefore it was surprising
449 that GSDME was dispensable for protecting against *Salmonella* replication. If CASP3/GSDME-
450 induced pyroptosis occurs without CASP1 activation, it could be detrimental to the host as it can
451 facilitate bacterial egress and infection of bystander cells without the full immune response
452 induced by CASP1 activation. In support of this idea, it was demonstrated that CASP11-mediated
453 cell death in the absence of CASP1 activation was detrimental and resulted in increased
454 susceptibility to *Salmonella* infection in mice[54]. Similarly, it was shown that GSDMD pores promote
455 the release of the fungal pathogen *Candida albicans* from mouse macrophages, and inhibiting
456 GSDMD was protective as it prevented *Candida albicans* escape while maintaining CASP1
457 activation and GSDMD-independent IL-1β release[83]. Alternatively, if GSDME pores are sublytic,
458 as has been suggested[45], it could facilitate pathogen entry and render *GSDME* KO cells less
459 susceptible to initial infection. We note that our GSDME KO cells were more resistant to infection
460 than WT and *GSDMD* KO cells and required higher MOIs to observe similar levels of infections.
461 Intriguingly, CASP3/GSDME-mediated pyroptosis appears to be critical for limiting viral
462 replication[35], suggesting that the impact on pathogen defense could be pathogen-specific.
463 Importantly, we demonstrate that CASP3/GSDME are processed in primary human macrophages
464 following non-canonical inflammasome activation, underscoring the physiological relevance of
465 this pathway.

466 In conclusion, we identify CASP3/7 as direct substrates of human non-canonical inflammasomes
467 (CASP4/5) in both immortalized and primary human macrophages. Mechanistically, CASP4/5
468 mediated cleavage of CASP3 initiates GSDME processing, but our data shows that CASP3 and
469 GSDMD, but not GSDME, are critical for limiting intracellular *Salmonella* replication. CASP3
470 activation can drive cell lysis that is independent of GSDMD and GSDME, likely through PANX
471 and NINJ1, which have both been demonstrated to be important in limiting *Salmonella*
472 replication[15,64]. Given that apoptosis has recently been reported to be important for infection
473 control, our data suggests that this seemingly immune silent form of cell death may be more
474 important than previously recognize in controlling infections. A key outstanding question is the
475 molecular mechanism by which CASP4/5 recognize and cleave CASP3/7. Given that
476 inflammatory caspases utilize exosites or tetrapeptide recognition sequences for substrate

477 specificity[16], it is likely that a combination of these factors underlies recognition. Future work is
478 needed to uncover the molecular mechanism of how CASP4/5 recognize and activate CASP3/7.

479 **Method Details**

480 **Antibodies and Reagents**

481 Antibodies used include: GSDMD Rabbit polyclonal Ab (Novus Biologicals, NBP2-33422), FLAG®
482 M2 monoclonal Ab (Sigma, F3165), GAPDH Rabbit monoclonal Ab (Cell Signaling Tech, 14C10),
483 CASP1 p12/10 Rabbit monoclonal Ab (Abcam, ab179515), CASP4 Rabbit polyclonal Ab (Cell
484 Signaling Tech, 4450S), CASP5 Rabbit monoclonal Ab (Cell Signaling Tech, 46680S), Myc Mouse
485 monoclonal Ab (Cell Signaling Tech, 2276S), CASP3 (Cell Signaling Tech 9664S), CASP3 (Cell
486 Signaling Tech, 9662), CASP7 (Cell Signaling Tech, 8438S), CASP7 (Cell Signaling Tech,
487 12827S), GSDME (Abcam, ab215191), hIL-1β Goat Polyclonal Ab (R&D systems, AF-201-NA),
488 hIL-18 Goat Ab (R&D systems, af2548), CASP8 (Cell Signaling Tech, 4790S), CASP9 (Cell
489 Signaling Tech, 9508S), Bid (Cell Signaling Technology, 2002), IL-1α (Novus, AF-200-NA), HA
490 Rabbit monoclonal Ab (Cell Signaling Tech, 3724S), FKBP12 Rabbit polyclonal Ab (Abcam,
491 ab24373). IRDye 800CW anti-rabbit (LICOR, 925-32211), IRDye 800CW anti-mouse (LI-COR,
492 925-32210), IRDye 680CW anti-rabbit (LI-COR, 925-68073), IRDye 680CW anti-mouse (LI-COR,
493 925-68072). Other reagents used include: LPS-EB Ultrapure (Invivogen, tlrl-3pelps), FuGENE
494 HD (Promega, E2311), AP20187 (Tocris™ 6297/5).
495

496 **Recombinant Caspase Assays**

497 All *in vitro* assays using recombinant caspases were performed in 20 µL reactions containing
498 caspase assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1mM EDTA, 0.1% CHAPS,
499 10% sucrose, pH 7.) as previously described[23]. 10 μg of plasmids encoding for GSDMD, CASP3,
500 CASP7, or GSDME were transfected into HEK 293T cells (10 cm plates) using FuGENE HD
501 Transfection Reagent (Promega, E2312) according to the manufacturer's protocol. After 24 hours,
502 cells were harvested and lysed by sonication for 10 second pulses for 60 seconds at 30%
503 amplitude in PBS and centrifuged at 12,000 g to remove cell debris. Lysates were diluted 10x in
504 caspase assay buffer and incubated with 0.25 activity units/µL of each indicated caspase. At the
505 indicated time points, samples were mixed 1:1 with 2x Licor Protein Sample Loading Buffer (Neta
506 Scientific, 928-40004) and boiled at 95 °C for 10 minutes, then analyzed by immunoblotting.

507 **Cell Culture**

508 All cells were grown at 37 °C in a 5% CO2 atmosphere incubator. HEK 293T cells were purchased
509 from ATCC and THP1 cells were obtained previously or acquired from the Bryant lab[21] or Abbott
510 lab[45]. HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-
511 glutamine and 10% fetal bovine serum (FBS). THP-1 cells were cultured in Roswell Park Memorial
512 Institute (RPMI) medium 1640 with L-glutamine and 10% fetal bovine serum (FBS). Purified
513 human monocytes from healthy donors were purchased from the University of Pennsylvania
514 Human Immunology Core. Purified monocytes were cultured in RPMI + 10% FBS and 25 ng/ml
515 rm-csf in 96 well plates at a density of 100,000 cells/well. Media was removed and replaced with
516 Opti-MEM containing Sytox Green and MCC950 as indicated for 30 minutes prior to LPS
517 transfection as previously described. Cell lines were negative for mycoplasma contamination.
518
519 **IncuCyte CASP3/7 activity and Sytox Green uptake assays**
520 The kinetics of CASP3/7 activity and Sytox Green uptake were measured in cells using the
521 IncuCyte S3 Live cell imaging system (Sartorius). THP-1 cells were resuspended in RPMI medium
522 containing 50 ng/mL phorbol 12-myristate 12-acetate (PMA) and plated in black, clear-bottom 96-
523 well plates at a density of 8 x 10^4 cells/well. After 48 h, the media was then replaced with Opti-
524 MEM (0.1 mL/well) supplemented with either the cell-permeable CASP3/7 green dye (final
525 concentration: 10 µM (1:200 dilution)) or Sytox Green (final concentration: 0.2-0.5 µM), as
526 indicated. A series of images were collected with a 10x objective at 1h intervals for 24 hours. The
527 number of Sytox Green or CASP3/7 dye-positive cells in each image was determined using the
528 IncuCyte S3 software with 3 different areas/well. Cell death was quantified relative to 0.1% Triton
529 treated macrophages.
530
531 **Cloning**
532 All plasmids were cloned using Gateway technology as previously described[21]. DNA encoding the
533 indicated proteins were inserted between the *attR* recombination sites and shuttled into modified
534 pLEX_307 vectors (Addgene) using Gateway technology (Thermo Fisher Scientific) according to
535 the manufacturer's instructions. Proteins expressed from these modified vectors contain an N-
536 terminal *att*B1 linker (GSTSLYKKAGFAT) after any N-terminal tag or a C-terminal *att*B2 linker
537 (DPAFLYKVVDI) preceding any C-terminal tag such as Myc or HA.
538
539 sgRNAs were designed using the Broad Institute's web portal and cloned into the
540 pXPR016_Hygro or plentiGuide-Puro vector (pXPR003) (Addgene #52963) as described
541 previously[29,84]. The sgRNA sequences used were:

542 h*CASP3 clone#1* 5′-CGTGGTACAGAACTGGACTG -3′

543 h*CASP3* clone#2 5′- TGTCGATGCAGCAAACCTCA -3′

544 h*CASP7* 5′- AGACAATCACGTCAAAACCC -3′

545 h*CASP9* 5′- ACATCGACTGTGAGAAGTTG -3′

546 *control (GFP)* 5′-GGGCGAGGAGCTGTTCACCG-3′

547

548 All plasmids were verified by DNA sequencing (Genewiz).

549

550 **THP1 Knockout cell lines**

551 Constructs were packaged into lentivirus in HEK 293T cells using the Fugene HD transfection
552 reagent (Promega) and 2 µg of the vector, 2 µg psPAX2, and 0.2 µg pMD2-G. THP-1 were
553 spinfected with virus for 2 h at 1000g at 30 °C supplemented 8 µg/mL polybrene. After 2 days,
554 cells were selected for stable expression of S. pyogenes Cas9 (Addgene #52962) using blasticidin
555 (5 µg/mL for THP-1) and for stable expression of sgRNAs using puromycin (0.5 µg/mL for THP-
556 1) or hygromycin (100 µg/mL for THP-1). After 10 d, single cells were isolated by serial dilution
557 and expanded. Cells were then harvested for immunoblotting or used in experiments.

558

559 **Generation of HEK 293T CASP3/7 double KO cells**

560 HEK 293T cells were transiently transfected with plasmids encoding for CAS9 and sgRNAs
561 targeting CASP3 (sgRNA Sequence: AATGGACTCTGGAATATCCC) and CASP7 (sgRNA
562 Sequence: GCATCTATCCCCCCTAAAGT). After 48, the cells were selected with puromycin (1
563 µg/mL) until all control cells were no longer viable. Single cell clones were then obtained by serial
564 dilution and were confirmed by immunoblotting.

565

566 **Transient Transfection**

567 HEK 293T cells were seeded in 12-well culture plates at 0.25×10^6 cells/well in DMEM. The
568 following day, the indicated plasmids were mixed in Opti-MEM and transfected using FuGENE
569 HD (Promega) according to the manufacturer's protocol.

570 **LPS Transfection of human macrophages**

571 THP-1 cells were resuspended in RPMI medium containing 50 ng/mL phorbol 12-myristate 12-
572 acetate (PMA). Cells were plated in 96-well plates at a density of 8×10^4 cells/well. After 48 h, the
573 media was then replaced with Opti-MEM (0.1 mLs/well). Where indicated, cells were treated with
574 MCC950 (10 µM) for 30 min at 37 °C 5% CO_2 before LPS transfection. The LPS solution was

575 prepared by adding LPS (25 mg/mL final concentration) and FuGENE (0.5% final concentration)
576 to Opti-MEM. This solution was gently mixed by flicking and incubated for 30 min at room
577 temperature before drop-wise addition to each well. The supernatants were collected separately,
578 and cells were lysed by sonication 24 h post transfection. Harvested supernatants were
579 precipitated by chloroform/methanol and analyzed by immunoblotting.

580 **HEK Blue IL-1α/β reporter assay**

581 HEK-Blue IL-1β (Invivogen) reporter cells were used according to manufacturer's protocols as
582 previously described[21] to quantify the amount of active IL-1α/β released. HEK Blue cells were
583 seeded in 96 well plates at 5 x 10^4 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) with
584 L-glutamine and 10% fetal bovine serum (FBS) to a total volume of 150 ml. 10 µL of supernatant
585 from control or treated THP1 cells were added, and the samples were incubated overnight at
586 37°C in a 5% CO2 atmosphere incubator. A serial dilution of recombinant IL-1β (Invivogen) was
587 included in all experiments to generate a standard curve which to calculate absolute levels of
588 active IL-1β. 50 µL of supernatant was mixed with 150 µL of freshly prepared Quanti-Blue solution
589 and incubated at room temperature for 10 minutes in the dark before reading absorbance at 620
590 nm using a Cytation 5 plate reader (Bio Tek).

591 ***Salmonella* Infection of human macrophages**

592 *Salmonella* Typhimurium SL1344 or SL1344 pDisC was streaked to isolation from frozen stock
593 on LB + Streptomycin (100µg/ml) agar plates. Carbenicillin (100µg/ml) was included for all studies
594 with SL1344 pDisC. For SPI-I induced bacteria, single colonies were inoculated in LB +
595 Streptomycin broth overnight (~18h). The overnight culture was back diluted 40-fold into LB +
596 Streptomycin broth containing 300 mM NaCl. After three hours bacteria were washed 2x in sterile
597 PBS and cell density was determined by quantifying OD_{600}. MOI was prepared according to OD
598 and bacterial cells were resuspended in PBS. 10µL suspension of bacteria was added to
599 macrophages in triplicate in 96-well plates. Macrophages were spun for 10 min at 400g to allow
600 infection and then placed in incubator for 1h. Media was then removed and replaced with fresh
601 RPMI without FBS containing gentamicin (100 μg/ml). After 1h, media was removed and wells
602 were washed 3x with PBS and replaced with fresh RPMI without FBS containing gentamicin (10
603 μg/ml). GFP fluorescence was monitored using IncuCyte live cell imaging for 24h.

604 For SL1344 (non-fluorescent) infection, after 1h 100µg/ml gentamicin, media was replaced with
605 fresh RPMI without FBS containing gentamicin (10 μg/ml) and Sytox Green (0.5 µM). Cell death
606 was quantified relative to 0.1% Triton treated macrophages.

607 For stationary phase bacterial infections, single colonies were inoculated in LB + Streptomycin for
608 2 days. MOI were prepared as described above and 10μL suspension of bacteria were added to
609 macrophages in triplicate in 96 well plates. Macrophages were spun for 10 min at 400g to allow
610 infection and then placed in incubator for 2h.

611 **LDH Assays**

612 Supernatants were harvested for LDH analyses at 24 h after bacterial infections and analyzed
613 using the Cyquant LDH Cytotoxicity Assay (Thermo Scientific) according to the manufacturer's
614 protocol. LDH activity was quantified relative to a lysis control where cells were lysed using Triton-
615 X at the beginning of each experiment.

616 **Western Blotting**

617 Protein samples were run on Nupage 4-12% Bis-Tris Midi Protein Gel (Thermo Scientific,
618 WG1403BOX) at 175 volts. Proteins were transferred to 0.45 μm nitrocellulose membranes
619 (BioRad,1704271) at 25 volts for 7 minutes on the Biorad Transblot Turbo System. Membranes
620 were blocked using LICOR Intercept blocking buffer (Neta scientific, 927-70010) for 1 hour and
621 stained with primary antibodies at 1:1000 concentration in 50% Licor blocking buffer and 50%
622 TBS buffer with 0.1% Tween for 1 hour at room temperature or overnight at 4C. Membranes were
623 washed 3x 15 minutes, followed by incubation with Donkey Anti-Mouse/Rabbit/Goat IgG
624 Polyclonal Antibody (IRDye® 800CW) for 1 hour at room temperature. Membranes were then
625 washed 3x10 minutes and imaged on an Odyssey M Imaging System (LI-COR Biosciences).
626 Images were analyzed with Empiria studio version 3.2 (Licor), and brightness, contrast, and tone
627 parameters were adjusted uniformly for entire membranes on Adobe Photoshop.

628 **Statistical Analysis**

629 Data and statistical analysis was performed using GraphPad Prism 10 software. Statistical
630 analysis was determined using One-way or Two-way ANOVA with multiple comparisons test.
631 Further details are listed under each figure legend.

632 **Author contributions CRediT:**

633 Conceptualization: CYT, Ma.Ku., CMB Methodology: CYT, Ma.Ku, CMB, Investigation: Ma.Ku.,
634 CMB, ABM, PME, CM, SC, Ma.Ka., Mi.Ka., WY, TJW, RCP, BMD Visualization: CYT, Ma.Ku.,
635 CMB, Funding acquisition: CYT, CMB, Project administration: CYT Data curation: CYT, Ma.Ku.,
636 CMB, Formal Analysis: CYT, Ma.Ku., CMB, ABM, PME, CM, SC, Ma.Ka., Mi.Ka., Resources:

637 CYT Supervision: CYT, Writing – original draft: CYT, Ma.Ku., CMB, Writing – review & editing:
638 CYT, Ma.Ku., CMB.
639

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651 1054907).

652 **Conflicts of Interest**

653 The authors declare no conflicts of interests.

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661 **FIGURES**

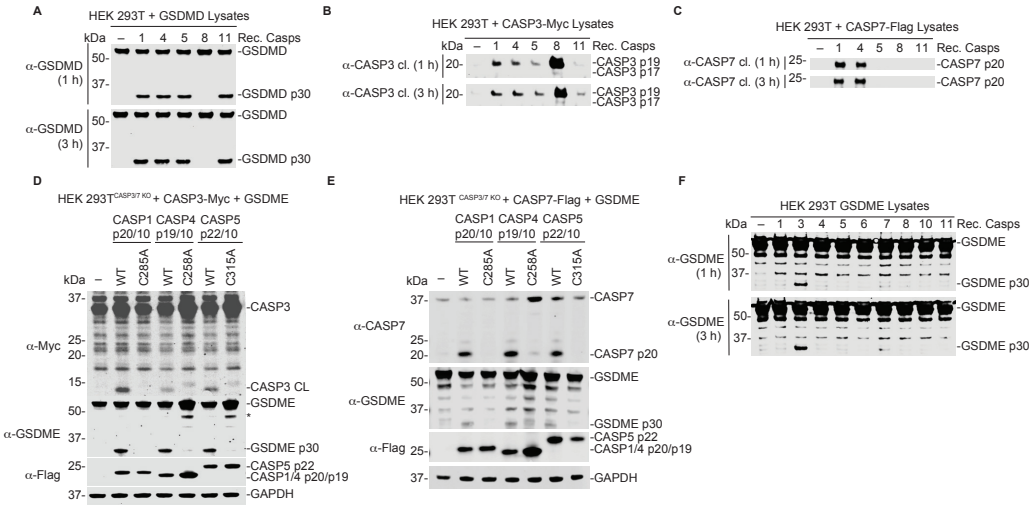
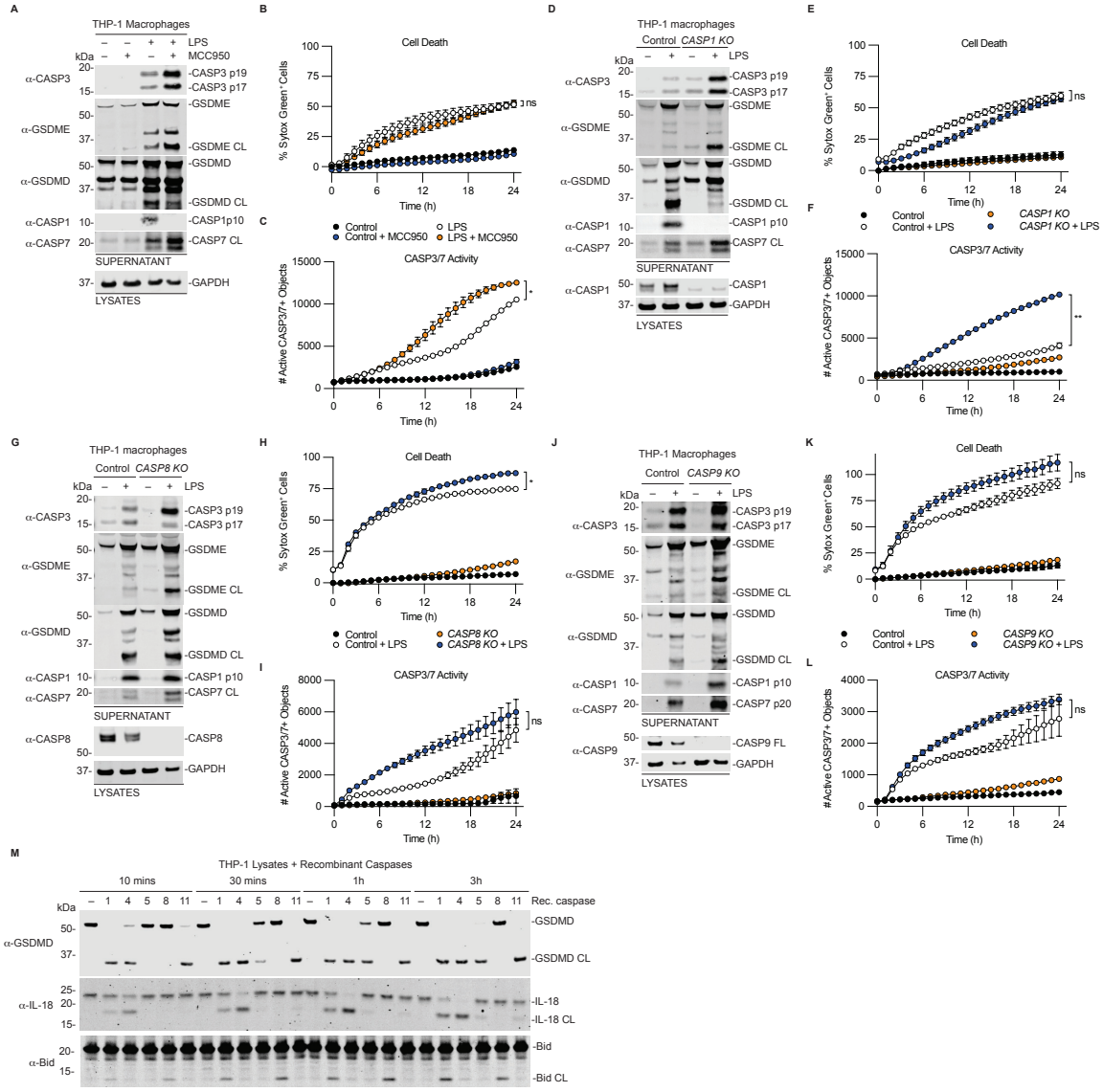


Figure 1. Caspases 1, 4, and 5 directly cleave and activate caspases-3 and -7. (A-C) HEK 293T cells were transiently transfected with plasmids coding for human GSDMD **(A)**, CASP3 **(B)**, or CASP7 **(C)**. Lysates were then incubated with 0.25 activity units/μL of each indicated recombinant caspase for 1 or 3 h and cleavage products were assessed by immunoblotting. **(D,E)** *CASP3/7* KO HEK 293T cells were transiently co-transfected with the indicated constructs coding for active CASP3 **(D)** or CASP7 **(E)** along with GSDME, and catalytically active or inactive CASP1, -4, or -5 for 24 h before the cell lysates were analyzed by immunoblotting. **(F)** HEK 293T cells were transiently transfected with a plasmid coding for GSDME for 24 h. Lysates were harvested and incubated with 0.25 activity units/μL of each recombinant caspase for 1 or 3 h. Cleavage products were assessed by immunoblotting. Data are representative of three or more independent experiments.



679

680 **Figure 2. CASP1, CASP8, and CASP9 are dispensable for CASP3/7 and GSDME processing**
681 **during non-canonical inflammasome activation by intracellular LPS.** (**A-C**) WT THP-1 cells
682 were transfected with 25 μg/mL LPS in the presence or absence of the NLRP3 inhibitor MCC950
683 (10 μM) for 24 h. Supernatants and lysates were then analyzed by immunoblotting (**A**), cell death
684 was measured by monitoring Sytox Green uptake (**B**), and CASP3/7 activity was determined
685 using CellTreat CASP3/7 detection reagent (**C**). (**D-F**) Control-matched *CASP1* KO (**D-F**), *CASP8*
686 KO (**G-I**), and *CASP9* KO (**J-L**) THP-1 cells were transfected with 25 μg/mL LPS for 24 h then
687 supernatants and lysates were then analyzed by immunoblotting (**D, G, J**), cell death was
688 measured by monitoring Sytox Green uptake (**E, H, K**), and CASP3/7 activity was determined
689 using CellTreat CASP3/7 detection reagent (**F, I, L**). (**M**) PMA-differentiated WT THP-1

690 macrophages were lysed and incubated with 0.25 activity units/μL of recombinant caspases for
691 the indicated times before immunoblot analysis. Data are means ± SEM of three independent
692 replicates, and representative of at least three independent experiments except for M (n = 2).
693 ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 by two-way ANOVA test with Tukey's
694 multiple comparison test comparing the control treated to KO treated samples at 24 h.

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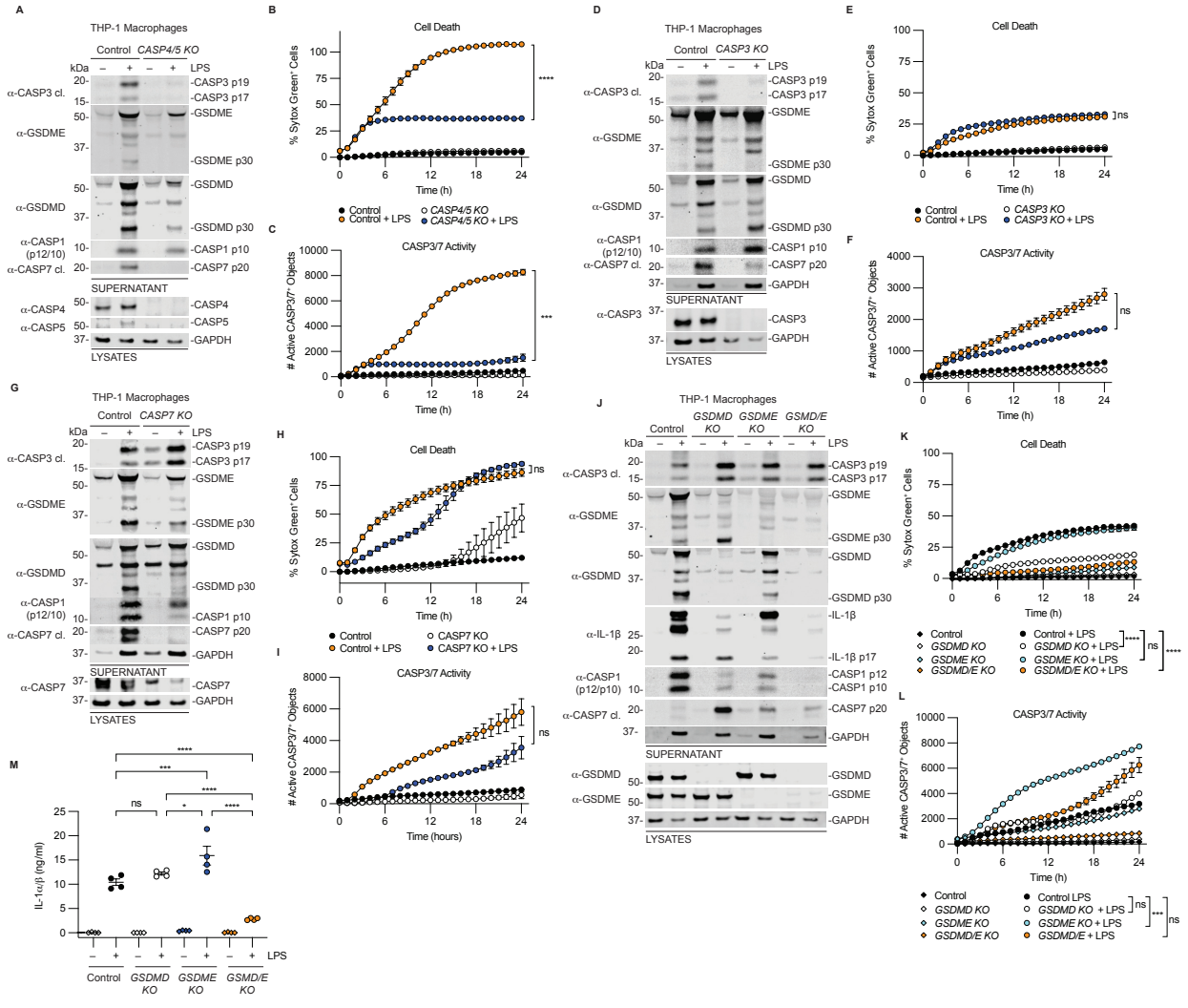

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711 **Figure 3. CASP3, and CASP4/5 are required to induce GSDME processing during non-**
712 **canonical inflammasome activation by intracellular LPS.** (**A-C**) WT and *CASP4/5* double KO
713 THP-1 cells were transfected with 25 μg/mL of LPS for 24 h then supernatants and lysates were
714 analyzed by immunoblotting (**A**), cell death was measured by monitoring Sytox Green uptake (**B**),
715 and CASP3/7 activity was determined using CellTreat CASP3/7 detection reagent (**C**). (**D-F**) WT
716 and *CASP3* KO THP-1 cells were transfected with 25 μg/mL of LPS for 24 h then supernatants
717 and lysates were then analyzed by immunoblotting (**H**), cell death was measured by monitoring
718 Sytox Green uptake (**I**), and CASP3/7 activity was determined using CellTreat CASP3/7 detection
719 reagent. (**G-I**) WT and *CASP7* KO THP-1 cells were transfected with 25 μg/mL of LPS for 24 h
720 then supernatants and lysates were then analyzed by immunoblotting (**H**), cell death was
721 measured by monitoring Sytox Green uptake (**I**), and CASP3/7 activity was determined using
722 CellTreat CASP3/7 detection reagent. (**J-M**) WT, *GSDMD* KO, *GSDME* KO and *GSDMD/E* KO

723 THP-1 macrophages were transfected with 25 μg/mL of LPS for 24 h then supernatants and
724 lysates were analyzed by immunoblotting (**J**), cell death was measured by Sytox Green uptake
725 (**K**), and CASP3/7 activity was determined using CellTreat CASP3/7 detection reagent (**L**), and
726 the amount of active IL-α/β released into the supernatants was quantified (**M**). Data are means ±
727 SEM of three independent replicates, and representative of at least three independent
728 experiments. ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 by two-way ANOVA test with
729 Tukey's multiple comparison test comparing the control treated to KO treated samples at 24 h.
730 Data in M were analyzed by One-way Anova with Tukey's multiple comparisons test.

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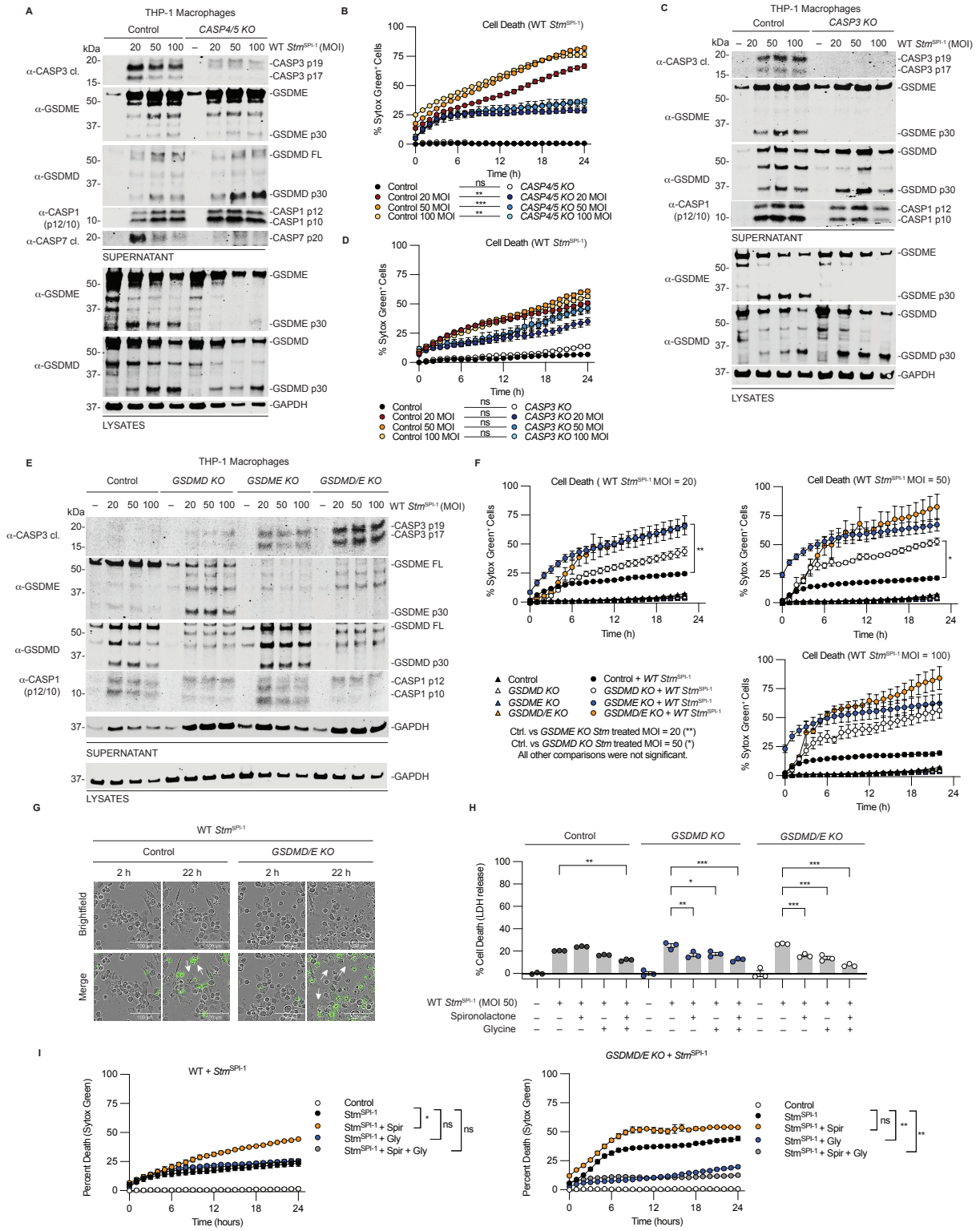


Figure 4. CASP3, and CASP4/5 are required for GSDME processing during non-canonical inflammasome activation by intracellular *Salmonella*. (A-D) WT and *CASP4/5* KO (A,B) or

WT and *CASP3* KO THP-1 macrophages (C,D) were treated with the indicated MOI of WT SPI1-induced *Salmonella* for 24 h then supernatants and lysates were analyzed by immunoblotting (**A,C**), and cell death was measured by monitoring Sytox Green uptake (**B,D**). **(E-G)** WT, *GSDMD* KO, *GSDME* KO and *GSDMD/E* KO THP-1 macrophages were treated with the indicated MOI of WT SPI1-induced *Salmonella* for 24 h then supernatants and lysates were analyzed by immunoblotting (**E**), and cell death was measured by monitoring Sytox Green uptake (**F**). Examples of Images of WT and *GSDMD/E* KO cells at MOI 20 used to quantify Sytox Green uptake are shown in **G.** White arrows show pyroptotic morphology. **(H,I)**. WT, *GSDMD* KO, and *GSDMD/E* KO THP-1 macrophages were infected with WT SPI1-induced *Salmonella* at MOI = 50 in the presence or absences of pretreatment with the PANX1 (20 μM spironolactone) or NINJ1(50 mM glycine) inhibitors for 24 h then LDH release (H) and Sytox Green (**I**) were assessed. Data in H were analyzed by One-way Anova with Tukey's multiple comparisons test. Data are means ± SEM of three independent replicates, and representative of at least three independent experiments.****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ by two-way ANOVA test with Tukey's multiple comparison test comparing the control treated to KO treated samples at 24 h.

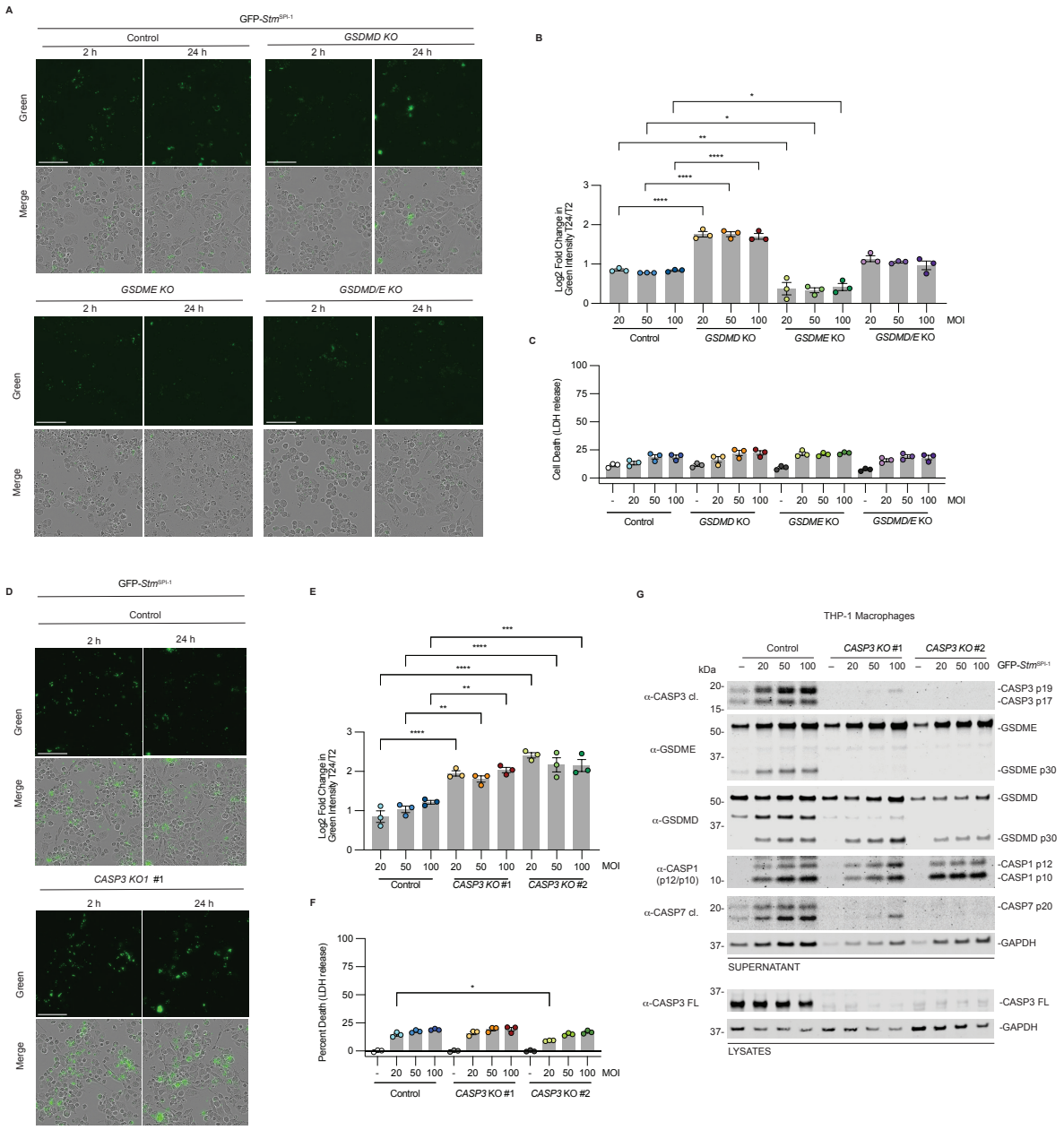


Figure 5. GSDMD and CASP3 are essential, but GSDME is dispensable for limiting *Salmonella* replication in human macrophages. (**A-C**) WT, *GSDMD* KO, *GSDME* KO and *GSDMD/E* KO THP-1 macrophages were treated with the indicated MOI of SPI1-induced GFP-expressing *Salmonella*. Representative images of the GFP-*Salmonella* (MOI = 20) are shown in **A**. Quantification of the fold change in green intensity is depicted in **B**. After 24 h, LDH assays were performed **C**. (**D-F**) WT or *CASP3* KO THP-1 macrophages were treated with the indicated MOI of SPI1-induced GFP expressing *Salmonella*. Representative images of the GFP-*Salmonella* (MOI = 20) are shown in **D.** Quantification of the fold change in green intensity is depicted in **E**.

After 24 h, LDH assays were performed **F**. Data are means ± SEM of three independent replicates, and representative of at least three independent experiments. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ by two-way ANOVA test with Tukey's multiple comparison test comparing the control treated to KO treated samples at 24 h.

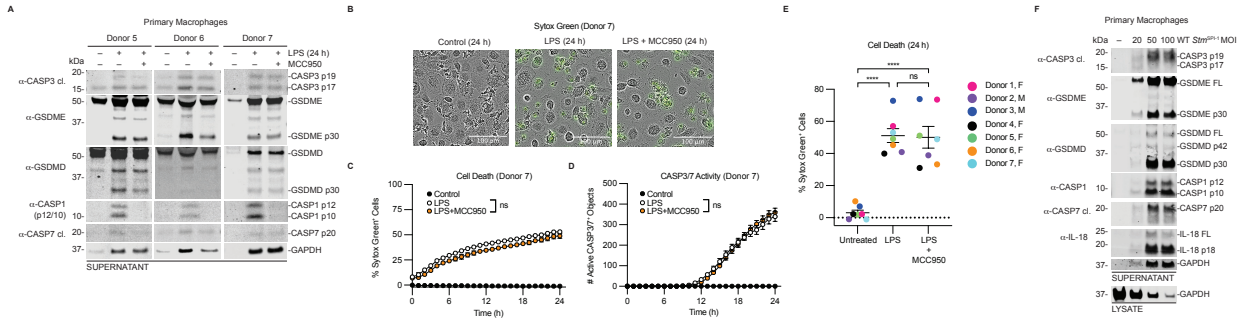
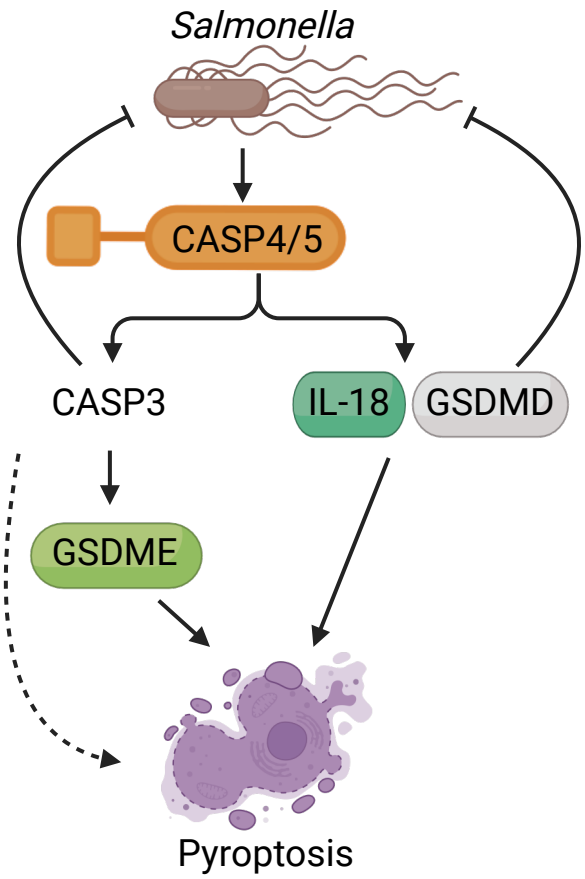


Figure 6. CASP3/7 and GSDME are cleaved in primary human macrophages during non-canonical inflammasome activation. (**A-E**) Primary monocytes were differentiated into macrophages and transfected with 25 μg/mL of LPS for 24 h. Some cells were pretreated with 10 μM MCC950 for 30 minutes before LPS transfection and supernatants were then analyzed by immunoblotting (**A**), cell death for a representative donor (Donor 7), as assessed by Sytox Green uptake is depicted in (**B**), and CASP3/7 activity CASP3/7 is depicted in (**C**). A summary of the Sytox Green uptake for all donors is shown in **E**. Data are means ± SEM of three or more independent experiments ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 by two-way ANOVA test with Tukey's multiple comparison test comparing the control vs treated samples at 24 h. Data in E were analyzed by One-way Anova with Tukey's multiple comparisons test.



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812 **Figure 7. Proposed model of how non-canonical inflammasomes contribute to limiting**
813 **intracellular *Salmonella* replication.** Upon sensing LPS or *Salmonella*, CASP4/5 directly cleave
814 CASP3/7 (CASP7 not shown), and CASP3 subsequently cleaves GSDME to induce pyroptosis.
815 In response to *Salmonella*, CASP3 activation can also induce rapid cell lysis resembling
816 pyroptosis independently of GSDMD and GSDME, likely mediated by NINJ1 and PANX1. CASP3
817 activation is important for limiting intracellular *Salmonella* replication. CASP4/5 can also directly
818 cleave GSDMD and IL-18 to induce pyroptosis, and GSDMD is also essential for limiting bacterial
819 replication. Figure generated in Biorender.

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