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 initiate innate immune signaling. 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.

Introduction

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

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

The substrates of inflammatory caspases are crucial mediators of immunity, yet their full repertoire and functional significance remain incompletely defined[16]. Human inflammatory caspases are known to cleave the interleukin family of cytokines, IL-1β and IL-18, and the pore-forming protein gasdermin D (GSDMD), leading to pyroptosis[17-23]. In this manner, pyroptosis protects the host from infection[24-28]. In the absence of GSDMD, CASP1 can process the apoptotic executioner caspases-3 and -7 (CASP3/7) to induce apoptosis[29]. A proteomic screen also identified CASP7 as a substrate of inflammasome-activated CASP1 in mice, but in the same study, CASP3 activation was found to be independent of CASP1[30]. It was later shown that, like human cells, CASP1 could mediate CASP3 processing in mice to induce apoptosis in the absence of GSDMD when infected with *Salmonella enterica* serovar Typhimurium or poly(dA:dT)[31,32]. This process was dependent on CASP1 cleaving Bid (BH3-interacting domain death agonist), which 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 CASP7. 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 stably expressing both a DmrB-caspase 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 cleavage activates the executioner caspases. To test
133 this, we co-expressed catalytically competent CASP3 (Fig. 1D) or CASP7 (Fig. 1E) with GSDME,
134 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 support that human CASP1/4/5 directly cleave CASP3/7 and promote their activation in
144 cellular and lysate systems, with CASP3 as 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 be activated via the NLRP3 inflammasome downstream of CASP4/5 due to K+ efflux
153 through GSDMD pores[8,9,44]. Prior work indicated that during CASP1 activation, both GSDME and
154 GSDMD contribute to cell death and cytokine release[45]. To rule out CASP1-mediated cleavage
155 of CASP3/7 downstream of CASP4/5, we pretreated THP1 cells with the NLRP3 inhibitor
156 MCC950[46] prior to LPS transfection (Fig. 2A). LPS treatment resulted in CASP3, CASP7, and
157 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 Sytox Green uptake remained unchanged in MCC950-treated
167 cells compared to LPS-only treated cells, likely due to elevated GSDME processing (Fig. 2B).

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

178 Both CASP8 and CASP9 are known to activate CASP3 and CASP7 to induce apoptosis, a
179 process constantly ongoing to maintain cellular homeostasis[47,48]. Like in *CASP1* KO cells, deletion
180 of CASP8 (Fig. 2G-I) or CASP9 (Fig. 2J-L) did not block CASP3/7 or GSDME processing, nor
181 alter GSDMD cleavage or pyroptosis, as assessed by Sytox Green uptake. In fact, CASP3/7
182 activity was modestly increased, suggesting that CASP8 or CASP9 may compete with CASP4/5
183 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. CASP3 is also known to cleave Bid[50]. To
187 rule out a contribution from CASP3 activated by CASP4/5 in cells, we incubated lysates from
188 THP-1 macrophages with equivalent activity units of recombinant caspases and assessed
189 processing of substrates at early time points preceding CASP3/7 activation by immunoblotting.

190 As expected, CASP1 and CASP8 processed Bid into the truncated form (Fig. 2M). However,
191 CASP4 and CASP5 failed to cleave Bid, but were active and able to process IL-18 and GSDMD
192 (Fig. 2M). In this assay, where multiple substrates are present simultaneously, IL-18 processing
193 appeared to be favored by CASP4 compared to other caspases while GSDMD processing was
194 favored by CASP1 (Fig. 2M). Taken together, these data indicate that CASP1, CASP8, and
195 CASP9 are dispensable for CASP3/7 activation during noncanonical inflammasome activation
196 and support a model in which CASP4/5 directly cleave CASP3/7 to promote CASP3/GSDME-
197 mediated pyroptosis.
198
199 **CASP3, and CASP4/5 are required to induce GSDME processing during non-canonical**
200 **inflammasome activation by intracellular LPS.**

201 Our findings above suggest that CASP4/5 activate the executioner CASP3/7, leading to
202 CASP3/GSDME-mediated pyroptosis. To directly test this, we transfected WT, *CASP4/5* KO,
203 *CASP3* KO, or *CASP7* KO cells with LPS (Fig. 3A-I). We then assessed CASP3/7 and GSDME
204 processing by immunoblotting, cell death by Sytox Green uptake, and CASP3/7 activity using a
205 cell permeable reporter dye. Loss of CASP4/5 significantly impaired CASP3/7 and GSDME
206 cleavage, cell death, and CASP3/7 activity compared to control cells in response to intracellular
207 LPS (Fig 3A-C). *CASP3* KO cells lacked GSDME cleavage but exhibited increased GSDMD
208 processing in LPS transfected cells (Fig. 3D). Despite loss of GSDME processing, pyroptosis was
209 not significantly diminished (Fig. 3E), suggesting functional compensation through enhanced
210 GSDMD activation. CASP3 deficiency slightly reduced overall CASP3/7 activity, likely due to
211 preserved CASP7 activity (Fig. 3F). Unexpectedly, *CASP7* KO cells retained CASP3 activation
212 and GSDME processing (albeit less than control cells), but CASP1 processing into the p10
213 species and GSDMD processing were nearly abolished, resulting in delayed cell death (Fig.
214 3G,H). *CASP7* KO cells also had attenuated CASP3/7 activity, as expected (Fig. 3I). Taken
215 together, these findings demonstrate that CASP4/5 activate CASP3/7, with CASP3 serving as the
216 primary protease that cleaves GSDME. While CASP3/GSDME activation can drive pyroptosis
217 downstream of the non-canonical inflammasome in part, loss of CASP3 is buffered by increased
218 GSDMD activity, highlighting plasticity in the execution of pyroptosis.
219
220 Mammals encode six gasdermins: gasdermin A, B, C, D, E, and PJVK (also known as
221 DFNB59)[36,51,52]. Typically, their N-terminal pore-forming domains are autoinhibited by the C-
222 termini, but upon cleavage, the N-termini are liberated and form pores to induce pyroptosis.

223 Inflammatory caspases cleave GSDMD to induce pyroptosis[17-19]. While CASP3 has been reported
224 to cleave GSDME to induce pyroptosis, this has primarily been described in the context of
225 chemotherapy-induced apoptosis or cytotoxic lymphocyte-mediated cell death[38,53,54]. Whether
226 CASP3/GSDME contributes to pyroptosis during non-canonical inflammasome activation has not
227 been established.

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

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

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

250 The non-canonical inflammasomes detect LPS from intracellular gram-negative bacteria. We next
251 asked if CASP4/5 cleave and activate CASP3/7 during bacterial infections. We infected WT,
252 *CASP4/5* KO, and *CASP3* KO cells with *Salmonella enterica* serovar Typhimurium (*Stm*) then
253 assessed CASP3/7 and GSDME processing by immunoblotting, cell death by Sytox Green

254 uptake, and CASP3/7 activity using a cell permeable reporter dye. Log-phase *Salmonella* induces
255 expression of the type 3 secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1
256 (SPI-1) that activates both canonical and non-canonical inflammasome signaling [55-58]. Thus, we
257 first infected WT and *CASP4/5* KO (Fig. S2A,B) or *CASP3* KO (Fig. S2C, D) THP1-1
258 macrophages with *Salmonella* grown to stationary phase ($Stm^{Stat.}$) to minimize the contribution
259 of the canonical caspase-1 signaling pathway then measured cell death and processing of
260 caspases and gasdermins by immunoblotting. Since CASP4 is thought to contribute to resolving
261 infection at later time in infection[15], we tested different multiplicity of infections (MOIs) to mimic
262 different infection burdens. In control cells, we observed dose-dependent cleavage of GSDME
263 and GSDMD (Fig. S2A,C). Importantly, CASP3/7 and GSDME processing was largely abrogated
264 in *CASP4/5* KO cells while GSDMD processing was retained, likely due to the activation of the
265 NLRP3 inflammasomes (Fig. S2A)[58-60]. GSDME, but not GSDMD processing, was also abrogated
266 in *CASP3* KO cells (Fig. S2C). Interestingly, despite cleavage of the pore-forming proteins, we
267 observed little to no cell death by Sytox Green (Fig. S2B,D). This suggests that CASP3/7
268 activation and subsequent GSDME cleavage is CASP4/5-dependent, but processing does not
269 generate enough pores to induce cell lysis with stationary phase *Salmonella*.

270 Given that there was little cell death observed with ($Stm^{Stat.}$) and the fact that SPI-1 is important
271 for *Salmonella* to invade and replicate in host cells[61,62], we focused on SPI-1-induced infection
272 models for further studies. We infected WT, *CASP4/5* KO and *CASP3* KO cells with SPI-1-
273 induced *Salmonella* (Stm^{SPI-1}) (Fig. 4A-D). Although CASP1 was activated and could contribute
274 to CASP3 activation, CASP3/7 activation and CASP3-dependent processing of GSDME were
275 predominantly mediated by CASP4/5 (Fig. 4A,C). However, consistent with the notion that CASP1
276 is the dominant protease that processes GSDMD in cells, SPI-1-induced *Stm* infection triggered
277 more cell death than stationary-phase *Stm* infections, which was attenuated in *CASP4/5* KO cells
278 compared to control cells, but unchanged between control and *CASP3* KO cells (Fig. 4B,D).

279 To determine the contribution of the gasdermin proteins to cell death, we infected WT, *GSDMD*,
280 *GSDME*, or *GSDMD/E* KO THP-1 macrophages with Stm^{SPI-1} (Fig. 4E,F). We observed similar
281 results in activation of CASP3/7 with *Salmonella* infection as with LPS transfections, but there
282 were some notable differences in cell death (Fig. 4E,F). GSDME processing was increased in
283 *GSDMD* KO cells and GSDMD processing was increased in *GSDME* KO cells unlike with LPS
284 treatment where GSDMD processing was attenuated in *GSDME* KO cells, likely due to the
285 activation of CASP1 by SPI-1-induced *Salmonella* (Fig. 4E). Indeed, CASP1 processing was
286 similar in WT and *GSDME* KO cells, but was attenuated in *GSDMD* and *GSDMD/E* KO cells

287 compared to WT cells. Strikingly, cell death, as assessed by Sytox Green uptake, was not
288 attenuated in *GSDMD*, *GSDME*, or *GSDMD/E* KO cells at 24h post infection compared to WT
289 cells and although not significant in all cases, trended higher (Fig. 4F).

290 Despite the loss of both pore-forming proteins, *GSDMD/E* KO cells displayed a lytic morphology
291 consistent with gasdermin-independent rupture (Fig. 4G). This implies that enhanced CASP3/7
292 activity may drive rapid cell lysis, potentially through alternative pore-forming mechanisms such
293 as Ninjurin-1 (NINJ1)-mediated membrane rupture[61,63] or pannexin-1 (PANX1), a substrate of
294 CASP3/7 that can form pores independently of GSDMD and GSDME[64]. To test this, WT, *GSDMD*
295 KO and *GSDMD/E* KO cells were infected with *Stm*$^{SPI-1}$ at MOI 50 after pretreatment with
296 spironolactone, a pannexin-1 inhibitor, or glycine, an osmoprotectant that inhibits NINJ1-mediated
297 lysis, or a combination of both[63,64]. Pretreatment with PANX1 or NINJ1 inhibitors separately had
298 a modes impact but together, significantly reduced LDH release in all genotypes (Fig. 4H),
299 supporting the notion that PANX1 channels and NINJ1-mediated rupture occurring downstream
300 of apoptosis contribute to infection-induced lysis, like recently described[15,65]. However, inhibiting
301 PANX1 and NINJ1 together had no impact on pore formation in WT cells, as assessed by Sytox
302 Green uptake, but significantly inhibited Sytox Green uptake in *GSDMD/E* KO cells (Fig. 4I).
303 PANX1 inhibition alone did not reduce Sytox Green uptake in *GSDMD/E* KO cells whereas NINJ1
304 inhibition did, hinting that lysis is mainly mediated by NINJ1. Collectively, these findings suggest
305 that during intracellular LPS sensing, GSDMD is the dominant pore-forming protein mediating cell
306 lysis, while both GSDMD and GSDME contribute to cytokine release. In contrast, during
307 *Salmonella* infection, gasdermin-independent pathways can drive cell rupture downstream of non-
308 canonical inflammasome mediated CASP3/7 activation.

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

311 Recent work revealed that CASP4 plays a role in limiting intracellular *Salmonella* replication,
312 particularly at late stages of infection, though the mechanism remains unclear[15]. Activation of
313 pyroptosis in response to infection is generally thought to be host-protective by limiting cellular
314 replicative niches and activating cytokines that potentiate innate and adaptive immune responses.
315 We hypothesized that CASP4 prevents replication by activating CASP3 to induce GSDME-
316 mediated pyroptosis once bacterial burdens become high. To test the contribution of GSDME in
317 preventing *Salmonella* replication, we infected WT, *GSDMD*, *GSDME,* and *GSDMD/E* KO THP-
318 1 macrophages with GFP-expressing *Salmonella (GFP-Salmonella*) at increasing MOIs (MOI =

319 20, 50, or 100) and measured the change in GFP intensity during infection as a proxy for bacterial
320 replication (Fig. 5). We collected images and quantified the fold change in *GFP-Salmonella*
321 intensity at 24 h post infection relative to the start of imaging (t = 2 h) infection (Fig. 5A).
322 Unexpectedly, GSDME was dispensable for limiting bacterial replication (Fig. 5B). Only *GSDMD*
323 KO cells had significantly higher GFP-*Salmonella* burdens relative to WT infected cells (Fig. 5B),
324 consistent with a recent study demonstrating that GSDMD is essential for limiting intracellular
325 *Salmonella* replication[61]. *GSDME* KO cells had decreased burdens compared to WT cells,
326 suggesting that GSDME may promote, instead of limit infection and replication. Consistent with
327 this idea, *GSDMD/E* KO cells had similar bacterial burdens to WT cells and had decreased
328 burdens compared to *GSDMD* KO cells. Cell death was assessed by LDH release and observed
329 to be largely independent of both GSDMD and GSDME (Fig. 5C), consistent with contributions
330 from PANX1 and NINJ1 activation.

331 We next focused on the role of CASP3 in restricting bacterial replication, as *CASP7* KO cells had
332 a confounding defect in GSDMD processing (Fig. 3G). WT and *CASP3* KO cells were infected
333 with GFP-*Salmonella* and images of infected cells were collected to quantify the fold change in
334 GFP intensity. In WT cells, GFP intensity remained stable, indicating replication control at all MOIs
335 (Fig. 5D,E). In contrast, *CASP3* KO cells showed a significant increase in bacterial burden across
336 all MOIs tested compared to WT cells (Fig. 5D,E). We tested two independent *CASP3* KO clones
337 and LDH release at MOI = 20 was decreased in one clone compared to WT cells but unaffected
338 at MOI = 50 and 100 (Fig. 5F). Together, these data indicate that in human macrophages,
339 GSDMD and CASP3 are essential for restricting intracellular *Salmonella*, whereas GSDME is
340 dispensable and may even facilitate replication.

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

343 To evaluate the physiological relevance of CASP4/5-induced CASP3/GSDME activation, we
344 transfected primary human monocyte-derived macrophages (differentiated with macrophage
345 colony stimulating factor (M-CSF)) with LPS [66]. Consistent with observations in THP-1 cells, LPS
346 transfection induced robust processing of CASP3/7 and GSDME in primary macrophages (Fig.
347 6A). Representative immunoblots from three independent donors are shown (Fig. 6A).
348 Pretreatment with the NLRP3 inhibitor MCC950 blocked CASP1 activation and reduced GSDMD
349 processing but did not alter CASP3/7 or GSDME cleavage, indicating that CASP3/7 activation is
350 primarily mediated by CASP4/5, whereas GSDMD processing is largely downstream of CASP1

351 (Fig. 6A). Intracellular LPS-induced cell death and CASP3/7 activity were comparable between
352 untreated and MCC950-treated cells (Fig. 6B-D), consistent with non-canonical inflammasome
353 activation. Representative images from Donor 7 illustrate pyroptotic morphology and Sytox Green
354 uptake used to monitor cell death kinetics (Fig. 6B). Aggregate analysis across all donors
355 confirmed that cell death was unchanged by MCC950 treatment (Fig. 6E). Importantly, *Salmonella*
356 infection induced CASP3/7, GSDME, and GSDMD processing in primary cells (Fig. 6F). Together,
357 these findings demonstrate that intracellular LPS and *Salmonella* primarily engage CASP4/5 to
358 induce GSDMD and CASP3/GSDME processing.

359 **Discussion**

360 Cell death is central to maintaining cellular homeostasis and resolving infections, with
361 inflammasome-mediated pyroptosis serving as a critical defense mechanism against intracellular
362 pathogens[67]. Previous studies have revealed complex crosstalk between apoptotic and pyroptotic
363 caspases, particularly in canonical inflammasome signaling, where CASP1 can indirectly or
364 directly activate executioner CASP3 and -7 [23,29,31,32,68-71]. These signaling cascades appears to be
365 both species- and stimulus-dependent. Direct cleavage of CASP3/7 by CASP1 has been reported
366 in human cells under certain conditions, such as dipeptidyl peptidase 8/9 inhibitor treatment[29],
367 while in mouse bone marrow–derived macrophages, CASP1-mediated CASP3/7 activation
368 requires upstream CASP8 and CASP9 activation induced by CASP1-dependent Bid cleavage in
369 response to inflammasome agonists like LPS plus nigericin (NLRP3 agonist), *Salmonella* (NLRC4
370 agonist), or poly(dA:dT) (AIM2 agonist) [31,32]. Whether human non-canonical inflammasomes
371 (CASP4/5) similarly activate apoptotic executioner caspases remained unclear.

372 In the present study, we demonstrate that human CASP4/5 directly cleave CASP3 and CASP7,
373 and CASP3 subsequently cleaves GSDME, but our data suggests that most of the cell death is
374 mediated by GSDMD. Unlike CASP1, CASP4/5 did not cleave Bid, and neither CASP8 nor
375 CASP9 contributed to CASP3 activation during non-canonical inflammasome signaling. These
376 data identify CASP4/5 as direct upstream activators of apoptotic executioner caspases in human
377 cells. Although previous *in vitro* studies suggested that murine CASP11 (the ortholog of human
378 CASP4) can cleave mouse CASP3/7, we did not observe significant CASP11 processing of
379 human CASP3[70,72]. Other work indicates that in mouse macrophages, CASP11 triggers CASP1
380 activation, that then cleaves CASP3[71]. This points to species-specific differences in non-canonical
381 inflammasome signaling, consistent with other observations that human CASP4/5, but not mouse
382 CASP11, can cleave IL-18[20-22].

383 Our data also reveal that pharmacological or genetic inhibition of CASP1 attenuated GSDMD
384 processing while increasing GSDME cleavage, indicating that CASP4/5 preferentially drive
385 CASP3/GSDME-mediated pore formation. Both GSDMD and GSDME contributed to IL-1β
386 release as measured by immunoblotting, in agreement with prior observations[45]. However, IL-1β
387 levels measured using an IL-1 receptor activation assay were not significantly reduced by single
388 GSDMD or GSDME deletion, likely reflecting contributions from IL-1α release downstream of pore
389 formation, which activates IL-1α via calpains[73]. Consistent with this, *GSDMD/E* double knockout
390 cells transfected with LPS showed a reduction in released IL-1α/β compared to wild-type or single
391 knockout cells, highlighting the collective role of these gasdermins in coordinating cytokine
392 release.

393 The CASP4/5–CASP3/GSDME axis may function as a backup pathway when pathogens inhibit
394 canonical CASP1-GSDMD signaling. For example, SARS-CoV-2 NSP5 protease cleaves
395 GSDMD N-term to inactivate the pore forming ability, but the protease also activates CASP1 and
396 CASP8, that subsequently activate CASP3 to induce GSDME-mediated pyroptosis[74]. Similarly,
397 Enterovirus 3C protease cleaves and inactivates GSDMD N-term[75], and gram-negative bacterial
398 pathogen *Shigella flexneri* can degrade GSDMD, thereby impairing canonical pyroptosis and
399 promoting infection[76]. Consistently, we observed increased CASP3/7 and GSDME cleavage in
400 GSDMD-deficient cells, highlighting the compensatory nature of this pathway, similar to
401 observations made by others[32,45].

402 While GSDMD has long been recognized as the primary effector of LPS-induced pyroptosis via
403 inflammatory caspases[17-19], our data suggest that in human macrophages, most GSDMD
404 processing is mediated by CASP1, which is activated downstream of the non-canonical pathway.
405 In contrast, CASP4/5 preferentially induce GSDME-mediated pore formation via CASP3
406 activation, but the GSDME pores are sublytic. As CASP4/5 can cleave and inactivate IL-1β[21] and
407 CASP3 is known to inactivate GSDMD[29], IL-1β[77,78], and IL-18[79], it is possible that this pathway
408 may have evolved to induce pyroptosis while limiting inflammation that could result in sepsis.
409 GSDME-mediated pyroptosis may also have a distinct role in promoting a sublytic phase that
410 enables rapid IL-1β release, which could have important ramifications for pathogen defense[45].

411 Our data suggest reciprocal regulation between CASP1 and CASP3-induced pore formation. In
412 CASP1-deficient cells, GSDME processing was increased, while GSDMD processing was
413 increased in CASP3-deficient cells, suggesting that these pathways can compensate for one
414 another to maintain overall cell lysis. This crosstalk may ensure robust inflammatory responses

415 even when pathogens attempt to inhibit one arm of the pathway. Notably, GSDMD appears to
416 play a dominant role in the kinetics of pyroptotic cell death, consistent with previous reports that
417 GSDMD pore formation precedes apoptotic caspase activation during canonical inflammasome
418 signaling[29,80]. However, this only applied in the context of when CASP4/5 were activated by LPS.
419 In this study, neither GSDMD nor GSDME were required for *Salmonella* induced cell lysis at 24h
420 post infection, contrasting with a recent study demonstrating that GSDMD and NINJ1 were
421 essential for LDH release in THP-1 macrophages infected with *Salmonella*[15]. Differences in
422 infection timepoint (6 h vs 24 h in this study), cell priming (e.g., Pam3CSK4 vs. unprimed in our
423 study) and contributions of alternative pore-forming proteins such as NINJ1 or PANX1 may explain
424 these discrepancies. Notably, recent work in mice found that LPS or *Escherichia coli* infection plus
425 heat shock induced cell lysis that was independent of the typical pore-forming proteins such as,
426 GSDMD, GSDME, and MLKL (mixed lineage kinase domain-like protein), but was critically
427 dependent on NINJ1, which mediates plasma membrane rupture downstream of several cell
428 death pathways[63,81]. Furthermore, this death only occurred with certain priming agents, such as
429 poly(I:C) and LPS, but not Pam3CSK4[63]. Hence, it's possible that under certain conditions,
430 *Salmonella* can induce rapid cell lysis that is independent of GSDMD and GSDME. More studies
431 using human cells are needed to understand the precise contributions of the different pore-
432 forming proteins in pathogen defense.

433 Much of our understanding of the innate immune response to *Salmonella* has stemmed from work
434 using mice, but emerging studies using human cells have begun to yield critical insights. Recent
435 work in human macrophages demonstrated that CASP1-dependent pyroptosis is important for
436 limiting intracellular *Salmonella* replication early during infection but CASP4 was important for
437 restricting replication at later stages of infection[15]. Our data suggests that CASP4 inhibits
438 replication in part by activating CASP3/7 to induce apoptosis and cell lysis that is likely dependent
439 on NINJ1 or PANX1. Indeed, NINJ was reported to be important for cell death in THP1
440 macrophages infected with *Salmonella*[15]. Recent work also revealed that CASP3 can activate
441 PANX1 to induce cell lysis independently of CASP1, CASP8, GSDMD, and GSDME[64]. Consistent
442 with this notion, we found that *GSDMD/E* KO cells exhibited lytic morphology and GSDME was
443 dispensable for inhibiting bacterial replication. Also in agreement, a recent report also noted that
444 GSDME was dispensable, and that GSDMD is the only gasdermin that confers protection against
445 acute *Salmonella* infection in the gut of mice[82]. Additional support for this mechanism stems from
446 observations that apoptosis is critical for restricting *Legionella* infection and replication in dendritic
447 cells[34], and apoptosis was reported to be essential for limiting *Salmonella* replication in mice[65,83].

448 Collectively, this implies that apoptosis may play a broader role in preventing intracellular
449 pathogen replication and future studies should focus on delineating the contributions of apoptosis
450 and pyroptosis in restricting infection.

451 Controlled activation of pyroptosis protects the host from infection, and therefore it was surprising
452 that GSDME was dispensable for protecting against *Salmonella* replication. If CASP3/GSDME-
453 induced pyroptosis occurs without CASP1 activation, it could be detrimental to the host as it can
454 facilitate bacterial egress and infection of bystander cells without the full immune response
455 induced by CASP1 activation. In support of this idea, it was demonstrated that CASP11-mediated
456 cell death in the absence of CASP1 activation was detrimental and resulted in increased
457 susceptibility to *Salmonella* infection in mice[55]. Similarly, it was shown that GSDMD pores promote
458 the release of the fungal pathogen *Candida albicans* from mouse macrophages, and inhibiting
459 GSDMD was protective as it prevented *Candida albicans* escape while maintaining CASP1
460 activation and GSDMD-independent IL-1β release[84]. Alternatively, if GSDME pores are sublytic,
461 as has been suggested[45], it could facilitate pathogen entry and render *GSDME* KO cells less
462 susceptible to initial infection. We note that our GSDME KO cells were more resistant to infection
463 than WT and *GSDMD* KO cells and required higher MOIs to observe similar levels of infections.
464 Intriguingly, CASP3/GSDME-mediated pyroptosis appears to be critical for limiting viral
465 replication[35], suggesting that the impact on pathogen defense could be pathogen-specific.
466 Importantly, we demonstrate that CASP3/GSDME are processed in primary human macrophages
467 following non-canonical inflammasome activation, underscoring the physiological relevance of
468 this pathway.

469 In conclusion, we identify CASP3/7 as direct substrates of human non-canonical inflammasomes
470 (CASP4/5) in both immortalized and primary human macrophages. Mechanistically, CASP4/5
471 mediated cleavage of CASP3 initiates GSDME processing, but our data shows that CASP3 and
472 GSDMD, but not GSDME, are critical for limiting intracellular *Salmonella* replication in THP-1
473 cells. Future work is needed to confirm the role of CASP4/5 and CASP3 on replication in primary
474 cells. CASP3 activation can drive cell lysis that is independent of GSDMD and GSDME, likely
475 through PANX and NINJ1, which have both been demonstrated to be important in limiting
476 *Salmonella* replication[15,65]. Given that apoptosis has recently been reported to be important for
477 infection control, our data suggests that this seemingly immune silent form of cell death may be
478 more important than previously recognize in controlling infections. A key outstanding question is
479 the molecular mechanism by which CASP4/5 recognize and cleave CASP3/7. Given that

480 inflammatory caspases utilize exosites or tetrapeptide recognition sequences for substrate
481 specificity[16], it is likely that a combination of these factors underlies recognition. Indeed, recent
482 complementary work using *in vitro* biochemical assays found that CASP3/7 are direct substrates
483 of CASP4 and CASP5, and the primary mode of recognition and processing was dominated by
484 the exosite interactions, offering critical insight into the molecular mechanism of recognition and
485 processing[85].

486 **Method Details**

487 **Antibodies and Reagents**

488 Antibodies used include: GSDMD Rabbit polyclonal Ab (Novus Biologicals, NBP2-33422), FLAG®
489 M2 monoclonal Ab (Sigma, F3165), GAPDH Rabbit monoclonal Ab (Cell Signaling Tech, 14C10),
490 CASP1 p12/10 Rabbit monoclonal Ab (Abcam, ab179515), CASP4 Rabbit polyclonal Ab (Cell
491 Signaling Tech, 4450S), CASP5 Rabbit monoclonal Ab (Cell Signaling Tech, 46680S), Myc Mouse
492 monoclonal Ab (Cell Signaling Tech, 2276S), CASP3 (Cell Signaling Tech 9664S), CASP3 (Cell
493 Signaling Tech, 9662), CASP7 (Cell Signaling Tech, 8438S), CASP7 (Cell Signaling Tech,
494 12827S), GSDME (Abcam, ab215191), hIL-1β Goat Polyclonal Ab (R&D systems, AF-201-NA),
495 hIL-18 Goat Ab (R&D systems, af2548), CASP8 (Cell Signaling Tech, 4790S), CASP9 (Cell
496 Signaling Tech, 9508S), Bid (Cell Signaling Technology, 2002), IL-1α (Novus, AF-200-NA), HA
497 Rabbit monoclonal Ab (Cell Signaling Tech, 3724S), FKBP12 Rabbit polyclonal Ab (Abcam,
498 ab24373). IRDye 800CW anti-rabbit (LICOR, 925-32211), IRDye 800CW anti-mouse (LI-COR,
499 925-32210), IRDye 680CW anti-rabbit (LI-COR, 925-68073), IRDye 680CW anti-mouse (LI-COR,
500 925-68072). Other reagents used include: LPS-EB Ultrapure (Invivogen, tlrl-3pelps), FuGENE
501 HD (Promega, E2311), AP20187 (Tocris™ 6297/5).
502

503 **Recombinant Caspase Assays**

504 All *in vitro* assays using recombinant caspases were performed in 20 µL reactions containing
505 caspase assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1mM EDTA, 0.1% CHAPS,
506 10% sucrose, pH 7.) as previously described[23]. 10 μg of plasmids encoding for GSDMD, CASP3,
507 CASP7, or GSDME were transfected into HEK 293T cells (10 cm plates) using FuGENE HD
508 Transfection Reagent (Promega, E2312) according to the manufacturer's protocol. After 24 hours,
509 cells were harvested and lysed by sonication for 10 second pulses for 60 seconds at 30%
510 amplitude in PBS and centrifuged at 12,000 g to remove cell debris. Lysates were diluted 10x in
511 caspase assay buffer and incubated with 0.25 activity units/µL of each indicated caspase. At the

512 indicated time points, samples were mixed 1:1 with 2x Licor Protein Sample Loading Buffer (Neta
513 Scientific, 928-40004) and boiled at 95 °C for 10 minutes, then analyzed by immunoblotting.

514 **Cell Culture**

515 All cells were grown at 37 °C in a 5% CO2 atmosphere incubator. HEK 293T cells were purchased
516 from ATCC and THP1 cells were obtained previously or acquired from the Bryant lab[21] or Abbott
517 lab[45]. HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-
518 glutamine and 10% fetal bovine serum (FBS). THP-1 cells were cultured in Roswell Park Memorial
519 Institute (RPMI) medium 1640 with L-glutamine and 10% fetal bovine serum (FBS). Purified
520 human monocytes from healthy donors were purchased from the University of Pennsylvania
521 Human Immunology Core. Purified monocytes were cultured in RPMI + 10% FBS and 25 ng/ml
522 rm-csf in 96 well plates at a density of 100,000 cells/well. Media was removed and replaced with
523 Opti-MEM containing Sytox Green and MCC950 as indicated for 30 minutes prior to LPS
524 transfection as previously described. Cell lines were negative for mycoplasma contamination.
525

526 **IncuCyte CASP3/7 activity and Sytox Green uptake assays**

527 The kinetics of CASP3/7 activity and Sytox Green uptake were measured in cells using the
528 IncuCyte S3 Live cell imaging system (Sartorius). THP-1 cells were resuspended in RPMI medium
529 containing 50 ng/mL phorbol 12-myristate 12-acetate (PMA) and plated in black, clear-bottom 96-
530 well plates at a density of 8 x 10^4 cells/well. After 48 h, the media was then replaced with Opti-
531 MEM (0.1 mL/well) supplemented with either the cell-permeable CASP3/7 green dye (final
532 concentration: 10 µM (1:200 dilution)) or Sytox Green (final concentration: 0.2-0.5 µM), as
533 indicated. A series of images were collected with a 10x objective at 1h intervals for 24 hours. The
534 number of Sytox Green or CASP3/7 dye-positive cells in each image was determined using the
535 IncuCyte S3 software with 3 different areas/well. Cell death was quantified relative to 0.1% Triton
536 treated macrophages.
537

538 **Cloning**

539 All plasmids were cloned using Gateway technology as previously described[21]. DNA encoding the
540 indicated proteins were inserted between the *attR* recombination sites and shuttled into modified
541 pLEX_307 vectors (Addgene) using Gateway technology (Thermo Fisher Scientific) according to
542 the manufacturer's instructions. Proteins expressed from these modified vectors contain an N-
543 terminal *att*B1 linker (GSTSLYKKAGFAT) after any N-terminal tag or a C-terminal *att*B2 linker
544 (DPAFLYKVVDI) preceding any C-terminal tag such as Myc or HA.

545

546 sgRNAs were designed using the Broad Institute's web portal and cloned into the
547 pXPR016_Hygro or plentiGuide-Puro vector (pXPR003) (Addgene #52963) as described
548 previously[29,86]. The sgRNA sequences used were:

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

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

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

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

553 Control *(GFP)* 5′-GGGCGAGGAGCTGTTCACCG-3′

554

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

556

557 **THP1 Knockout cell lines**

558 Constructs were packaged into lentivirus in HEK 293T cells using the Fugene HD transfection
559 reagent (Promega) and 2 µg of the vector, 2 µg psPAX2, and 0.2 µg pMD2-G. THP-1 were
560 spinfected with virus for 2 h at 1000g at 30 °C supplemented 8 µg/mL polybrene. After 2 days,
561 cells were selected for stable expression of S. pyogenes Cas9 (Addgene #52962) using blasticidin
562 (5 µg/mL for THP-1) and for stable expression of sgRNAs using puromycin (0.5 µg/mL for THP-
563 1) or hygromycin (100 µg/mL for THP-1). After 10 d, single cells were isolated by serial dilution
564 and expanded. Cells were then harvested for immunoblotting or used in experiments.

565

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

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

572

573 **Transient Transfection**

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

577 **LPS Transfection of human macrophages**

578 THP-1 cells were resuspended in RPMI medium containing 50 ng/mL phorbol 12-myristate 12-
579 acetate (PMA). Cells were plated in 96-well plates at a density of 8 x 10^4 cells/well. After 48 h, the
580 media was then replaced with Opti-MEM (0.1 mLs/well). Where indicated, cells were treated with
581 MCC950 (10 μM) for 30 min at 37 °C 5% CO_2 before LPS transfection. The LPS solution was
582 prepared by adding LPS (25 mg/mL final concentration) and FuGENE (0.5% final concentration)
583 to Opti-MEM. This solution was gently mixed by flicking and incubated for 30 min at room
584 temperature before drop-wise addition to each well. The supernatants were collected separately,
585 and cells were lysed by sonication 24 h post transfection. Harvested supernatants were
586 precipitated by chloroform/methanol and analyzed by immunoblotting.

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

588 HEK-Blue IL-1β (Invivogen) reporter cells were used according to manufacturer's protocols as
589 previously described[21] to quantify the amount of active IL-1α/β released. HEK Blue cells were
590 seeded in 96 well plates at 5 x 10^4 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) with
591 L-glutamine and 10% fetal bovine serum (FBS) to a total volume of 150 ml. 10 μL of supernatant
592 from control or treated THP1 cells were added, and the samples were incubated overnight at
593 37°C in a 5% CO2 atmosphere incubator. A serial dilution of recombinant IL-1β (Invivogen) was
594 included in all experiments to generate a standard curve which to calculate absolute levels of
595 active IL-1β. 50 μL of supernatant was mixed with 150 μL of freshly prepared Quanti-Blue solution
596 and incubated at room temperature for 10 minutes in the dark before reading absorbance at 620
597 nm using a Cytation 5 plate reader (Bio Tek).

598 ***Salmonella* Infection of human macrophages**

599 *Salmonella* Typhimurium SL1344 or SL1344 pDiGc[87] was streaked to isolation from frozen stock
600 on LB + Streptomycin (100μg/ml) agar plates. Carbenicillin (100μg/ml) was included for all studies
601 with SL1344 pDiGc. For SPI-I induced bacteria, single colonies were inoculated in LB +
602 Streptomycin broth overnight (~18h). The overnight culture was back diluted 40-fold into LB +
603 Streptomycin broth containing 300 mM NaCl. After three hours bacteria were washed 2x in sterile
604 PBS and cell density was determined by quantifying OD_{600}. MOI was prepared according to OD
605 and bacterial cells were resuspended in PBS. 10μL suspension of bacteria was added to
606 macrophages in triplicate in 96-well plates. Macrophages were spun for 10 min at 400g to allow
607 infection and then placed in incubator for 1h. Media was then removed and replaced with fresh
608 RPMI without FBS containing gentamicin (100 μg/ml). After 1h, media was removed and wells

609 were washed 3x with PBS and replaced with fresh RPMI without FBS containing gentamicin (10
610 μg/ml). GFP fluorescence was monitored using IncuCyte live cell imaging for 24h.

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

614 For stationary phase bacterial infections, single colonies were inoculated in LB + Streptomycin for
615 2 days. MOI were prepared as described above and 10μL suspension of bacteria were added to
616 macrophages in triplicate in 96 well plates. Macrophages were spun for 10 min at 400g to allow
617 infection and then placed in incubator for 2h.

618 **LDH Assays**

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

623 **Western Blotting**

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

635 **Statistical Analysis**

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

Author contributions CRediT:

Conceptualization: CYT, Ma.Ku., CMB Methodology: CYT, Ma.Ku, CMB, Investigation: Ma.Ku., CMB, ABM, PME, CM, SC, Ma.Ka., Mi.Ka., WY, TJW, RCP, BMD Visualization: CYT, Ma.Ku., CMB, Funding acquisition: CYT, CMB, Project administration: CYT Data curation: CYT, Ma.Ku., CMB, Formal Analysis: CYT, Ma.Ku., CMB, ABM, PME, CM, SC, Ma.Ka., Mi.Ka., Resources: CYT Supervision: CYT, Writing – original draft: CYT, Ma.Ku., CMB, Writing – review & editing: CYT, Ma.Ku., CMB.

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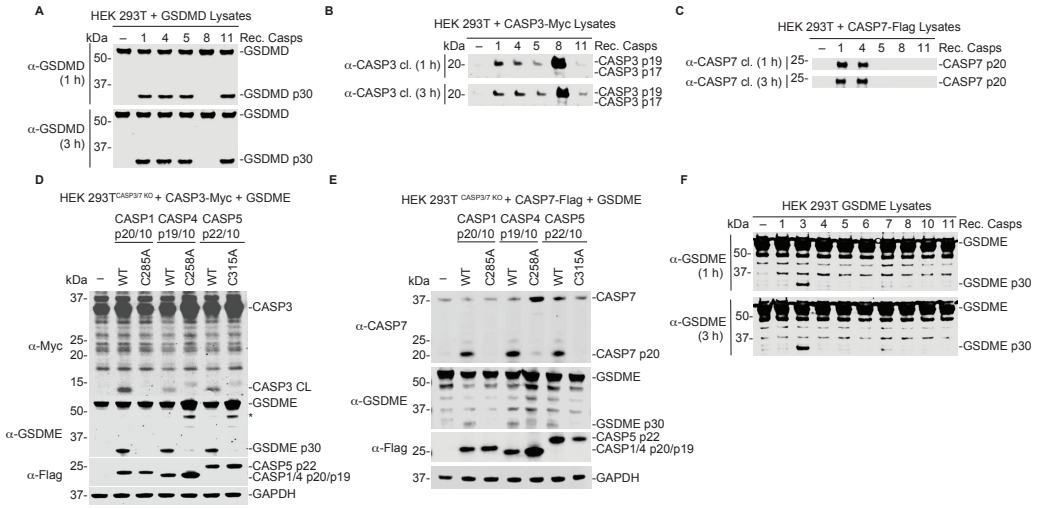
Conflicts of Interest

The authors declare no conflicts of interests.

668 **FIGURES**



669

670 **Figure 1. Caspases-1, -4, and -5 directly cleave and activate caspases-3 and -7. (A-C)** HEK
671 293T cells were transiently transfected with plasmids coding for human GSDMD **(A)**, CASP3 **(B)**,
672 or CASP7 **(C)**. Lysates were then incubated with 0.25 activity units/μL of each indicated
673 recombinant caspase for 1 or 3 h and cleavage products were assessed by immunoblotting. **(D,E)**
674 *CASP3/7* KO HEK 293T cells were transiently co-transfected with the indicated constructs coding
675 for active CASP3 **(D)** or CASP7 **(E)** along with GSDME, and catalytically active or inactive CASP1,
676 -4, or -5 for 24 h before the cell lysates were analyzed by immunoblotting. **(F)** HEK 293T cells
677 were transiently transfected with a plasmid coding for GSDME for 24 h. Lysates were harvested
678 and incubated with 0.25 activity units/μL of each recombinant caspase for 1 or 3 h. Cleavage
679 products were assessed by immunoblotting. Data are representative of three or more independent
680 experiments.

681

682

683

684

685

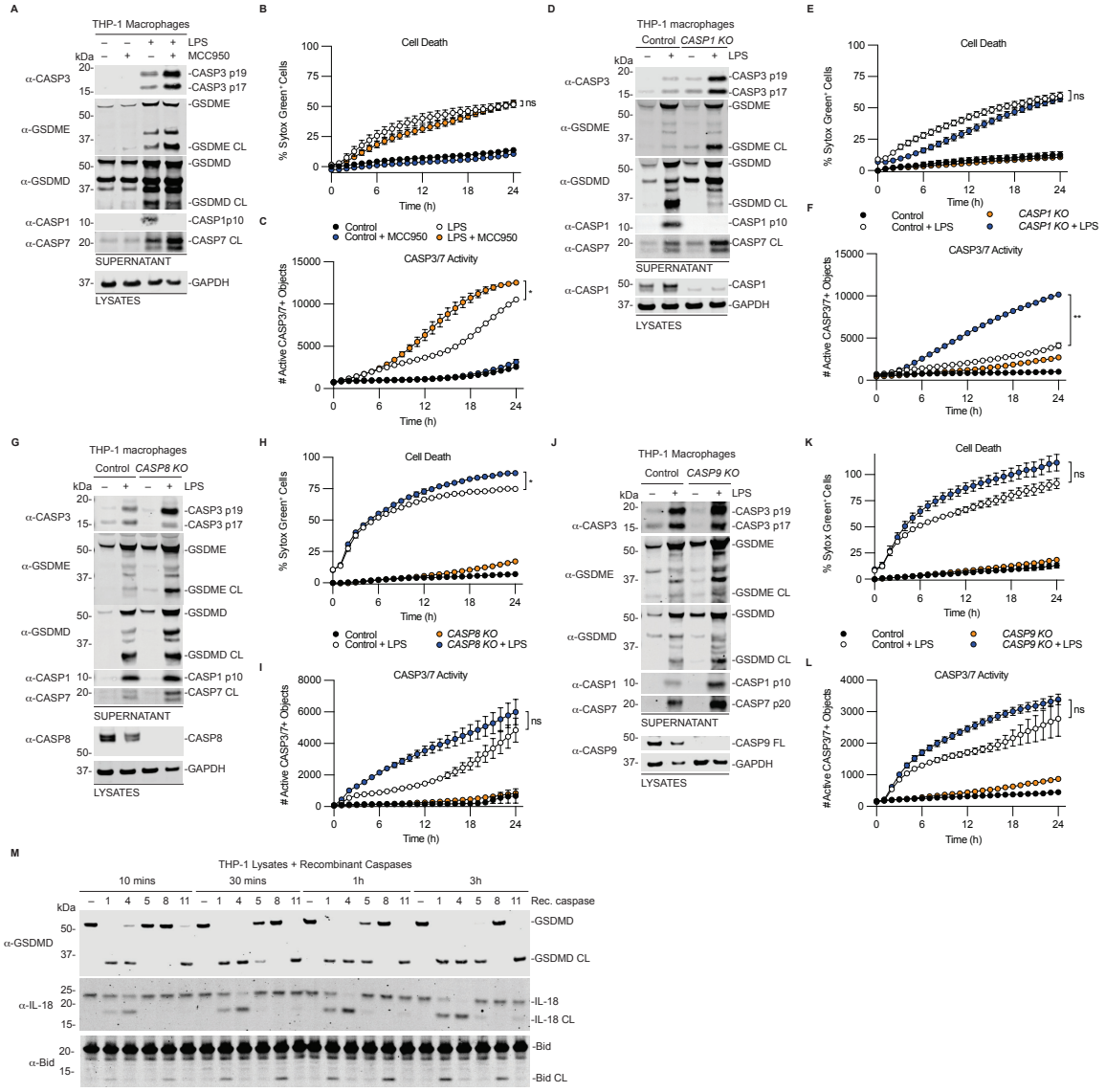


Figure 2. CASP1, CASP8, and CASP9 are dispensable for CASP3/7 and GSDME processing during non-canonical inflammasome activation by intracellular LPS. (**A-C**) WT THP-1 cells were transfected with 25 μg/mL LPS in the presence or absence of the NLRP3 inhibitor MCC950 (10 μM) for 24 h. Supernatants and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

macrophages were lysed and incubated with 0.25 activity units/μL of recombinant caspases for the indicated times before immunoblot analysis. Data are means ± SEM of three independent replicates, and representative of at least three independent experiments except for M (n = 2). ****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.

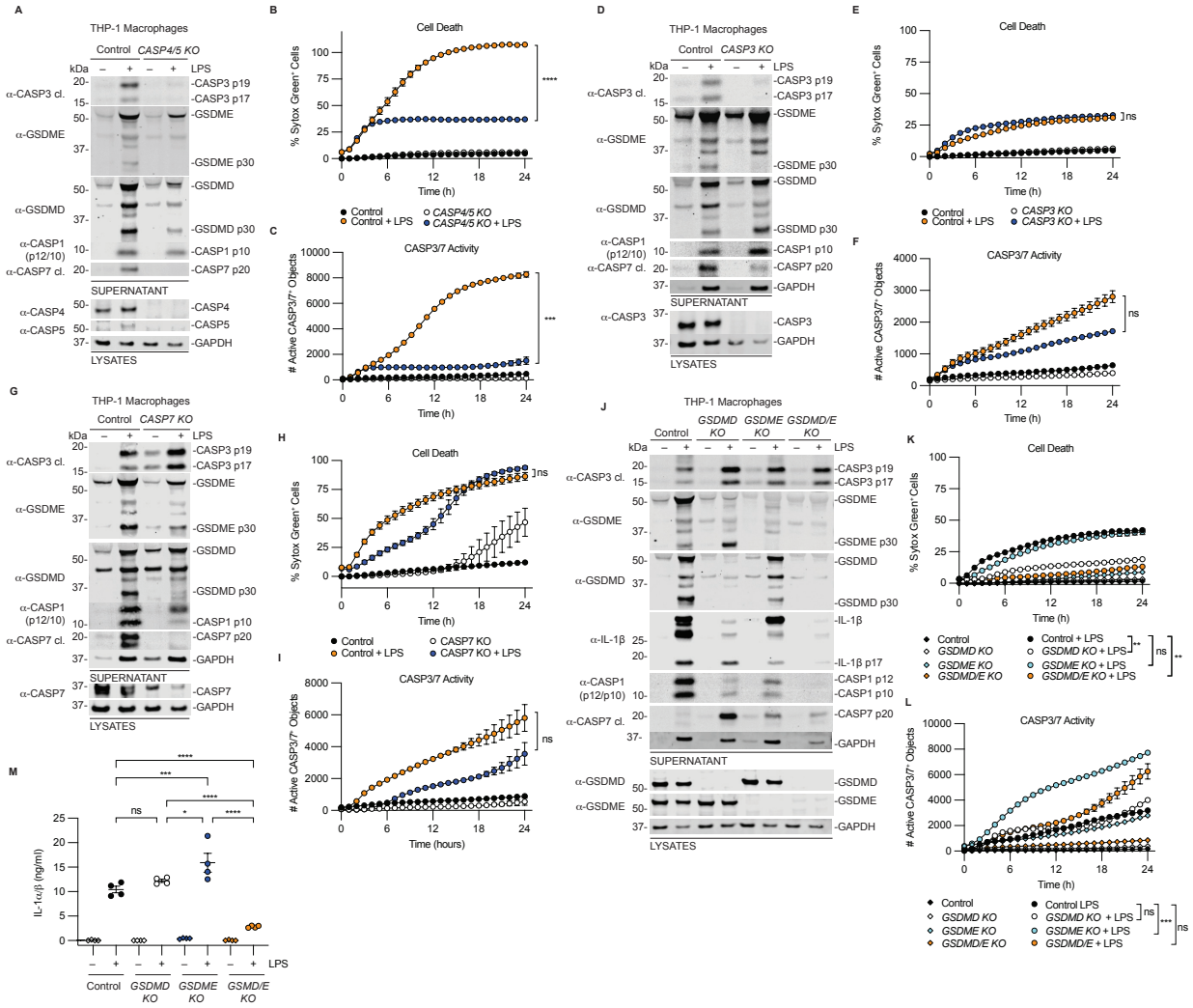


Figure 3. CASP3, and CASP4/5 are required to induce GSDME processing during non-canonical inflammasome activation by intracellular LPS. (**A-C**) WT and *CASP4/5* double KO THP-1 cells were transfected with 25 μg/mL of LPS for 24 h then supernatants and lysates were analyzed by immunoblotting (**A**),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

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

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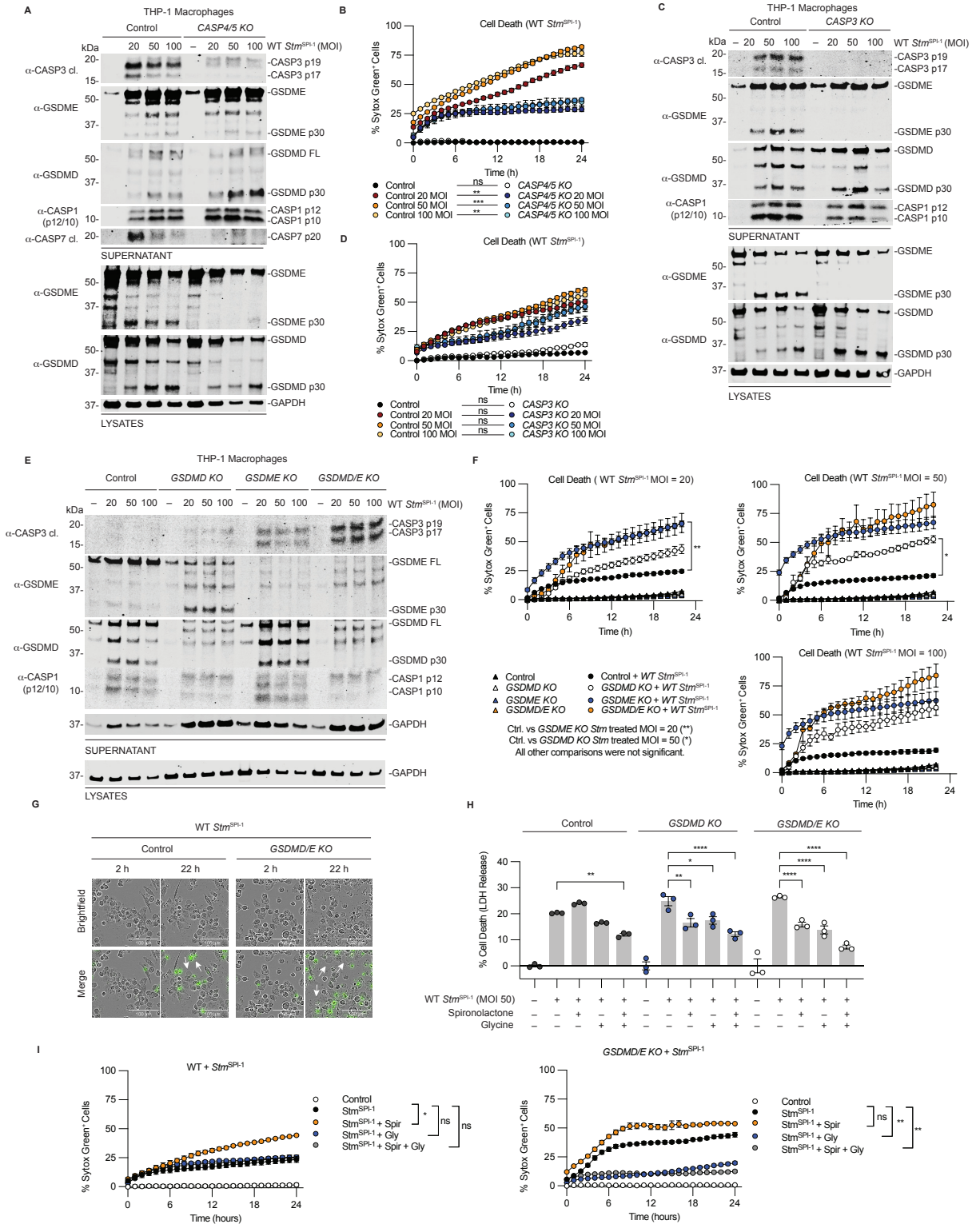

Figure 4. CASP3, and CASP4/5 are required for GSDME processing during non-canonical inflammasome activation by intracellular *Salmonella*. (A-D) WT and *CASP4/5* KO (A,B) or WT and *CASP3* KO THP-1 macrophages (C,D) were treated with the indicated MOI of WT SPI1-

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

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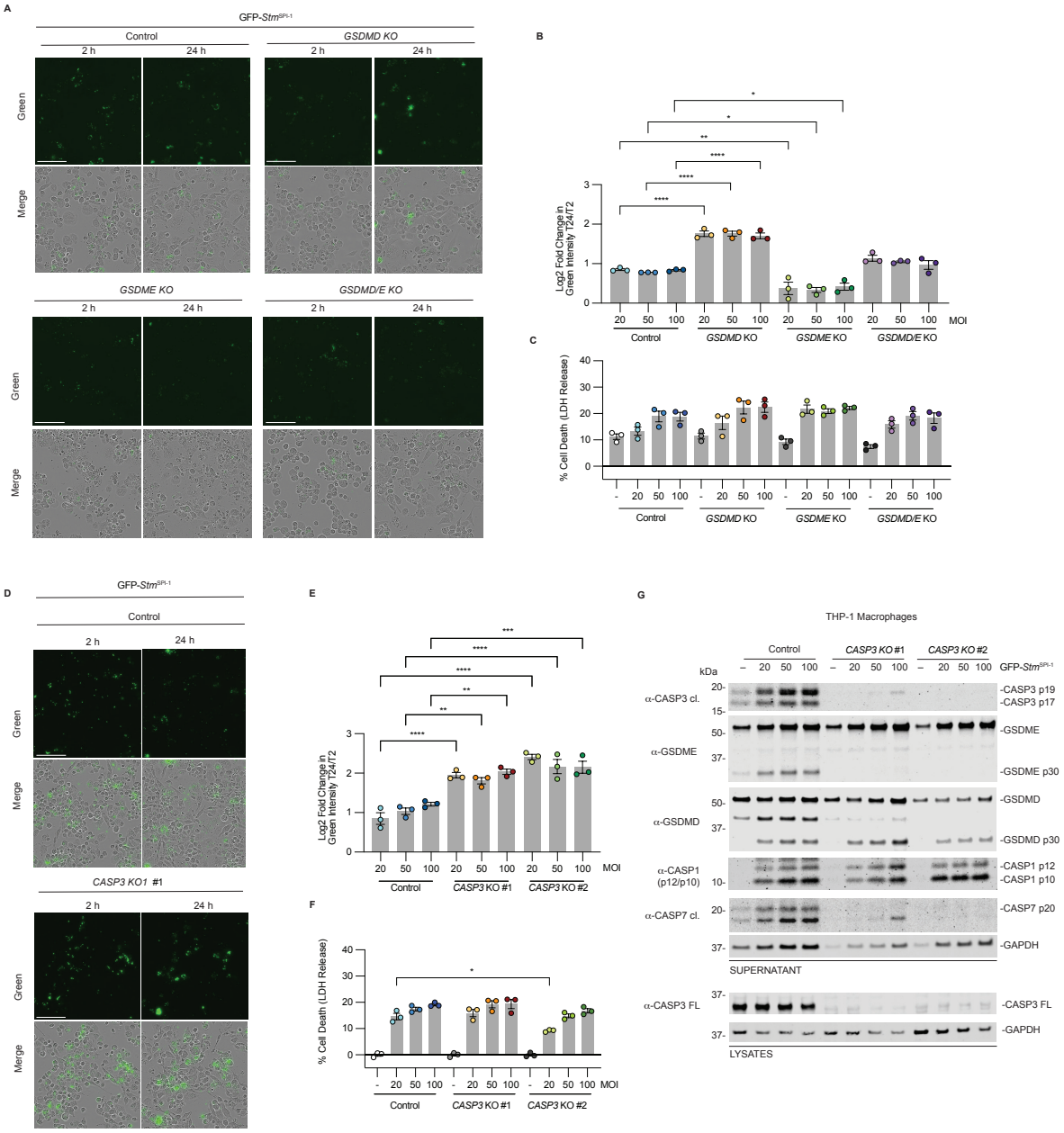


Figure 5. GSDMD and CASP3 are essential, but GSDME is dispensable for limiting *Salmonella* replication in human macrophages. (**A-C**) WT, *GSDMD* KO, *GSDME* KO and *GSDMD/E* KO THP-1 macrophages were treated with the indicated MOI of SPI-1-induced GFP-expressing *Salmonella*. Representative images 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.

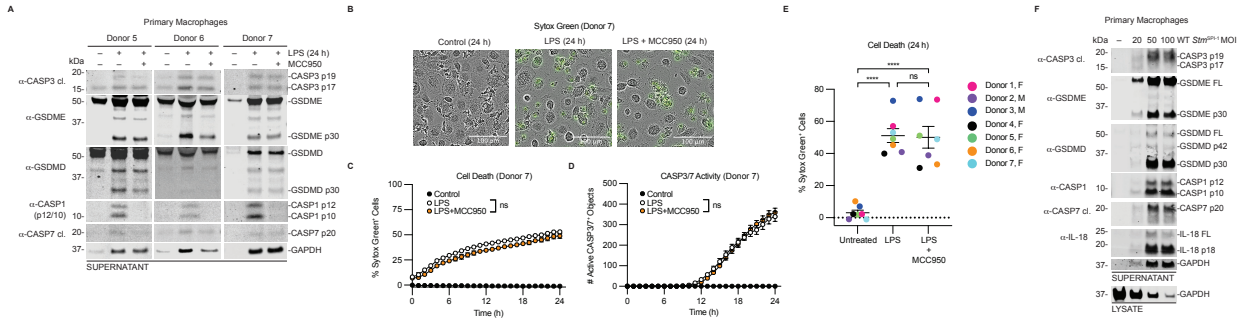
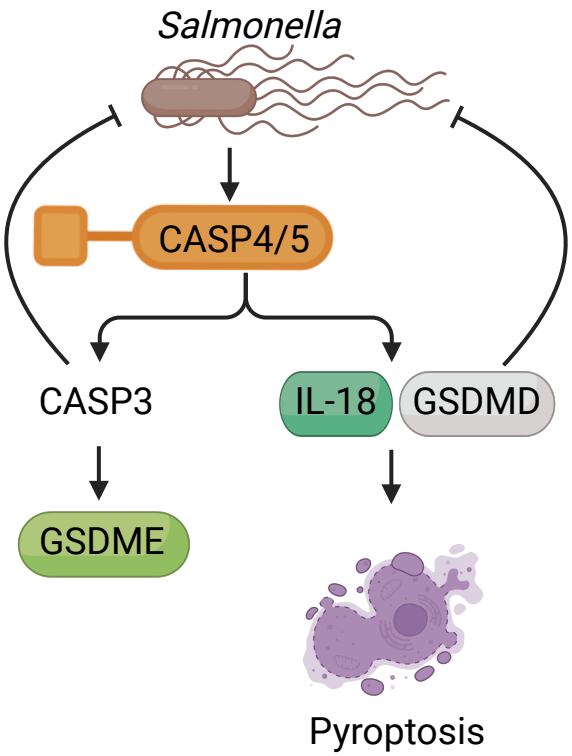


Figure 6. CASP3/7 and GSDME are cleaved in primary human macrophages during non-canonical inflammasome activation. (**A-E**) Primary monocytes were differentiated into macrophages and transfected with 25 μg/mL of LPS for 24 h. Some cells were pretreated with 10 μM MCC950 for 30 minutes before LPS transfection 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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817 **Figure 7. Proposed model of how non-canonical inflammasomes contribute to limiting**
818 **intracellular *Salmonella* replication.** Upon sensing LPS or *Salmonella*, CASP4/5 directly cleave
819 CASP3/7 (CASP7 not shown), and CASP3 subsequently cleaves GSDME to activate it. However,
820 GSDME does not appear to impact cell lysis, and is dispensable for controlling *Salmonella*
821 replication. Notably, CASP3 activation is important for limiting intracellular *Salmonella* replication.
822 CASP4/5 can also directly cleave GSDMD and IL-18 to induce pyroptosis, and GSDMD is
823 essential for limiting bacterial replication. Figure generated in Biorender.

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