

1 **Title: Sequentially activated death complexes regulate pyroptosis and IL-1 β**
2 **release in response to *Yersinia* blockade of immune signaling**

3

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16 **Abstract:** The *Yersinia* virulence factor YopJ potently inhibits immune signaling in macrophages
17 by blocking activation of the signaling kinases TAK1 and IKK. In response, macrophages trigger
18 a backup pathway of host defense that mediates cell death via the apoptotic enzyme caspase-8 and
19 pyroptotic enzyme caspase-1. While caspase-1 is normally activated within multiprotein
20 inflammasome complexes that contain the adaptor ASC and NLRs, which act as sensors of
21 pathogen virulence, caspase-1 activation following *Yersinia* blockade of TAK1/IKK surprisingly
22 requires caspase-8 and is independent of all known inflammasome components. Here, we report
23 that caspase-1 activation by caspase-8 requires both caspase-8 catalytic and auto-processing
24 activity. Intriguingly, while caspase-8 serves as an essential initiator of caspase-1 activation,
25 caspase-1 amplifies its own activation through a feed-forward loop involving auto-processing,
26 caspase-1-dependent cleavage of the pore-forming protein GSDMD, and subsequent activation of
27 the canonical NLRP3 inflammasome. Notably, while caspase-1 activation and cell death are
28 independent of inflammasomes during *Yersinia* infection, IL-1 β release requires the canonical
29 NLRP3 inflammasome. Critically, activation of caspase-8 and activation of the canonical
30 inflammasome are kinetically and spatially separable events, as rapid caspase-8 activation occurs
31 within multiple foci throughout the cell, followed by delayed subsequent assembly of a single

32 canonical inflammasome. Importantly, caspase-8 auto-processing normally serves to prevent
33 RIPK3/MLKL-mediated necroptosis, and in caspase-8's absence, MLKL triggers NLPR3
34 inflammasome activation and IL-1 β release. Altogether, our findings reveal that functionally
35 interconnected but temporally and spatially distinct death complexes differentially mediate
36 pyroptosis and IL-1 β release to ensure robust host defense against pathogen blockade of TAK1
37 and IKK.

38

39 **One Sentence Summary:** *Yersinia*-induced cell death and IL-1 β release are driven by spatially
40 and temporally distinct but functionally connected death complexes.

41

42 **Main Text:**

43 INTRODUCTION

44 The innate immune system is critical for host defense against bacterial pathogens, as it detects
45 pathogen-associated molecular patterns (PAMPs) as well as pathogen-mediated perturbations of
46 host biological pathways^{1,2}. Apoptosis, pyroptosis and necroptosis are distinct forms of regulated
47 cell death that mediate anti-microbial host defense^{3,4}. Apoptosis is classically viewed as a
48 developmentally programmed or homeostatic, non-inflammatory cell death, whereas pyroptosis is
49 a lytic form of cell death accompanied by release of inflammatory IL-1 family cytokines that takes
50 place in response to microbial infection⁵⁻⁷. Apoptosis and pyroptosis are both driven through
51 activation of caspases, pro-enzyme cysteine proteases that undergo proteolytic activation
52 following recruitment to multiprotein complexes⁸, while necroptosis is caspase-independent^{9,10}.

53 Apoptosis and pyroptosis require engagement of distinct signaling complexes and effector
54 caspases, and are traditionally thought to be mutually exclusive and cross-inhibitory³. However,
55 disruption of core immune signaling pathways by pathogen virulence factors can trigger cell death
56 that exhibits biochemical features of both apoptosis and pyroptosis¹¹. Indeed, recent studies have
57 proposed the existence of a cell death pathway involving simultaneous activation of pyroptosis,
58 apoptosis, and necroptosis, termed PANoptosis^{12,13} following microbial infection or disruption of
59 immune signaling pathways. However, as the morphologic and physiologic consequences of
60 distinct cell death pathways are unique, and the effector enzymes of one death pathway typically
61 cross-inhibit the others, how an individual cell might simultaneously undergo multiple distinct
62 forms of cell death is unclear.

63 During apoptosis, processing of executioner caspases-3 and -7 by the initiator caspase-8
64 results in the cleavage of numerous caspase-3/7-dependent substrates, leading to the organized
65 breakdown of the cell into membrane-enclosed ‘blebs’ that are rapidly phagocytosed by
66 neighboring cells with minimal inflammation⁵. Conversely, during pyroptosis, caspase-1 is
67 activated by its recruitment into inflammasomes, multiprotein signaling complexes that form in
68 response to microbial contamination of the cytosol^{6,7}, and are nucleated by sensor NLR proteins
69 and the adaptor protein ASC (apoptosis-associated speck like protein containing a caspase
70 activation and recruitment domain)¹⁴. Active caspase-1 processes the inflammatory cytokine pro-
71 IL-1 β and pore-forming protein Gasdermin D (GSDMD). The N-terminal fragment of GSDMD
72 (p30) oligomerizes and inserts into the plasma membrane, releasing mature IL-1 β as well as other
73 intracellular alarmins through membrane rupture and cell lysis¹⁴⁻¹⁶. However, pathogenic
74 *Yersinia* inject a variety of virulence factors known as *Yersinia* outer proteins (Yops) into the
75 cytoplasm of host cells through their Type 3 Secretion Systems (T3SS)¹⁷⁻¹⁹ to disrupt innate
76 immune responses. Among these is the acetyl-transferase YopJ, which blocks IKK and TAK1
77 signaling²⁰⁻²². Such blockade leads to the combined activation of caspase-1 and caspase-8, and
78 elicits caspase-1 and caspase-8-dependent cleavage of GSDMD and IL-1 β ^{23,24}. Interestingly,
79 caspase-1 activation following *Yersinia pseudotuberculosis* (*Yptb*) infection is independent of all
80 currently known inflammasome components, including NLRP3, NLRC4 and the inflammasome
81 adaptor protein ASC²⁵, but is instead dependent on caspase-8²⁵. Intriguingly, despite the lack of a
82 requirement for ASC in caspase-8 or -1 activation or cell death²⁵, ASC forms large oligomers in
83 response to YopJ activity, suggesting that ASC complexes play an as-yet-undefined role in
84 *Yersinia* infection²⁴. While the ASC pyrin (PYD) domain interacts with the caspase-8 death-
85 effector (DED) domain^{24,26,27}, whether these distinct pathways are activated simultaneously or
86 sequentially within infected cells and their role in promoting programmed cell death and
87 inflammatory responses is poorly defined.

88 Here, we find that caspase-8-dependent caspase-1 activation requires both caspase-8 and
89 caspase-1 activity. Surprisingly, despite the ability of caspase-8 to cleave caspase-1 directly,
90 caspase-1 catalytic activity was required for its own processing downstream of caspase-8
91 activation, indicating that caspase-1 acts as a feed-forward amplifier of caspase-8-dependent
92 pyroptosis. Macrophages that express an uncleavable caspase-8 (*Casp8*^{D387A/D387A}) are sensitized
93 to RIPK3-mediated necroptosis, which triggers a backup pathway of caspase-1 activation to enable

94 pyroptotic cell death and IL-1 β release even in the absence of active caspase-8. Additionally,
95 although ASC is not required for caspase-1 activation during *Yptb* infection, IL-1 β release requires
96 the canonical NLRP3 inflammasome. These findings indicate that secondary NLRP3
97 inflammasome activation subsequent to GSDMD cleavage and potassium-efflux mediates IL-1 β
98 release. Indeed, caspase-8 activation preceded assembly of ASC puncta, and ASC puncta and
99 active caspase-8 were differentially localized within macrophages. Altogether, this work
100 demonstrates that functionally linked, but temporally and spatially distinct death complexes
101 mediate pyroptosis and IL-1 β release in response to pathogen blockade of innate immune
102 signaling.

103 **RESULTS**

104 **Caspase-8 activity is required for cell death and caspase-1 processing**

105 During *Yersinia pseudotuberculosis* (*Yptb*) infection, cell death and caspase-1 processing occur
106 independently of all known inflammasome components²⁵. Consistent with previous findings from
107 our group and others^{25,28}, cell death in response to *Yersinia* YopJ activity is dependent on caspase-
108 8, as in contrast to either *Ripk3*^{-/-} or C57BL/6 bone marrow-derived macrophages (BMDMs),
109 *Casp8*^{-/-}*Ripk3*^{-/-} BMDMs remain viable following *Yptb* infection (**FIG. 1A**). Furthermore,
110 processing of caspase-1 into its active p20 fragment is dependent on caspase-8, as in contrast to
111 *Ripk3*^{-/-} BMDMs, it is not observed in *Casp8*^{-/-}*Ripk3*^{-/-} BMDMs, (**FIG. 1B**). Consistent with prior
112 findings^{23,24,29}, GSDMD processing also requires caspase-8 but not RIPK3 (**FIG. 1B**). To
113 determine if caspase-8 is sufficient for caspase-1 activation, as well as to define its molecular
114 requirements, we co-expressed caspase-1 with various caspase-8 constructs in which the caspase-
115 8 death-effector domains (DEDs) were replaced with an inducible dimerizable domain that
116 promotes its activation upon addition of the dimerizer AP20187³⁰ (**FIG. 1C**). Addition of AP20187
117 to induce dimerization of caspase-8 triggered robust caspase-1 processing into its active p20
118 fragment, which was undetectable in the absence of dimerizer (**FIG. 1D**). Critically, dimerizable
119 constructs containing catalytic mutant caspase-8 (C360A) or uncleavable caspase-8 lacking five
120 aspartate processing sites³⁰ were unable to promote caspase-1 cleavage, indicating that both
121 caspase-8 catalytic activity and auto-processing are required for caspase-1 cleavage (**FIG. 1D**). To
122 determine if auto-processed caspase-8 functions solely as a scaffold to recruit caspase-1, or
123 whether its catalytic activity is necessary for caspase-1 processing, we expressed dimerizable
124 caspase-8 constructs in which the interdomain auto-processing site at position D384 was replaced

125 with the cleavage sequence for the tobacco etch virus (TEV) protease. While addition of dimerizer
126 to cells co-transfected with caspase-8-TEV and caspase-1 led to some baseline caspase-1
127 processing, co-expression of TEV protease to allow for caspase-8 cleavage resulted in maximal
128 caspase-1 processing (**FIG. 1E**). Notably, caspase-8 catalytic activity was essential for caspase-1
129 activation even when caspase-8 was dimerized and exogenously cleaved by TEV, demonstrating
130 that both caspase-8 cleavage and enzymatic activity are absolutely required for caspase-1
131 processing (**FIG. 1E**).

132

133 **Caspase-1 activation by caspase-8 requires caspase-1 catalytic activity**

134 Our findings indicate that caspase-8 acts as an apical initiator caspase to activate caspase-1 in
135 response to blockade of TAK1 and IKK signaling by pathogens. Caspase-1 autoproteolysis is
136 required for its activation within canonical inflammasomes³¹⁻³³. In contrast, during apoptosis,
137 caspase-8 processes caspase-3 into its mature form, but caspase-3 does not undergo
138 autoproteolysis, thus limiting its feed-forward amplification capacity^{33,34}. Unexpectedly, however,
139 catalytically inactive caspase-1 (Casp1^{C284A}) failed to undergo processing in response to inducible
140 dimerization of caspase-8 in HEK293T cells, indicating that caspase-1 catalytic activity was
141 required for its own processing in the setting of caspase-8 activation (**FIG. 2A**). Notably, while
142 *Casp1*^{-/-} immortalized BMDMs (iBMDMs) stably expressing WT caspase-1 robustly processed
143 caspase-1 upon infection with *Salmonella* Typhimurium or *Yptb* (**FIG. 2B**), iBMDMs expressing
144 catalytically inactive caspase-1 DEAD (C284A)³² were unable to process caspase-1 during either
145 *Salmonella* or *Yptb* infection. These observations support our findings that caspase-1 catalytic
146 activity is necessary for its own processing and activation downstream of YopJ-induced caspase-
147 8 activation (**FIG. 2B**). Consistent with previous findings that *Yptb*-induced cell death does not
148 require caspases-1 or -11^{24,25}, caspase-1 DEAD cells exhibited wild-type levels of LDH release
149 upon infection with *Yptb* (**FIG. 2C**), but failed to induce LDH release upon infection with *S.*
150 Typhimurium, as expected (**FIG. 2C, S1A**). Critically, primary BMDMs from knock-in mice
151 lacking caspase-1 catalytic activity (*Casp1*^{mlt/mlt})³⁵ also failed to process caspase-1, and had
152 significantly reduced processing of GSDMD, as well as reduced IL- β processing and release in
153 response to *Yptb* infection (**FIG. 2D-F**). Similarly to iBMDMs expressing catalytically inactive
154 caspase-1 DEAD (C284A) however, cytotoxicity responses to *Yptb* infection were normal, despite
155 being unable to undergo cytotoxicity in response to *Salmonella* (**FIG. 2G, S1B**). In contrast to IL-

156 1 β release, IL-12 secretion by *Casp1^{mlt/mlt}* BMDMs was largely intact (**FIG. S1C**). Taken together,
157 our results show that caspase-8 and caspase-1 enzymatic activities are both critical for caspase-1
158 processing in response to *Yptb* infection, and that caspase-1 catalytic activity is required for IL-1 β
159 secretion, even in the presence of sufficient caspase-8 activity.

160

161 **Caspase-8 auto-processing limits RIPK3-mediated necroptosis**

162 *Yersinia* infection or TAK1 blockade have been proposed to induce a combined form of cell death
163 termed PANoptosis, involving the simultaneous activation of pyroptosis, apoptosis, and
164 necroptosis, as assessed by phosphorylation of RIPK3 and the Mixed Lineage Kinase Domain Like
165 Pseudokinase (MLKL) pore-forming protein, coincident with activation of apoptotic and
166 pyroptotic caspases^{12,13,36}. However, in the absence of caspase-8 auto-processing, cells undergo
167 RIPK3-dependent necroptosis mediated by RIPK3-dependent activation of MLKL^{37,38}. Because
168 MLKL pore formation can promote potassium efflux, a common trigger of the NLRP3
169 inflammasome^{37,39,40}, we hypothesized that caspase-1 activation in the absence of caspase-8 auto-
170 processing could result from NLRP3 activation downstream of RIPK3-and MLKL-mediated
171 necroptosis. We therefore monitored cell death and caspase-1 processing in *Casp8^{D387A/D387A}*
172 BMDMs, which express an uncleavable caspase-8, either after infection with *Yptb* or treatment
173 with LPS/IKK inhibitor (IKKi), which pharmacologically mimics the activity of YopJ⁴¹. In
174 contrast to the HEK293T system or in our previous studies in which non-cleavable caspase-8 was
175 expressed in cells lacking RIPK3²⁵, we found that *Casp8^{D387A/D387A}* BMDMs infected with *Yptb* or
176 treated with LPS/IKKi exhibited comparable LDH release and caspase-1 processing as WT
177 BMDMs (**FIG. 3A-C**). Moreover, *Casp8^{D387A/D387A}* only processed GSDMD into the active p30
178 fragment, whereas WT BMDMs processed GSDMD into both p30 and p20 fragments (**FIG. 3C**).
179 The GSDMD p20 fragment is generated by caspase-3-mediated cleavage⁴², indicating that both
180 caspase-1 and caspase-3 are active in WT macrophages, but only caspase-1 is active in
181 *Casp8^{D387A/D387A}* macrophages. Moreover, the RIPK3 inhibitor GSK'872 inhibited both cell lysis
182 and caspase-1 processing in *Casp8^{D387A/D387A}* but not WT BMDMs following *Yptb* infection or
183 LPS/IKKi treatment, suggesting that caspase-8 auto-processing during *Yersinia* infection or
184 IKK/TAK1 blockade normally limits RIPK3-mediated necroptosis and subsequent activation of
185 caspase-1 (**FIG. 3A-C**). Notably, while the NLRP3-specific inhibitor MCC950 did not inhibit cell
186 lysis in the *Casp8^{D387A/D387A}* BMDMs, it completely blocked caspase-1 processing in *Yptb*-infected

187 or LPS/IKKi-treated *Casp8*^{D387A/D387A} BMDMs, indicating that caspase-1 processing downstream
188 of RIPK3 activation is mediated by NLRP3 (**FIG. 3D-F**). Importantly, *Casp8*^{D387A/D387A}*Mkl1*^{-/-}
189 BMDMs⁴³ exhibited neither cell lysis, caspase-1, caspase-8, nor GSDMD processing, indicating
190 that MLKL activation occurs upstream of caspase-1 activation, GSDMD processing, and cell lysis
191 in *Casp8*^{D387A/D387A} BMDMs (**FIG 3A-F**). Notably, we did not observe RIPK3 or MLKL
192 phosphorylation in WT BMDMs following *Yptb* infection (**FIG. 3G**), consistent with the lack of
193 requirement for RIPK3 in *Yptb*-induced death of BMDMs²⁵, but in contrast to the reported
194 phosphorylation of RIPK3 and MLKL during PANoptosis^{12,13,36}. Instead, our findings indicate
195 that caspase-8 auto-processing is responsible for direct activation of caspase-1 and limits a backup
196 caspase-1 activation pathway that occurs via RIPK3- and MLKL-dependent activation of NLRP3
197 in the absence of caspase-8 auto-processing.

198

199 **ASC speck formation is GSDMD- and NLRP3-dependent and is required for IL-1 β** 200 **processing and release**

201 While our findings indicate that NLRP3 activates caspase-1 downstream of RIPK3/MLKL when
202 caspase-8 activation is disrupted, whether and how NLRP3 might contribute to anti-*Yptb* responses
203 in wild-type BMDMs is unclear. Although the NLRP3 inflammasome is activated in response to
204 *Yersinia* infection or IKK/TAK1 blockade^{24,44}, NLRP3 and the adaptor ASC do not contribute to
205 either caspase-8 or caspase-1 activation, GSDMD processing, or cytotoxicity²⁵. It has been
206 suggested that co-assembly of ASC, NLRP3, RIPK3, caspase-1 and -8 triggers PANoptosis during
207 *Yersinia* infection^{12,13}. However, activation of GSDMD can lead to formation of pores that mediate
208 potassium efflux, a common trigger of the NLRP3 inflammasome that can promote feed-forward
209 activation of caspase-1 downstream of other stimuli⁴⁵. Intriguingly, while we did not observe any
210 differences between WT and *Asc*^{-/-} BMDMs in the extent or kinetics of caspase-1, -8, or GSDMD
211 processing, robust caspase-8 processing occurred substantially earlier than processing of caspase-
212 1 or GSDMD (**FIG. 4A**), consistent with a model in which caspase-8 activation occurs upstream
213 of NLRP3-dependent caspase-1 activation. To determine if NLRP3 inflammasome activation
214 occurs downstream of GSDMD pore formation following *Yptb* infection, we assessed NLRP3
215 activation by the formation of large ASC oligomers that can be visualized via fluorescence
216 microscopy⁴⁶ (**FIG. S2A**). Indeed, transgenic BMDMs expressing ASC-citrine⁴⁷ exhibited robust
217 formation of ASC specks following *Yptb* infection (**FIG. 4B-D, S2B**). The NLRP3-specific

218 inhibitor MCC950 abrogated ASC speck formation (**FIG. 4B, C**), as expected. During *Yersinia*
219 infection, caspase-8 is activated at endosomal membranes by recruitment to RAGulator
220 complexes⁴⁸. Critically, both caspase-8 activation and ASC speck formation were dependent on
221 YopJ activity (**FIG. 4D**). Cytotoxicity remained unchanged in infected WT and *Asc*^{-/-} BMDMs
222 even with MCC950 treatment, suggesting that NLRP3 inflammasome activation and ASC speck
223 formation occur downstream of GSDMD pore formation and induction of cell lysis following *Yptb*
224 infection (**FIG. S2C**). To test this hypothesis, we assayed ASC speck formation in *Gsdmd*^{-/-}
225 BMDMs following *Yptb* infection (**FIG. 4E, F**). Critically, *Gsdmd*^{-/-} BMDMs infected with *Yptb*
226 had a significantly lower frequency of ASC specks relative to wild-type cells in response to *Yptb*
227 infection (**FIG. 4E, F**). Furthermore, MCC950 treatment, or loss of either ASC or GSDMD
228 significantly reduced levels of IL-1 β release in response to *Yersinia* infection (**FIG. 4G**). Taken
229 together, our results show that NLRP3 inflammasome activation downstream of caspase-8 and
230 caspase-1-dependent GSDMD pore formation mediates ASC oligomerization and IL-1 β release.

231

232 **Caspase-8 and ASC form separate but functionally linked death complexes**

233 Our findings that ASC speck formation occurs downstream of caspase-8-dependent caspase-1
234 activation, and that caspase-8 processing precedes caspase-1 processing, suggest that rather than
235 simultaneous engagement of multiple death pathways within a single complex, sequential
236 activation of distinct death complexes occurs during *Yptb* infection. In support, whereas robust
237 caspase-8 activation was detected as early as 2 hours post infection and increased by 4 hours, ASC
238 specks were undetectable at 2h and were only detected at 4 hours post infection (**FIG. 5A-D,**
239 **S33A**). In addition to activation of caspase-8 and ASC puncta formation being temporally distinct,
240 active caspase-8 and ASC puncta assembly were also spatially distinct, as we observed virtually
241 no colocalization between active caspase-8 puncta and the ASC speck (**FIG. 5C**). Moreover, both
242 caspase-8 activity and ASC speck formation were abrogated upon treatment with the RIPK1 kinase
243 inhibitor Nec-1, whereas the NLRP3 inhibitor MCC950 abrogated ASC speck formation but not
244 active caspase-8 puncta formation (**FIG. 5A, C**). These data indicate that ASC speck formation
245 occurs downstream of caspase-8 activation and is dependent on NLRP3. As both caspase-8 and
246 caspase-1 cleavage of GSDMD can promote NLRP3 activation and ASC speck formation, whether
247 caspase-8 is sufficient, in the absence of caspase-1, to fully activate the NLRP3 inflammasome is
248 unclear. Notably, *Casp1/11*^{-/-} ASC-citrine BMDMs exhibited a significant decrease in ASC specks

249 compared to WT ASC-citrine BMDMs in responses to *Yptb* infection, but not in response to
250 LPS/ATP (FIG. S3B-D). These data support a model whereby ASC speck formation is upstream
251 of caspase-1 activation in response to LPS/ATP, but downstream of caspase-8-dependent caspase-
252 1 activation in response to YopJ-dependent blockade of immune signaling. Altogether, these data
253 show that during *Yptb* infection, active caspase-8 and ASC complex assembly occur in a kinetically
254 and spatially separable manner.

255

256 **DISCUSSION**

257 Cell death following blockade of immune signaling kinases TAK1 and IKK by pathogenic
258 *Yersinia* or pharmacological inhibitors is accompanied by activation of both apoptotic and
259 pyroptotic caspases, raising questions about how seemingly distinct forms of cell death can occur
260 simultaneously^{11,28}. The activation of pyroptotic and apoptotic caspases, along with the activation
261 of necroptosis when caspase-8 is absent or inhibited, has led to a proposed model in which a unified
262 complex containing regulators of multiple death pathways (pyroptosis, apoptosis, and necroptosis)
263 mediates *Yersinia*- and TAK1 blockade-induced cell death^{12,13}. Our findings support an alternative
264 model in which two spatially and temporally distinct, yet functionally linked death complexes
265 assemble in response to *Yptb* infection. Overall our data indicate that caspase-8 initiates
266 downstream responses via direct cleavage of caspase-1, followed by auto-amplification of caspase-
267 1 activation. Caspase-1 activation in response to *Yersinia* infection requires FADD and RIPK1²⁵,
268 and the formation of the FADD/RIPK1/caspase-8-containing complex IIa downstream of TAK1
269 inactivation^{49,50} suggests that caspase-1 activation initially takes place within this complex⁵¹⁻⁵³.
270 Caspase-1 and caspase-8 activation within complex IIa also mediates GSDMD cleavage, for which
271 our findings suggest that caspase-1 serves as the primary activator. Our data further demonstrate
272 that GSDMD-dependent activation of the canonical NLRP3-ASC-caspase-1 inflammasome,
273 presumably via potassium efflux, is kinetically and spatially distinct from caspase-8 activation,
274 and is not required for cell death, but is required for secretion of IL-1 β . Although caspase-8 can
275 cleave GSDMD to induce pyroptosis in the absence of caspase-1, caspase-8 cannot compensate
276 for lack of caspase-1 or NLRP3 inflammasome activation with respect to IL-1 β secretion. Why
277 caspase-1 and the NLRP3 inflammasome are required for IL-1 β secretion despite upstream
278 activation of caspase-8 is not clear, but they may enable enhanced or accelerated IL-1 β release
279 following *Yptb* infection.

280 As caspase-1 is cleaved and activated in the absence of inflammasome components during
281 *Yptb* infection^{25,49}, we hypothesized that caspase-8 might directly activate caspase-1. Indeed,
282 caspase-8 auto processing and catalytic activity were required for caspase-1 processing in a
283 HEK293 co-expression system. Surprisingly, caspase-1 catalytic activity was also required for its
284 own processing and activation downstream of IKK blockade. This was the case in HEK293 cells
285 as well as in immortalized and primary macrophages from *Casp1*^{C284A} mice. Our data suggest that
286 the enzymatic activity of caspase-8 is required to generate a catalytically active scaffold which can
287 then recruit and cleave caspase-1. Our data further indicate that caspase-1 activity is required for
288 its own processing, which likely occurs first within complex IIa, and subsequently within NLRP3
289 inflammasomes, thereby amplifying the response to enable maximal cleavage of GSDMD, IL-1 β ,
290 and pyroptosis.

291 Consistent with prior findings that caspase-1 and -11 are not required for death of BMDMs
292 in response to *Yersinia*²⁵, caspase-1 catalytic activity is dispensable for cell death, likely due to
293 caspase-8-dependent cleavage of caspase-3 and -7. GSDME, which is activated by caspase-3 and
294 mediates pyroptosis in other settings⁵⁴⁻⁵⁶, also does not contribute to cell death during *Yersinia*
295 infection²⁹, indicating that other caspase-3/7 targets are likely responsible. Additionally, consistent
296 with previous reports^{23,24}, in the absence of caspase-1, caspase-8-dependent cleavage of GSDMD
297 also occurs and contributes to pyroptosis, although GSDMD cleavage is significantly blunted in
298 the absence of caspase-1.

299 Simultaneous activation of multiple cell death pathways involving RIPK3 and caspase-
300 8/caspase-1 is proposed to occur during infection by a number of pathogens including *Legionella*,
301 *Francisella*, Influenza, and *Yersinia* infection^{11,57-59}. How such a complex assembles remains
302 mysterious, particularly when caspase-8 activity represses RIPK3-dependent necroptosis^{9,10}.
303 RIPK3 makes no detectable contribution to *Yersinia*-induced cell death^{25,28,60} and we do not
304 observe any evidence for RIPK3-mediated necroptosis during *Yersinia* infection in the presence
305 of functional caspase-8 (**FIG. 1A, B**). In addition, neither RIPK3 nor MLKL undergo
306 phosphorylation in wild-type cells following *Yersinia* infection or IKK blockade (**FIG. 3G**).
307 Rather, our data favor a model wherein caspase-8 activation and auto-processing downstream of
308 IKK blockade restrains necroptosis, as *Casp8*^{D387A/D387A} BMDMs undergo rapid RIPK3 and MLKL
309 phosphorylation, and the cell death that occurs in *Casp8*^{D387A/D387A} BMDMs shifts from being
310 RIPK3/MLKL-independent in WT BMDMs to entirely MLKL- and RIPK3 kinase-dependent

311 (FIG. 3A-C, G). RIPK3/MLKL-induced programmed necrosis also activates NLRP3, presumably
312 via potassium efflux, thereby providing another route to caspase-1 engagement during *Yersinia*
313 infection, even when caspase-8 cannot be activated (FIG. 3D-F). Thus, the coupling of NLRP3
314 activation to multiple types of lytic pores indicates an important role for backup mechanisms to
315 ensure IL-1 β releases and inflammation when immune signaling is inhibited or blocked by
316 pathogen activity.

317 Our inability to observe RIPK3 and MLKL phosphorylation during *Yersinia* infection of
318 wild-type BMDMs coupled with our observations that caspase-8 activation precedes assembly of
319 ASC specks and detectable caspase-1 activation, suggest that distinct apoptotic and pyroptotic cell
320 death complexes are activated sequentially during *Yersinia* infection. Importantly, caspase-1 is
321 processed in a caspase-8-dependent manner even in *Asc*^{-/-} or NLRP3-inhibited BMDMs, indicating
322 that its initial activation takes place within caspase-8-containing complex IIa. Critically, while we
323 observed punctate areas of active caspase-8 throughout the cell following *Yptb* infection, consistent
324 with previous reports⁴⁸, active caspase-8 did not colocalize with ASC specks. Finally, the reduced
325 frequency of ASC specks we observe in the absence of caspase-1 suggests that it serves as the
326 primary activator of GSDMD, which then enables NLRP3 inflammasome activation. Altogether,
327 our study reveals new insight into mechanisms of caspase-8-dependent activation of caspase-1, as
328 well as new understanding of how pyroptotic and apoptotic cell death pathways communicate to
329 mediate anti-microbial host defense.

330 MATERIALS AND METHODS

331 Cell culture and differentiation of bone marrow-derived macrophages

332 Bone marrow derived macrophages were isolated and differentiated as previously described^{25,49},
333 in adherence to the NIH Guide for the Care and Use of Laboratory Animals. Briefly, isolated bone
334 marrow cells from 6–10-week-old male and female mice were grown at 37°C, 5% CO₂ in 30%
335 macrophage media (30% L929 fibroblast supernatant, complete DMEM). BMDMs were harvested
336 in cold PBS on day 7 and replated in 10% macrophage media onto tissue culture (TC)-treated
337 plates or glass coverslips in TC-treated plates. Transduced iBMDMs from *Casp1*^{-/-} mice
338 containing either WT caspase-1, caspase-1 DEAD, or empty vector were previously described and
339 provided by Denise Monack³². Primary *Casp1*^{mlt/mlt} BMDMs were previously described³⁵ and
340 provided by Dr. Olaf Groß. *Casp8*^{D387A/D387A} *Mkl1*^{-/-} BMDMs were previously described⁴³ and
341 provided by Dr. Doug Green and Dr. Bart Tummers. HEK293T were grown in complete DMEM

342 (supplemented with 10% FBS, 10 mM HEPES, 10 mM Sodium pyruvate, 1%
343 Penicillin/Streptomycin), and maintained in a 37°C incubator with 5% CO₂.

344

345 **Bacterial culture and *in vitro* infections**

346 Bacterial strains: *Yersinia pseudotuberculosis* (*Yptb*) strain IP2666⁶¹, *Yptb* ΔYopJ⁶², *Salmonella*
347 *enterica* serovar Typhimurium strain SL1344 (*S. Tm*)⁶³ were all grown as previously described²⁵.

348 Briefly, bacteria were grown with aeration and appropriate antibiotics at 28°C (*Yptb*, irgasan) or
349 37°C (*Salmonella*, streptomycin). *Yptb* strains were induced prior to infection by diluting
350 stationary phase overnight cultures 1:40 in 3 mL of inducing media (2xYT broth, 20 mM Sodium
351 Oxalate, 20 mM MgCl₂). Cultures were grown at 28°C for 1 hour and shifted to 37°C for two
352 hours with aeration. *Salmonella* strains were induced prior to infection by diluting the overnight
353 culture 1:40 in 3 mL inducing media (LB broth, 300 mM NaCl), and grown standing for 3 hours
354 at 37°C. Bacterial growth was measured by absorbance at OD₆₀₀ using a spectrophotometer.
355 Bacteria were pelleted, washed, and resuspended in DMEM or serum-free media for infection. *In*
356 *vitro* infections were performed at MOI 20 unless otherwise noted. Gentamycin (100 µg/mL) was
357 added one hour post infection for all infections.

358

359 **LDH cytotoxicity assay and ELISA**

360 Triplicate wells of BMDMs were seeded in TC-treated 96 well plates. BMDMs were infected with
361 indicated bacterial strains as indicated above. BMDMs were primed with 100 ng/mL LPS for 3
362 hours followed by 2.5 mM ATP treatment or 5h 10µM IKKi (BMS-345541, Sigma-Aldrich)
363 treatment. BMDMs were primed with 400 ng/mL Pam3CSK4 O/N. BMDMs were treated with
364 1µM GSK'872 (Invivogen), 1µM MCC950 (Tocris), 60µM Necrostatin-1 (Invivogen) for 30
365 minutes, 1 hour, and 1 hour prior to infection, respectively. 100 µg/mL gentamycin was added 1
366 hour post treatment to all infectious experimental conditions. At indicated time points, plates were
367 spun down at 250g, and supernatants were harvested. Supernatants were combined with LDH
368 substrate and buffer (Sigma-Aldrich) according to the manufacturer's instructions and incubated
369 in the dark for 35 min. Plates were read on a spectrophotometer at 490 nm. Percent cytotoxicity
370 was calculated by background subtraction and normalizing to maximal cell death (1% triton X).
371 To assess IL-1β release, supernatants were diluted 4-fold and applied to Immulon ELISA plates
372 (ImmunoChemistry Technologies) pre-coated with anti-IL-1β capture antibody (eBioscience).

373 Following blocking (1% BSA in 1x PBS), plates were incubated with biotin-linked anti-IL-1 β
374 detection antibody (R&D Systems, 1:1000), followed by horseradish peroxidase-conjugated
375 streptavidin. As read-out for IL-1 β levels, peroxidase enzymatic activity was determined by
376 exposure to o-phenylenediamine hydrochloride (Sigma) in citric acid buffer. Reactions were
377 stopped with sulfuric acid and absorbance values were read at 490 nm, normalized to mock-
378 transfected cells (negative control).

379

380 **HEK293T transfections**

381 Mammalian expression plasmids containing indicated DNA constructs were transfected into
382 HEK293T cells using Lipofectamine 2000 (ThermoFischer) at 1:1 ratio (w/w
383 DNA:Lipofectamine) in Opti-MEM (Gibco). Media was changed to complete DMEM (10% v/v
384 FBS) 6h post-transfection. 24h post-transfection, cells were treated with 1 μ M AP20187 (dimerizer,
385 ApexBio) in serum-free DMEM for 6h in a humidified incubator at 37°C and 5% CO₂ prior to
386 subsequent analysis.

387

388 **Western Blotting**

389 BMDMs were seeded in TC-treated 24 well plates (3.0 x10⁵ cells/well). HEK293T cells were
390 seeded in poly-L-lysine-coated TC-treated 24-well plates (2.0 x 10⁵ cells/well) and transiently
391 transfected with appropriate gene constructs as described above. Following infection or treatment
392 in serum-free media, supernatants were harvested, and TCA precipitated overnight at 4°C.
393 Precipitated proteins were pelleted and washed with acetone. The pellet was resuspended in 5X
394 sample buffer (125 mM Tris, 10% SDS, 50% glycerol, 0.06% bromophenol blue, 1% β -
395 mercaptoethanol). BMDMs were lysed in lysis buffer (20 mM HEPES, 150 mM NaCl, 10%
396 glycerol, 1% triton X, 1mM EDTA, pH7.5) plus 1x complete protease inhibitor cocktail and 1x
397 sample buffer (25 mM Tris, 2% SDS, 10% glycerol, 0.012% bromophenol blue, 0.2% β -
398 mercaptoethanol). Lysates and supernatants were boiled and centrifuged at full speed for 5
399 minutes, were run on 4–12% polyacrylamide gels and transferred to PVDF membrane. Membranes
400 were immunoblotted using the following primary antibodies: β -Actin (Sigma-Aldrich, 1:5000),
401 caspase-1 (gift of Vishva Dixit, Genentech, 1:500), caspase-8 (Enzo, 1:1000), cleaved-caspase-8
402 (Cell signaling, 1:1000) GSDMD (Abcam, 1:1000), and IL-1 β (R&D Systems, 1:1000). Species
403 specific HRP-conjugated secondary antibodies were used for each antibody (1:5000). Membranes

404 were developed using Pierce ECL Plus and SuperSignal West Femto Maximum Sensitivity
405 Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions. Western blot
406 time-courses were performed in parallel with cytotoxicity assays to accurately interpret protein
407 release before and after overt cell death.

408

409 **Fluorescence and confocal microscopy**

410 BMDMs were seeded on circular glass coverslips (Thorlabs, #CG15NH) and allowed to adhere
411 overnight. Cells were then infected or transfected with the indicated DNA constructs (HEK293Ts).
412 At the indicated time points, cells were fixed with 4% PFA for 15 minutes, permeabilized with
413 0.2% triton X for 10 minutes, and blocked with 5% BSA for 1-2h. BMDMs were stained for
414 cleaved caspase-8 (#8592S Cell signaling, 1:1000) or ASC (#04-147 Millipore, 1:160) overnight
415 at 4°C, Alexa Fluor 647-conjugated anti-rabbit (1:1000), Alexa Fluor 488-conjugated anti-mouse
416 (1:1000) at RT for 1h, and Hoechst at RT for 30 min. Cells were mounted on glass slides with
417 Fluoromount-G (Southern Biotech). Slides were imaged using a Leica SP5-FLIM Inverted
418 confocal microscope with a single z-plane taken per field. Lasers were optimized for GFP (green)
419 Cy5 (far-red), Citrine (yellow), and DAPI (blue). Scale bar = 15 um for all images.

420

421 **Image quantification and analysis**

422 Each experiment was conducted in three technical replicates. Within each replicate, 20-30 fields
423 of view were analyzed, with 80-200 cells (BMDMs) per field of view. Specks were defined as
424 distinct high-fluorescence perinuclear clusters of citrine or Alexa Fluor 488 signal. Speck
425 formation frequency was determined as the percentage of citrine-expressing cells that contained
426 one or more specks, using custom macros from ImageJ.

427

428 **Statistical analysis**

429 Data were graphed and analyzed using GraphPad Prism. Mean values (\pm SEM) were compared
430 across triplicate conditions and P values were determined using the appropriate test and are
431 indicated in each figure legend. Studies were conducted without blinding or randomization. Values
432 of $p < 0.05$ were considered statistically significant.

433

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580
581

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609

610 **Figures:**

611 **Figure 1: Caspase-8 activity is required for cell death and caspase-1 processing.** (A) C57BL/6,
612 *Ripk3^{-/-}*, and *Casp8^{-/-}Ripk3^{-/-}* BMDMs were infected with WT *Yptb* and percent cytotoxicity was
613 measured 4 hours post-infection as described in materials and methods. (B) Lysates collected 3
614 hours post-infection were immunoblotted for caspase-1 and GSDMD. (C) Schematic
615 representation of FKBP constructs of caspase-8 employed in this study. (D) HEK293T cells
616 transfected with caspase-1 and WT, catalytically inactive (C360A), or uncleavable (D5A) FKBP-
617 caspase-8 and induced to dimerize with AP20187 (dimerizer) 24 hours post transfection. Lysates
618 were collected for western blotting 6 hours after adding AP20187. (E) HEK293T cells transfected
619 with caspase-1, TEV, and WT or catalytically inactive (C360A) FKBP-caspase-8-TEV and treated
620 with dimerizer as indicated 24 hours post transfection. Lysates were collected for western blotting
621 6 hours after AP20187 addition. Nd — not detected, **** p < 0.0001 by two-way ANOVA. Error
622 bars represent the mean +/- SEM of triplicate wells and are representative of three independent
623 experiments.

624

625 **Figure 2: Caspase-1 activation by caspase-8 requires caspase-1 catalytic activity.**

626 (A) HEK293T cells were transfected with FKBP-caspase-8 and WT or catalytically inactive
627 (C284A) caspase-1 and treated with dimerizer. Lysates were collected for western blotting as
628 described in materials and methods. (B) *iC57BL/6*, *iCasp1^{-/-} + Casp1^{WT}*, *iCasp1^{-/-} + Casp1^{DEAD}*,
629 and *iCasp1^{-/-} + EV* immortalized BMDMs were infected with WT *Yptb* as described in materials
630 and methods. Lysates collected 3 hours post-infection were immunoblotted for caspase-1, caspase-
631 8, GSDMD, and β -actin as indicated. (C) Percent cytotoxicity was assayed 4 hours post-infection
632 as described in materials and methods. (D) C57BL/6, *Casp1^{ml/ml}* BMDMs were infected with WT
633 *Yptb* as described. Lysates collected 3 hours post-infection were immunoblotted for caspase-1,
634 caspase-8, GSDMD, and β -actin. (E) Percent cytotoxicity was measured 4 hours post-infection.
635 (F) Release of IL-1 β into the supernatant was measured by ELISA 4 hours post-infection. (G)
636 Lysates and supernatants collected 3 hours post-infection were immunoblotted for IL-1 β . ns —
637 not significant, **** p < 0.0001 by two-way ANOVA. Error bars represent the mean +/- SEM of
638 triplicate wells and are representative of three independent experiments.

639

640 **Figure 3: Caspase-8 auto-processing limits RIPK3-mediated necroptosis.** (A) C57BL/6,

641 *Casp8^{D387A/D387A}*, and *Casp8^{D387A/D387A}Mik1^{-/-}* BMDMs were treated with GSK'872 or vehicle
642 control as indicated and infected with WT *Yptb*. Percent cytotoxicity was measured 4 hours post-
643 infection as described. (B) C57BL/6, *Casp8^{D387A/D387A}*, and *Casp8^{D387A/D387A}Mik1^{-/-}* BMDMs were
644 primed with LPS followed by IKK inhibitor. Prior to IKK inhibitor treatment, BMDMs were
645 treated with GSK'872 or vehicle control. Percent cytotoxicity was measured 5 hours post-
646 infection. (C) Lysates collected 3 hours post-infection were immunoblotted for caspase-1, caspase-
647 8, GSDMD, and β -actin. (D) C57BL/6, *Casp8^{D387A/D387A}*, and *Casp8^{D387A/D387A}Mik1^{-/-}* BMDMs were

648 treated with MCC950 or vehicle control and were infected with WT *Yptb*. Percent cytotoxicity was
649 measured 4 hours post-infection. (E) C57BL/6, *Casp8^{D387A/D387A}*, and *Casp8^{D387A/D387A}Mlkl^{-/-}*
650 BMDMs were primed with LPS followed by IKK inhibitor. Prior to IKK inhibitor treatment,
651 BMDMs were treated with MCC950 or vehicle control. Percent cytotoxicity was measured 5 hours
652 post-infection. (F) Lysates collected 3 hours post-infection were immunoblotted for caspase-1,
653 caspase-8, GSDMD, and β -actin. (G) C57BL/6, *Casp8^{D387A/D387A}*, and *Ripk3^{-/-}* BMDMs were
654 treated with MCC950, GSK'872, or vehicle control and were infected with WT *Yptb*. Lysates
655 collected 3 hours post-infection were immunoblotted for total RIPK3, pRIPK3, total MLKL,
656 pMLKL, and β -actin. Nd — not detected, **** $p < 0.0001$ by two-way ANOVA. Error bars
657 represent the mean \pm SEM of triplicate wells and are representative of three independent
658 experiments.

659

660 **Figure 4: ASC speck formation is GSDMD and NLRP3 dependent and is required for IL-1 β**
661 **processing and release.** (A) C57BL/6, and *Asc^{-/-}* BMDMs were infected with WT *Yptb*. Lysates
662 collected 3 hours post-infection were immunoblotted for caspase-1, caspase-8, GSDMD, and β -
663 actin. (B) C57BL/6 ASC-citrine BMDMs were treated with MCC950 or vehicle control and were
664 infected with WT *Yptb*. ASC speck formation was analyzed at 4 hours post-infection. (C) Percent
665 of cells with ASC specks was quantified. (D) C57BL/6 ASC-citrine BMDMs were infected with
666 WT and *$\Delta yopJ$ Yptb*. Caspase-8 cleavage and ASC speck formation were analyzed at 4 hours post-
667 infection. (E) C57BL/6, and *Gsdmd^{-/-}* BMDMs were treated with MCC950 or vehicle control and
668 infected with WT *Yptb*. ASC speck formation was analyzed at 4 hours post-infection via
669 immunofluorescence staining. (F) Percent of cells with ASC specks was quantified. (G) Release
670 of IL-1 β into the supernatant was measured by ELISA at 4 hours post-infection in C57BL/6, and
671 *Asc^{-/-}*, *Gsdmd^{-/-}* BMDMs. ns — not significant, **** $p < 0.0001$, ** $p < 0.001$ by two-way
672 ANOVA. Error bars represent the mean \pm SEM of triplicate wells and are representative of three
673 independent experiments.

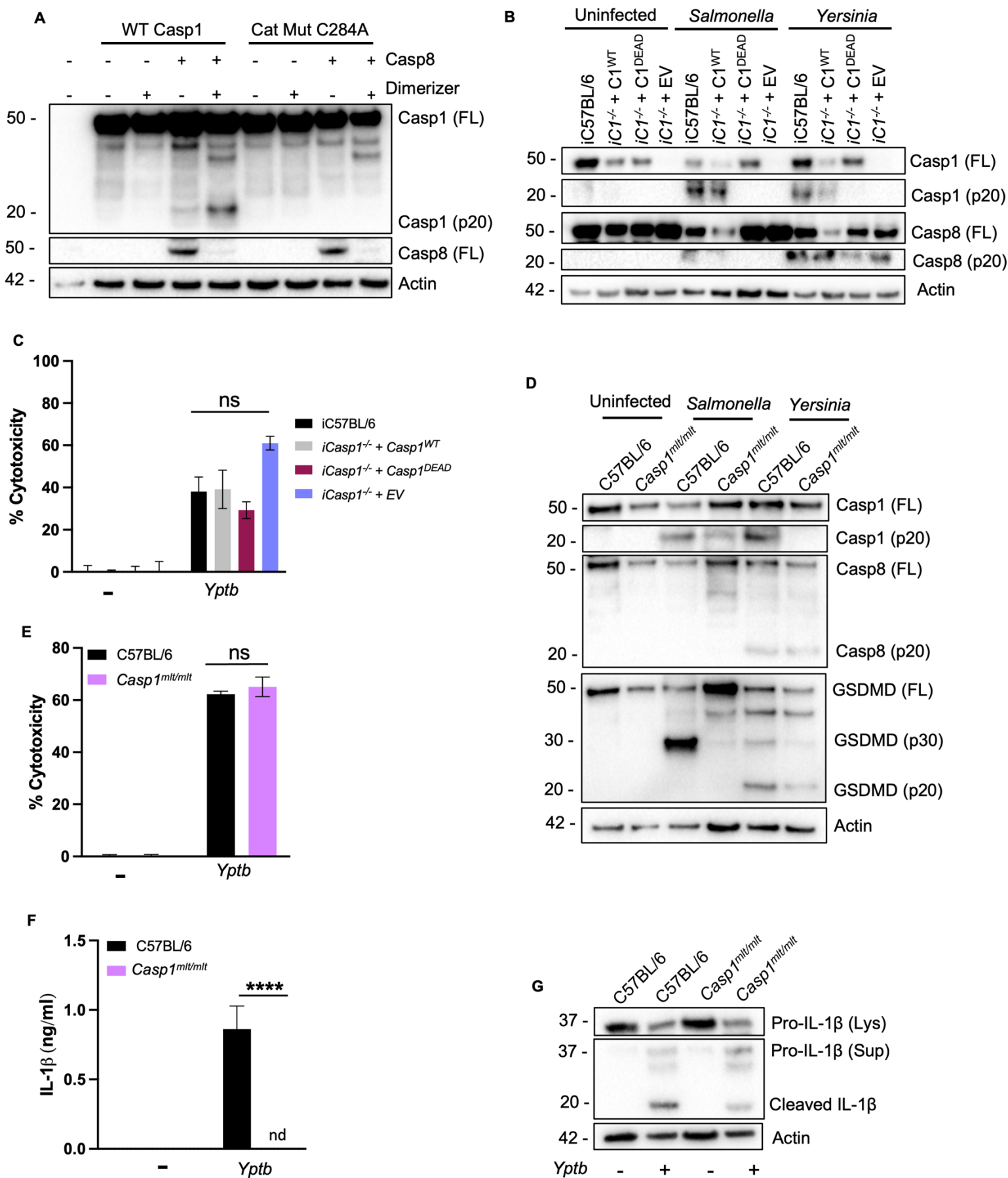
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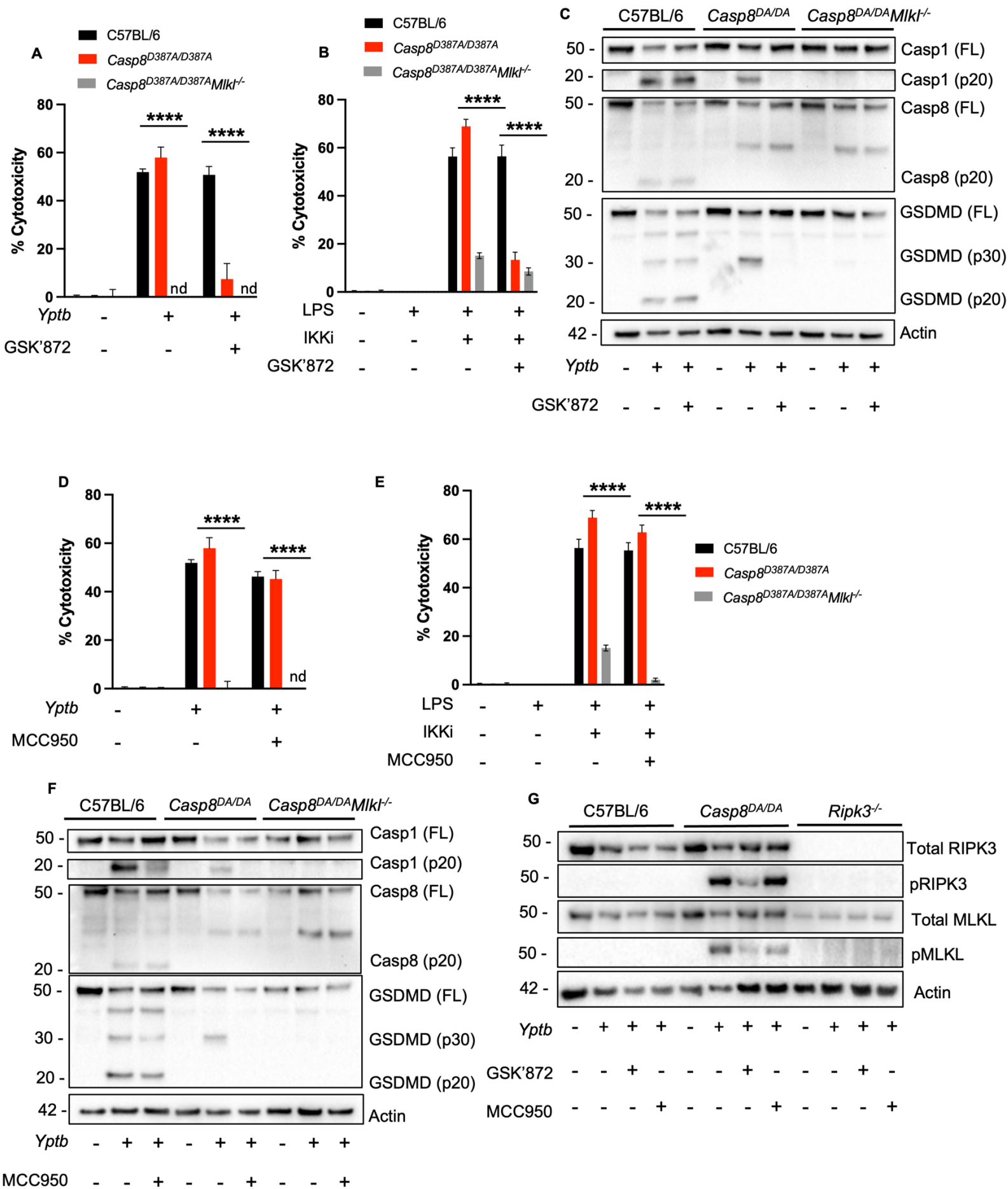
675 **Figure 5: Caspase-8 and ASC form separate but functionally linked death complexes.** (A)
676 C57BL/6 ASC-citrine BMDMs were treated with MCC950, Nec-1, or vehicle control and were
677 infected with WT *Yptb*. Caspase-8 cleavage and ASC speck formation were analyzed at 2 hours
678 post-infection. (B) Percent of cleaved caspase-8 positive cells was quantified at 2- and 4 hours
679 post-infection. (C) C57BL/6 ASC-citrine BMDMs were treated with MCC950, Nec-1, or vehicle
680 control and were infected with WT *Yptb*. Caspase-8 cleavage and ASC speck formation were
681 analyzed at 4 hours post-infection. (D) Quantification of percent of cells with ASC specks at 2-
682 and 4 hours post-infection. (E) Graphical representation of findings. **** $p < 0.0001$ by two-way
683 ANOVA, ** $p < 0.05$ by unpaired t-test. Error bars represent the mean \pm SEM of triplicate wells
684 and are representative of three independent experiments.

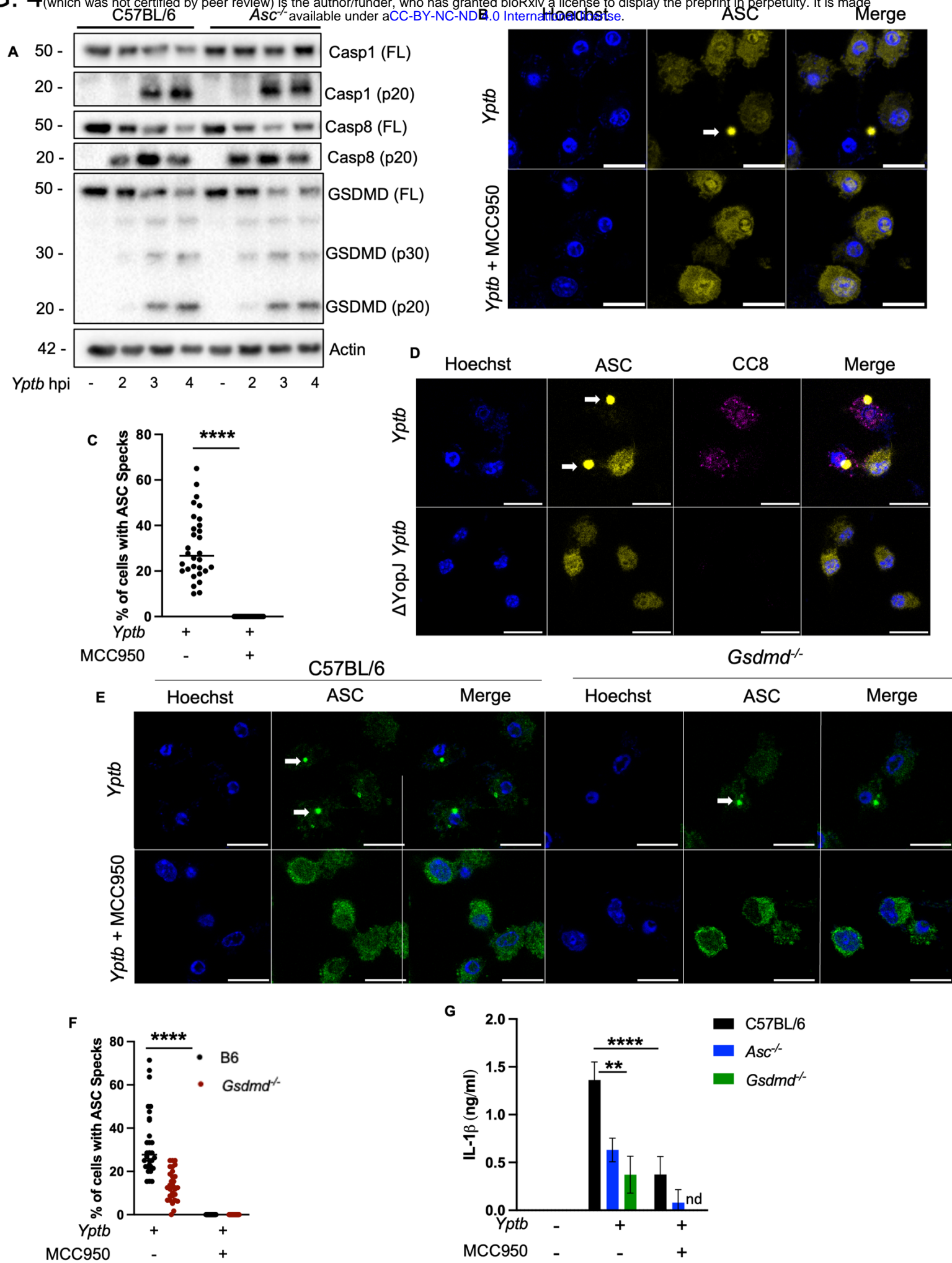
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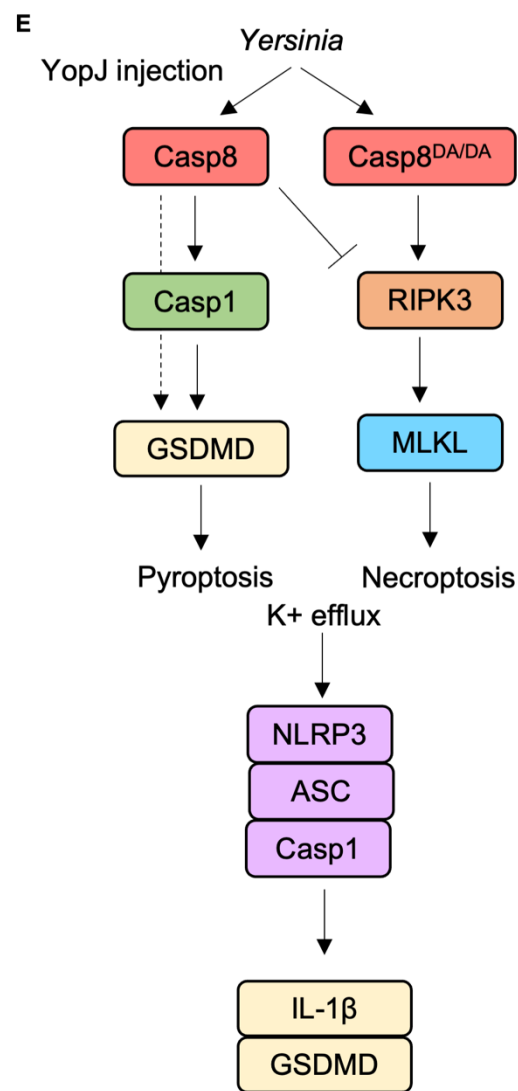
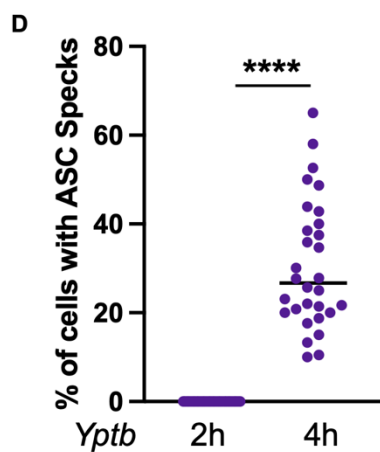
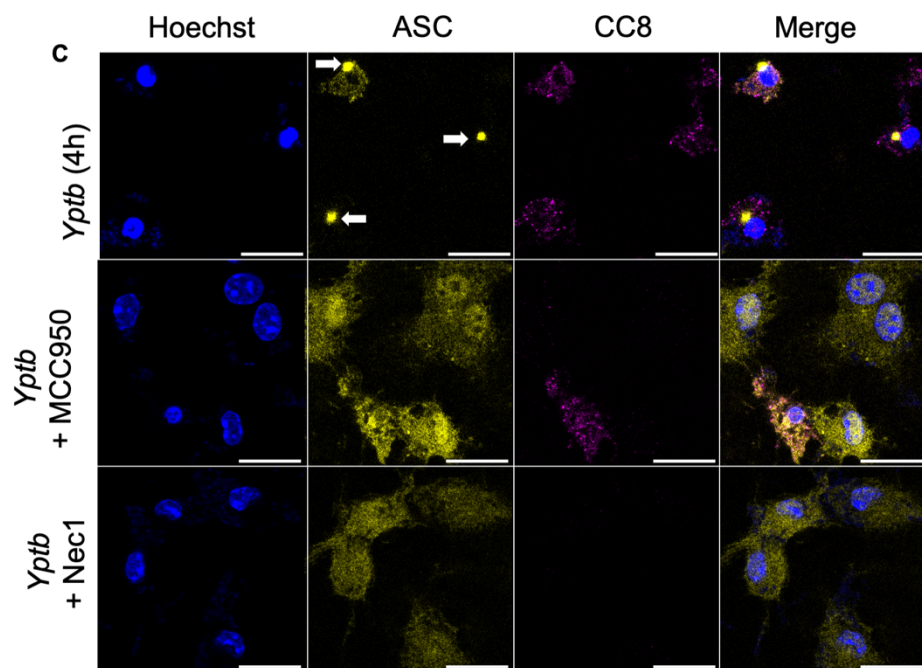
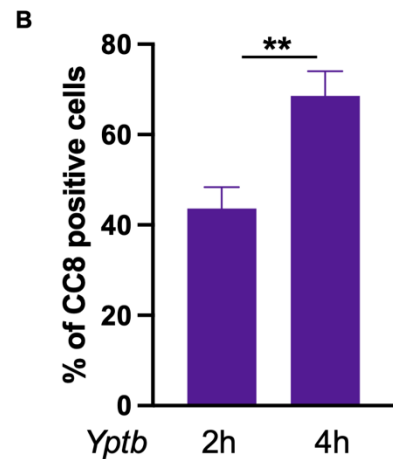
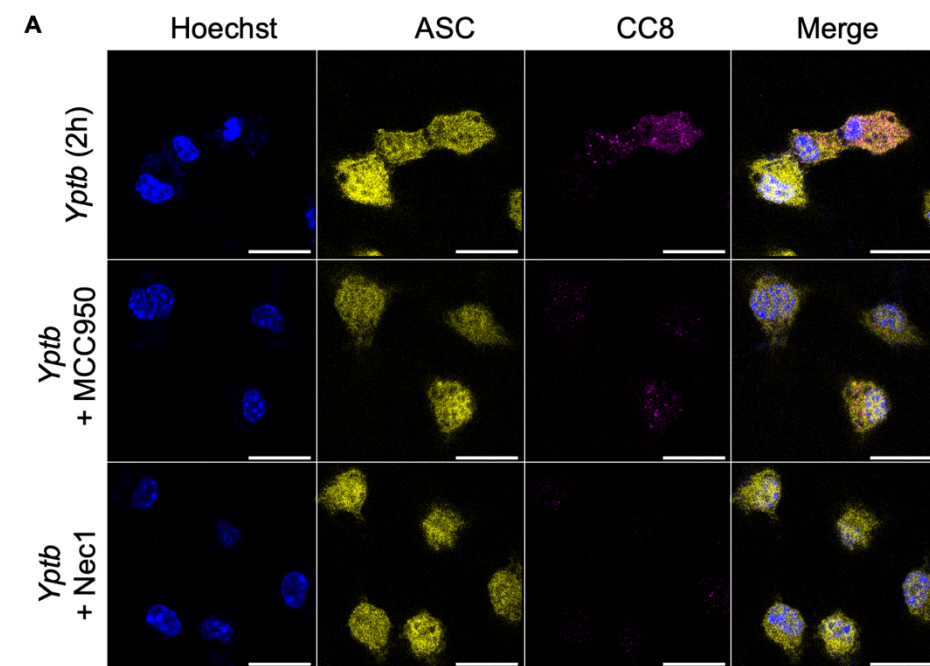
686 **Competing interests:** Authors declare that they have no competing interests.

687 **Data and materials availability:** All data are available in the main text or the supplementary
688 materials.









Supplementary Materials

Figure S1 (related to Figure 2)- Caspase-1 catalytic activity is required for cell death during

Stm* infection:** (S1A) iC57BL/6, *iCasp1*^{-/-} + *Casp1*^{WT}, *iCasp1*^{-/-} + *Casp1*^{DEAD}, and *iCasp1*^{-/-} + *EV* iBMDMs were infected with WT *Stm*. Percent cytotoxicity was measured 4 hours post-infection. (S1B) C57BL/6, *Casp1*^{mlt/mlt} BMDMs were infected with WT *Stm*. Percent cytotoxicity was measured 4 hours post-infection. (S1C) C57BL/6, *Casp1*^{mlt/mlt} BMDMs were infected with WT *Yptb*. Release of IL-12 into the supernatant was measured by ELISA at 4 hours post-infection. ns — not significant. *p < 0.0001 by two-way ANOVA. Error bars represent the mean +/- SEM of triplicate wells and are representative of three independent experiments.

Figure S2 (related to figure 4)- ASC specks can be visualized by fluorescence microscopy:

(S2A) C57BL/6 ASC-citrine BMDMs were primed with LPS followed by ATP treatment. ASC speck formation was analyzed 1-hour post ATP treatment. (S2B) C57BL/6, and *Asc*^{-/-} BMDMs were infected with WT *Yptb*. ASC speck formation was analyzed 4 hours post-infection. (S2C) C57BL/6, and *Asc*^{-/-} BMDMs were treated with MCC950 or vehicle control and were infected with WT *Yptb*. Percent cytotoxicity was measured 4 hours post-infection. ns — not significant. Error bars represent the mean +/- SEM of triplicate wells and are representative of three independent experiments.

Figure S3 (related to Figure 5)- Absence of casp1/11 results in reduced ASC speck formation:

(S3A) C57BL/6, and *Casp8*^{-/-}*Ripk3*^{-/-} BMDMs were infected with WT *Yptb*. Caspase-8 cleavage was analyzed 4 hours post-infection. (S3B) 1 hour prior to infection *Casp1/11*^{-/-} ASC-citrine BMDMs were treated with MCC950, Nec-1, or vehicle control and were infected with WT *Yptb*.

Caspase-8 cleavage and ASC speck formation were analyzed at 4 hours post-infection. (S3C)
Quantification of percent of cells with ASC specks for all conditions. (S3D) *Casp1/11*^{-/-} C57BL/6
ASC-citrine BMDMs were primed with LPS followed by ATP treatment. ASC speck formation
was analyzed 1-hour post ATP treatment. **** $p < 0.0001$ by two-way ANOVA. Error bars
represent the mean +/- SEM of triplicate wells and are representative of three independent
experiments.

FIG. S1

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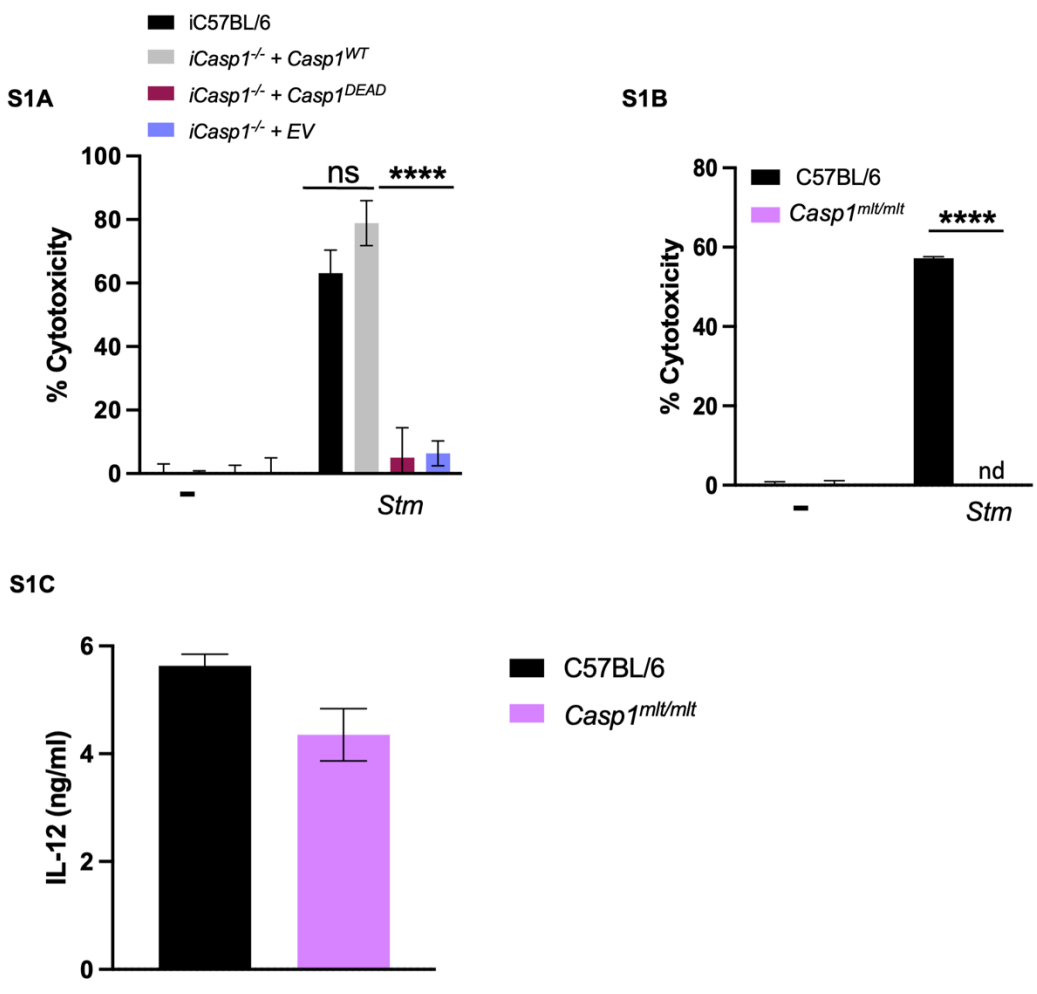


FIG. S2

