Caspase-1 cleaves Bid to release mitochondrial SMAC and drive secondary necrosis in absence of GSDMD

Rosalie Heilig[1], Marisa Dilucca[1], Dave Boucher[1], Kaiwen W. Chen[1], Dora Hancz[1], Benjamin Demarco[1], Kateryna Shkarina[1] and Petr Broz[1,*]

Affiliations:

[1]Department of Biochemistry, University of Lausanne, Chemin des Boveresses 155, 1066 Epalinges, Switzerland

* Corresponding Author: Department of Biochemistry, University of Lausanne, Chemin des Boveresses 155, 1066 Epalinges, Switzerland, phone: 0041 21 692 5656, petr.broz@unil.ch

SUMMARY (198/200)

Caspase-1 activation in GSDMD-deficient cells induces a rapid form of caspase-3-dependent secondary necrosis that is licenced by caspase-1-induced Bid cleavage and the release of mitochondrial SMAC.

ABSTRACT (175/175)

1   Caspase-1 drives a lytic inflammatory cell death named pyroptosis by cleaving the pore-
2   forming cell death executor gasdermin-D (GSDMD). *Gsdmd*-deficiency however only delays
3   cell lysis, indicating that caspase-1 controls alternative cell death pathways. Here we show that
4   in absence of GSDMD caspase-1 activates apoptotic initiator and executioner caspases and
5   triggers a rapid progression into secondary necrosis. GSDMD-independent cell death required
6   direct caspase-1-driven truncation of Bid and generation of caspase-3 p19/p12 by either
7   caspase-8 or caspase-9. tBid-induced mitochondrial outer membrane permeabilization
8   (MOMP) was also required to drive SMAC release and relieve IAP inhibition of caspase-3
9   thereby allowing caspase-3 auto-processing to the fully active p17/p12 form. Our data reveal
10 that cell lysis in inflammasome-activated *Gsdmd*-deficient cells is caused by a synergistic
11 effect of rapid caspase-1-driven activation of initiator caspases-8/-9 and Bid cleavage, resulting
12 in an unusually fast activation of caspase-3 and immediate transition into secondary necrosis.
13 This pathway might be advantageous for the host in counteracting pathogen-induced inhibition
14 of GSDMD, but also has implications for the use of GSDMD inhibitors in immune therapies for
15 caspase-1-dependent inflammatory disease.

16

INTRODUCTION

Inflammasomes are cytosolic signalling platforms assembled after the recognition of host- or pathogen-derived danger signals by cytosolic pattern recognition receptors, such as pyrin, AIM2 and members of the NLR protein family (Broz and Dixit, 2016). These complexes serve as activation platforms for caspase-1, the prototypical inflammatory caspase. Active caspase-1 cleaves the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 to their mature bioactive form, and induces a lytic form of cell death known as pyroptosis, by processing the cell death executor gasdermin-D (GSDMD) (Shi et al., 2015; Kayagaki et al., 2015). Caspase cleavage at the residue D276 in mouse (D275 in human), removes the inhibitory GSDMD[CT] and allows GSDMD[NT] to translocate to cellular membranes and form permeability pores, that disrupt ion homeostasis and the electrochemical gradient (Aglietti et al., 2016; Shi et al., 2015; Ding et al., 2016; Liu et al., 2016; Sborgi et al., 2016; Kayagaki et al., 2015). GSDMD is also cleaved by caspase-11 in mice and by caspases-4 and -5 in humans, which are activated by the so-called non-canonical inflammasome pathway in response to lipopolysaccharide (LPS) stemming from infections with cytosolic Gram-negative bacteria (Shi et al., 2014; Kayagaki et al., 2011; Hagar et al., 2013; Kayagaki et al., 2013). Uncontrolled inflammasome activation by gain-of-function mutations in inflammasome receptors or in the context of sterile inflammatory disease has been linked to a number of hereditary and acquired inflammatory diseases, such as Cryopyrin-associated periodic syndrome (CAPS) Muckle-wells, but also gout, Alzheimer's disease and atherosclerosis (Masters et al., 2009). It is thus of high interest to target and inhibit inflammasome assembly or downstream effector processes like GSDMD pore formation and IL-1β release.

While *Gsdmd*-deficiency results in complete abrogation of caspase-11 (-4)-induced lytic cell death, it only delays caspase-1-induced cell lysis (He et al., 2015; Kayagaki et al., 2015). Caspase-1 activation in *Gsdmd*^{−/−} cells correlates with high levels of caspase-3/7 and caspase-8 activity, but whether these apoptotic caspases trigger lysis of *Gsdmd*-deficient cells after caspase-1 activation has not been proven (He et al., 2015), and activation of apoptotic caspases has been observed to occur even in inflammasome-activated WT cells (Lamkanfi et al., 2008; Sagulenko et al., 2018). The lytic death of *Gsdmd*^{−/−} cells is also in contrast to the notion that apoptosis is non-lytic and thus immunologically silent. However, it is also known that prolonged apoptotic caspase activity will result in apoptotic cells losing membrane integrity, a process termed 'secondary necrosis'. Apoptosis is executed by caspase-3/-7, which themselves are activated by either caspase-8 (extrinsic apoptosis pathway) or caspase-9 (intrinsic or mitochondrial apoptosis pathway). Ligation of death receptors at the plasma membrane (FasR, TNFR, Trail) results in the assembly of the DISC (Death inducing signalling complex) or TNFR complex IIa/b, which activate caspase-8, the initiator caspase of the extrinsic pathway. In type I cells caspase-8 activity is sufficient to activate the executioner

54 caspases, while in type-II cells caspase-8 requires activation of the intrinsic pathway (Jost et
55 al., 2009). Here, caspase-8 cleaves the Bcl-2 family protein Bid to generate a truncated version
56 (tBid), which triggers Bax/Bak induced mitochondrial outer membrane permeabilization
57 (MOMP). MOMP results in the release of second mitochondria-derived activator of caspases
58 (SMAC), ATP and cytochrome C to promote intrinsic apoptosis via formation of the
59 apoptosome. This complex consists of Apoptotic Protease Activating Factor 1 (APAF1),
60 cytochrome C, ATP and caspase-9 and serves as an activation platform for caspase-9 which
61 in turn cleaves caspase-3. Apoptosis is a tightly regulated process, disturbance of the
62 equilibrium of cytosolic pool of pro- and anti-apoptotic Bcl-2 family proteins can result in
63 MOMP, apoptosis induction and cell death (Vince et al., 2018). To prevent accidental activation
64 of apoptosis, Inhibitor of Apoptosis Proteins (IAPs), in particular XIAP, supresses caspase-3/7
65 and -9 activation by direct binding to the caspases via BIR domains (Bratton et al., 2002;
66 Takahashi et al., 1998; Roy et al., 1997; Scott et al., 2005). SMAC, that is released during
67 MOMP, antagonizes IAPs thus removing the brake on caspase auto-processing and allowing
68 full activity of the executioner caspases and apoptotic cell death (Du et al., 2000; Wilkinson et
69 al., 2004; Verhagen et al., 2000).

70 Here we investigated the mechanism that induces lytic cell death after caspase-1 activation in
71 *Gsdmd*-deficient cells. We show that cell death in *Gsdmd*$^{-/-}$ macrophages requires caspase-
72 1, Bid-dependent mitochondrial permeabilization and the executioner caspase-3. Remarkably,
73 *Gsdmd*-deficient cells form apoptotic blebs and bodies only transiently, before shifting rapidly
74 to a necrotic phenotype that is characterized by extensive membrane ballooning.
75 Unexpectedly, we found that Bid cleavage and subsequent MOMP is driven directly by
76 caspase-1 independently of caspase-8, even though high levels of cleaved caspase-8 p18 are
77 found in inflammasome-activated *Gsdmd*-deficient cells. Upon investigating the steps
78 downstream of MOMP, we observed that knocking-out *Casp9* in *Gsdmd*$^{-/-}$ cells had only a
79 small effect on cell death, while removing both *Casp8* and *Casp9* abrogated GSDMD-
80 independent cell death. The redundancy in caspase-8 and -9 requirement was explained by
81 the observation that either caspase was sufficient to process caspase-3 between the large and
82 small catalytic domains thereby generating the intermediate caspase-3 p19 and p12
83 fragments. Caspase-1-dependent Bid cleavage and SMAC release is then required to remove
84 IAP inhibition, thereby allowing auto-cleavage of caspase-3 to the p17/p12 fragments and full
85 caspase activation (Kavanagh et al., 2014). Thus, cell lysis in absence of GSDMD is driven by
86 the synergistic effect of both rapid caspase-1-driven activation of initiator caspases-8/-9 and
87 Bid cleavage, which results in an unusually fast activation of caspase-3 and immediate
88 transition into secondary necrosis.

89   RESULTS

90   **Canonical inflammasomes trigger a rapid secondary necrosis in absence of GSDMD**

91   The canonical and non-canonical inflammasome pathways converge on the caspase-
92   dependent cleavage and activation of the pyroptosis executor GSDMD (Shi et al., 2015;
93   Kayagaki et al., 2015). However, while GSDMD is essential for lytic cell death (pyroptosis) after
94   LPS-induced non-canonical inflammasome activation (**Fig. S1A**), *Gsdmd*-deficiency only
95   delays cell lysis after engagement of canonical inflammasome receptors, such as AIM2 (**Fig.**
96   **1A, S1B-D**), NLRC4 (**Fig. S1B**) and NLRP3 (**Fig. S1B**) (Kayagaki et al., 2015). The absence
97   of caspase-1 and -11 in primary bone marrow derived macrophages (BMDMs), by contrast,
98   showed a much stronger reduction in LDH release and PI influx; and *Asc*-deficiency completely
99   abrogated cell lysis after AIM2 or NLRP3 activation, in line with the reported ASC-dependent
100   activation of apoptosis in absence of caspase-1 (Sagulenko et al., 2013; Pierini et al., 2012;
101   Chen et al., 2015; Man et al., 2013; Vajjhala et al., 2015).

102   We next tested a number of cell death inhibitors for their ability to block cell lysis in *Gsdmd*^{-/-}
103   immortalized BMDMs (iBMDMs) transfected with poly(dA:dT), an activator of the AIM2
104   inflammasome (**Fig. S2**). Neither 7-Cl-O-Nec1 (RIPK1 kinase inhibitor) nor GSK872 (RIPK3
105   kinase inhibitor) were able to delay cell death in *Gsdmd*^{-/-} iBMDMs, thereby excluding a role
106   for necroptosis or complex IIb-dependent apoptosis which require the kinase activity of RIPK3
107   or RIPK1 respectively (Tenev et al., 2011; Feoktistova et al., 2011; He et al., 2009; Zhang et
108   al., 2009; Cho et al., 2009). Similarly, we ruled out the involvement of calpains, calcium-
109   dependent proteases (PD150606, Calpeptin) or cathepsins (pan-cathepsin inhibitor K777),
110   which were previously shown to induce apoptosis through a caspase-3 dependent or
111   independent mechanisms (Chwieralski et al., 2006; Stennicke et al., 1998; Momeni, 2011).
112   Finally, we also tested if caspase inhibitors delayed death in *Gsdmd*^{-/-} or WT iBMDMs.
113   Remarkably, we found that whereas the pan-caspase inhibitor VX765 delayed PI uptake in
114   both WT and *Gsdmd*^{-/-} poly(dA:dT)-transfected cells, the specific caspase-3/-7 inhibitor I only
115   blocked cell death in *Gsdmd*^{-/-} but not in WT cells (**Fig. 1B, Fig. S2**). VX765 failed to prevent
116   cell death in WT cells at later timepoints in accordance with previous studies that showed that
117   pyroptosis is difficult to block pharmaceutically (Schneider et al., 2017).

118   While this suggested that apoptotic executioner caspases were necessary for cell death in
119   *Gsdmd*-deficient cells but dispensable for cell death in WT cells, the speed by which *Gsdmd*-
120   deficient cells underwent apoptosis and subsequently cell lysis was remarkable. *Gsdmd*^{-/-}
121   BMDMs displayed DNA-laddering and processing of caspase-3 to the mature p17 fragment
122   within one hour after poly(dA:dT) transfection, which was faster than even the highest
123   concentrations of either extrinsic or intrinsic apoptosis stimuli tested (**Fig. 1C-D**). It is
124   noteworthy that the highest concentration regularly used to induce apoptosis is yet 20-times

125 lower than the concentration used in our study (Vince et al., 2018). Phenotypically, this rapid
126 activation of caspase-3 resulted in a very fast lytic cell death as measured by PI influx (**Fig.**
127 **1E**) and morphological analysis (**Fig. 1F**). Of note, inflammasome-stimulated $Gsdmd^{-/-}$
128 BMDMs initiated membrane blebbing and apoptotic body formation initially, but rapidly lost this
129 morphology and transitioned into a necrotic state, characterized by extensive membrane
130 ballooning (**Fig. 1F**), similarly to the end-stage of GSDMD-induced pyroptosis (**Fig. S3A-C**).
131 We conclude that inflammasome activation in absence of GSDMD results in rapid cell lysis,
132 which we propose to refer to as 'GSDMD-independent secondary necrosis' to reflect both the
133 rapid transition to the necrotic state and the requirement for the activity of the apoptotic
134 executioner caspases-3/-7.

135

136 **GSDMD-independent secondary necrosis is mainly driven by caspase-3**

137 We next investigated which executioner caspase was required for GSDMD-independent
138 secondary necrosis after caspase-1 activation. High levels of caspase-3/-7 activity was
139 detected in poly(dA:dT)-transfected and $Salmonella$-infected $Gsdmd^{-/-}$ and to a lesser degree
140 in $Casp1^{-/-}/Casp11^{-/-}$ BMDMs, whereas WT or $Asc^{-/-}$ BMDMs showed minimal to no activity
141 (**Fig. 2A, Fig. S4A**). Since both caspase-3 and -7 cleave the DEVD peptidic substrate, we next
142 determined which executioner caspase was cleaved in $Gsdmd^{-/-}$ cells, but found that both
143 caspase-3 and caspase-7 were rapidly cleaved (**Fig. 2B**). Although, caspase-7 was found in
144 WT and $Gsdmd$-deficient cells, only $Gsdmd^{-/-}$ cells display detectable caspase-3/-7 activity
145 and caspase-3 cleavage (**Fig. 2A**). We therefore hypothesized that caspase-3 must account
146 for the DEVDase activity in $Gsdmd^{-/-}$ BMDMs.

147 To confirm our hypothesis genetically, we used CRISPR/Cas9 genome engineering to delete
148 either $Casp3$ or $Casp7,$ or both $Casp-3/7$ in $Gsdmd^{-/-}$ BMDMs (**Fig. S4B**) and determined the
149 impact of the deletion on GSDMD-independent secondary necrosis after AIM2 inflammasome
150 activation (**Fig. 2C, Fig. S4C**). $Gsdmd^{-/-}/Casp3^{-/-}$ as well as $Gsdmd^{-/-}/Casp3^{-/-}/Casp7^{-/-}$
151 iBMDMs were strongly protected against cell death after poly(dA:dT) transfection, while $Casp7$
152 single-deficiency did not provide protection, despite previous reports that caspase-3 and -7
153 function in redundant manner (**Fig. 2C, Fig. S4C**) (Lamkanfi and Kanneganti, 2010; Walsh et
154 al., 2008). Caspase-7 appeared to mainly contribute to the cell death observed in $Gsdmd^{-/-}$
155 $/Casp3^{-/-}$ iBMDMs, as these had higher LDH levels than $Gsdmd^{-/-}/Casp3^{-/-}/Casp7^{-/-}$ iBMDMs
156 (**Fig. 2C**). These data were further corroborated by knock-down of caspase-3 or -7 in $Gsdmd^{-}$
157 $^{/-}$ iBMDMs **(Fig. S4D)**. Finally, we also examined cell morphology after poly(dA:dT)
158 transfection. $Casp7$ knock-out in $Gsdmd^{-/-}$ iBMDMs failed to reduce necrotic features and cell
159 lysis, whereas $Gsdmd^{-/-}/Casp3^{-/-}$ as well as $Gsdmd^{-/-}/Casp3^{-/-}/Casp7^{-/-}$ iBMDMs remained
160 alive and intact (**Fig. 2D**) at 3 hours post treatment. In summary, these results demonstrate

161 that even though both executioner caspases are cleaved during cell death, it is caspase-3 that

162 drives GSDMD-independent secondary necrosis in inflammasome-activated cells.

163 Because caspase-3 was shown to cleave gasdermin-E (GSDME), another member of the

164 gasdermin- family, and was proposed to drive secondary necrosis during prolonged apoptosis,

165 we asked whether lack of GSDMD drives an alternative pathway via caspase-3 mediated

166 GSDME cleavage and pore formation. We thus measured LDH release and PI influx in WT,

167 $Gsdmd^{-/-}$, $Gsdme^{-/-}$, $Gsdmd^{-/-}/Gsdme^{-/-}$ BMDMs upon activation of the AIM2 inflammasome

168 (**Fig. 2E, S5A**). Surprisingly, although GSDME was cleaved in $Gsdmd^{-/-}$ at 1h post poly(dA:dT)

169 transfection, we did not find a contribution of GSDME to cell death in $Gsdmd^{-/-}$ BMDMs, since

170 *double* GSDMD/GSDME-*deficiency* did not conferred any additional protection (**Fig. 2E, S5A-**

171 **B**). Furthermore, BMDMs lacking only GSDME were comparable to WT BMDMs, overall

172 suggesting that GSDME does neither contribute to pyroptosis nor GSDMD-independent

173 necrosis.

174

175 **Caspase-1 is required to cause GSDMD-independent secondary necrosis in**

176 **inflammasome-activated cells**

177 Since the ASC speck has been reported to control activation of apoptotic caspases

178 independently of caspase-1 (Sagulenko et al., 2013; Pierini et al., 2012; Schneider et al., 2017;

179 Van Opdenbosch et al., 2017; Lee et al., 2018; Mascarenhas et al., 2017), we next generated

180 $Gsdmd^{-/-}/Casp1^{-/-}$ BMDMs to determine if caspase-1 was required for GSDMD-independent

181 secondary necrosis (**Fig. 3A**). Deletion of caspase-1 in *Gsdmd*-deficient BMDMs strongly

182 reduced LDH release, caspase-3 processing and caspase-3 activity (**Fig. 3A-C**). LDH levels

183 after 3 and 5 hours of poly(dA:dT) transfection were comparable to $Casp1^{-/-}/Casp11^{-/-}$

184 BMDMs, but not as low as in $Asc^{-/-}$, confirming that *Casp1* deletion did not affect the cell death

185 that is caused through the ASC-Caspase-8 axis **(Fig. 3B)**. It would theoretically be possible

186 that GSDMD-independent secondary necrosis is not driven by the catalytic activity of caspase-

187 1, but by the formation of a caspase-1-containing scaffold and the assembly of an unknown

188 death inducing complex, in analogy to the scaffolding function of caspase-8 (Henry and Martin,

189 2017). However, we found that poly(dA:dT)-induced PI influx in BMDMs from $Casp1^{C284A/C284A}$

190 mice, which express a catalytically dead caspase-1, was comparable to $Casp1^{-/-}/Casp11^{-/-}$ or

191 $Casp1^{-/-}$ BMDMs, and much lower than PI influx in $Gsdmd^{-/-}$, we formally excluded this

192 possibility (**Fig. 3D**), we formally concluded that caspase-1 enzymatic-activity is required to

193 drive GSDMD-independent secondary necrosis.

194

Bid cleavage is required for mitochondrial damage and GSDMD-independent secondary necrosis

While examining the morphology of inflammasome activated BMDMs by confocal microscopy, we found that *Gsdmd*⁻/⁻ cells were characterized by mitochondrial fragmentation and loss of mitochondrial membrane potential **(Fig. S6A)** and a rapid drop of cellular ATP levels **(Fig. 4A, Fig. S6B-D)** as early as 30 min after inflammasome activation. Given this rapid loss of mitochondrial integrity we hypothesized that it was linked to the rapid onset of caspase-3 activation and induction of secondary necrosis in *Gsdmd*-deficient cells.

An imbalance of pro- and anti-apoptotic Bcl2 family members results in activation of Bax/Bak pore formation and loss of mitochondrial integrity during apoptosis. Often, degradation and/or cleavage of anti-apoptotic Bcl2 proteins as well as activating cleavage of BH3-only protein are responsible for mitochondrial outer membrane permeabilization (MOMP). To identify which pro-apoptotic Bcl2 proteins are processed in *Gsdmd*⁻/⁻ BMDMs, we made use of Stable Isotope Labeling with Amino acids in Cell culture (SILAC) mass spectrometry approach (Ong et al., 2002). Differentially isotope-labelled immortalized *Gsdmd*⁻/⁻ and *Asc*⁻/⁻ BMDMs were transfected with poly(dA:dT), proteins separated by molecular weight using SDS-PAGE, cut according to MW and each slice analyzed by mass spectrometry (Slice-SILAC). The differential analysis of the heavy versus light fraction enabled a comparison between the non-responsive *Asc*⁻/⁻ and the responsive *Gsdmd*⁻/⁻, wherein appearance of smaller fragments in *Gsdmd*⁻/⁻ indicated potential cleavage. We focused on potential cleavage of Bcl-2 family proteins that indicate their inability to inhibit BH3-only proteins or promote BH3-only proteins to induce mitochondrial outer membrane permeabilization (MOMP) (Bock and Tait, 2019). The anti-apoptotic protein Mcl-1(of Bcl-2, Mcl-1 and Bcl-XL) and the pro-apoptotic proteins Bax, Bak, Bid (but not Bim) were found to be cleaved in *Gsdmd*⁻/⁻, but not in *Asc*⁻/⁻ cells **(Fig. 4B)**. Since in type-II cells caspase-8-cleaved tBid translocates to the mitochondria to promote Bax/Bak-dependent pore formation and intrinsic apoptosis, we investigated whether Bid cleavage promoted GSDMD-independent secondary necrosis. Confirming the SILAC data, Bid was found to be rapidly cleaved in *Gsdmd*⁻/⁻ cells but not in *Asc*⁻/⁻ after inflammasome activation **(Fig. 4C)**. However, since Bid cleavage was also observed in WT and *Casp1*⁻/⁻/*Casp11*⁻/⁻ BMDMs, we proceeded to assess its contribution to GSDMD-independent secondary necrosis genetically by generating *Gsdmd*⁻/⁻/*Bid*⁻/⁻ iBMDMs **(Fig. S7A).** Knocking out *Bid* in *Gsdmd*⁻/⁻ cells significantly reduced the levels of caspase-3 activity (**Fig. S7B**) after poly(dA:dT) transfection and in agreement with that strongly reduced LDH release and PI uptake were observed **(Fig. 4D, Fig. S7C)**. Strikingly, *Gsdmd*⁻/⁻/*Bid*⁻/⁻ cells looked adhered and elongated comparable to untreated iBMDMs upon transfection with poly(dA:dT) which is in contrast to *Gsdmd*⁻/⁻ iBMDMs which displayed typical necrotic features such as rounding up, permeabilization, shrinkage and blebbing **(Fig. S7D)**. In summary, these results show that Bid

232 is an essential mediator of GSDMD-independent secondary necrosis, and suggest that Bid

233 cleavage is required to drive this cell death.

Caspase-1 cleaves Bid to promote caspase-3 activation and cell lysis

235 Since proteolytic cleavage of Bid precedes MOMP and is required for cell death we next

236 enquired which upstream caspase is responsible for Bid activation. Immunoblotting for the

237 cleaved p18 fragment of caspase-8 suggested that $Gsdmd^{-/-}$ BMDMs contain active caspase-

238 8 at 15-30 minutes after poly(dA:dT) transfection, while very little cleaved caspase-8 p18 was

239 found in WT, $Asc^{-/-}$ or $Casp1^{-/-}/Casp11^{-/-}$ BMDMs (**Fig. 5A, S8A**). Interestingly, the relatively

240 low levels of caspase-8 cleavage in $Casp1^{-/-}/Casp11^{-/-}$ compared to $Gsdmd^{-/-}$ BMDMs

241 suggested that direct activation of caspase-8 by the ASC speck was negligible and that instead

242 caspase-8 activation in $Gsdmd^{-/-}$ cells depended on the presence caspase-1. However,

243 whether caspase-1 would cleave and activate caspase-8 directly or by an indirect pathway

244 could not be deduced.

245 We next assessed the role of caspase-8 in causing GSDMD-independent secondary necrosis

246 by generating $Gsdmd^{-/-}/Casp8^{-/-}$ iBMDM lines (**Fig. S8B**). Of note, while $Casp8$-deficiency in

247 mice results in embryonic lethality due to the unchecked activation of RIP3-dependent

248 necroptosis (Oberst et al., 2011; Kaiser et al., 2011), $Casp8$-deficient macrophages were

249 reported to be viable unless stimulated with extrinsic apoptotic triggers (Kang et al., 2004;

250 Cuda et al., 2015; Kaiser et al., 2011). Indeed, when testing if $Gsdmd^{-/-}/Casp8^{-/-}$ BMDMs

251 showed reduced levels of cell death after induction of apoptosis with the extrinsic apoptosis

252 stimulus TNFα/SMAC we found that cell death was reduced, but not completely abrogated

253 (**Fig. S8C**). The remaining cell death, however, was block when TNFα/SMAC was combined

254 with the RIPK3 kinase inhibitor GSK'872 (**Fig. S8C**). These results confirmed that the cells

255 were indeed $Casp8$ knock-outs, and that the necroptotic pathway was only initiated when death

256 receptors were engaged. We next compared LDH release in $Gsdmd^{-/-}$ and $Gsdmd^{-/-}/Casp8^{-/-}$

257 BMDMs after transfection of the AIM2 inflammasome activator poly(dA:dT). Unexpectedly, we

258 found no difference in LDH release nor PI uptake between these two genotypes (**Fig. 5B,**

259 **S8D**). Furthermore, we were still able to detect Bid cleavage and caspase-3 processing to the

260 active p17 fragment in inflammasome-activated $Gsdmd^{-/-}/Casp8^{-/-}$ BMDMs (**Fig. 5C-D**).

261 Previous work has implied that Bid can also be a substrate of caspase-1 (Li et al., 1998), since

262 caspase-1 and caspase-8 have partially overlapping substrate spectrum that includes also

263 GSDMD and IL1β (Orning et al., 2018; Chen et al., 2019; Sarhan et al., 2018; Maelfait et al.,

264 2008). In line with caspase-1 controlling Bid cleavage directly and independently of caspase-

265 8, we found that tBid generation after AIM2 activation was completely abrogated in $Gsdmd^{-/-}$

266 $/Casp1^{-/-}$ BMDMs at early timepoint and strongly reduced after prolonged incubation (**Fig. 5E**),

267 and that caspase-1 was able to efficiently convert Bid to tBid in an *in vitro* cleavage assay (**Fig.**

268 **5F**). In summary our data thus far suggest that while Bid cleavage is essential for GSDMD-
269 independent secondary necrosis and high-levels of active caspase-8 are found in these cells,
270 it is caspase-1 and not caspase-8 that processes Bid and induces mitochondrial
271 permeabilization.

272

273 **GSDMD-independent secondary necrosis requires both caspase-8 and caspase-9**

274 Having identified caspase-1, Bid and caspase-3 as the essential drivers of GSDMD-
275 independent secondary necrosis, we next asked if activation of caspase-9 downstream of
276 mitochondrial permeabilization and cytochrome c release provides the link between Bid and
277 caspase-3 activation. We thus generated $Gsdmd^{-/-}/Casp9^{-/-}$ iBMDM lines by CRISPR/Cas9
278 genome targeting and verified that they lacked caspase-9 expression and no longer responded
279 to intrinsic apoptosis induction (**Fig. S9A-B**). However, we found that in analogy to $Casp8$-
280 deficiency, knocking out of $Casp9$ in $Gsdmd^{-/-}$ had only a small impact on poly(dA:dT)-induced
281 secondary necrosis after 5 hours of treatment, while no impact was detectable at earlier
282 timepoint (**Fig. 6A, S9C**). Furthermore, caspase-3 processing was also found to be unaffected
283 in these cell lines (**Fig. 6B**).

284 These results raised the possibility that caspase-8 and caspase-9 activity was redundant or
285 that caspase-1 was driving Bid cleavage and caspase-3 activation somehow independently of
286 both initiator caspases. We addressed these two scenarios by creating $Gsdmd^{-/-}/Casp8^{-/-}$
287 $/Casp9^{-/-}$ iBMDM lines (**Fig. S9D**) and compared their phenotype after AIM2 inflammasome
288 activation to our other knockout lines. Poly(dA:dT)-transfected $Gsdmd^{-/-}/Casp8^{-/-}/Casp9^{-/-}$
289 BMDMs displayed significantly reduced levels of LDH release compared to $Gsdmd^{-/-}$, $Gsdmd^{-/-}/Casp8^{-/-}$
290 $^{-/-}/Casp8^{-/-}$ or $Gsdmd^{-/-}/Casp9^{-/-}$ cells (**Fig. 6C**). Consistent with the reduced levels of cell lysis,
291 we also found that caspase-3 processing was significantly reduced in $Gsdmd^{-/-}/Casp8^{-/-}$
292 $/Casp9^{-/-}$ when compared to the other genotypes (**Fig. 6D**), confirming that activity of either
293 initiator caspase was sufficient to drive caspase-3 activation and GSDMD-independent
294 secondary necrosis.

295

296 **Bid-induced mitochondrial permeabilization is required to release SMAC and promote**
297 **conversion of caspase-3 p19 to p17**

298 The finding that single deficiency in either caspase-8 or caspase-9 had no impact on caspase-
299 3 activation and GSDMD-independent secondary necrosis, while double-deficiency abrogated
300 cell lysis was unexpected and puzzling. Since Bid was essential for GSDMD-independent
301 secondary necrosis while caspase-9 was not, we hypothesized that other factors released from
302 permeabilized mitochondria were required. Besides cytochrome C, which activates Apaf-1 to

303   assemble the apoptosome and promote caspase-9 activity, mitochondria also release ATP
304   and SMAC. SMAC binds IAPs, in particular XIAP, which normally supresses caspase-3/7 and
305   -9 activity, and thus relieves the block on apoptosis induction (Deveraux et al., 1997; Wu et al.,
306   2000). We thus closely examined caspase-3 processing between poly(dA:dT)-transfected
307   *Gsdmd*^{-/-} and *Gsdmd*^{-/-}/*Bid*^{-/-}, and found that while only the p17 fragment of caspase-3 was
308   found in *Gsdmd*^{-/-} BMDMs, *Gsdmd*^{-/-}/*Bid*^{-/-} featured two cleaved caspase-3 bands, at 19 and
309   17 kDa (**Fig. 6E, S9E**). Previous studies showed that p19 fragment is generated by apical
310   caspases cleaving in the linker domain between the large and small subunit, while the p17 is
311   generated by auto-processing of the pro-peptide by caspase-3 itself (Kavanagh et al., 2014)
312   (**Fig. 6F**). We hypothesised that *Bid*-deficiency delayed IAP release and thus conversion from
313   the p19 to the p17 fragment and full activity of caspase-3 and that this was a critical factor for
314   GSDMD-independent secondary necrosis. Indeed, treatment with the SMAC mimetic AZD
315   5582 increased generation of caspase-3 p17 (**Fig. S9F**), and partially restored cells death in
316   *Gsdmd*^{-/-}/*Bid*^{-/-} BMDMs (**Fig. 6G**). These results suggest that during GSDMD-independent
317   secondary necrosis, Bid cleavage and mitochondrial permeabilization are mainly required for
318   the release of SMAC and subsequent binding to XIAP, but not to drive caspase-9 activation.
319   However, since either caspase-8 or caspase-9 are needed to process caspase-3 (**Fig. 6D**),
320   caspase-1 cannot induce GSDMD-independent secondary necrosis in absence of both initiator
321   caspases (**Fig. 7**).

322

DISCUSSION

Here we show that the cell lysis that occurs in *Gsdmd*-deficient cells upon activation of canonical inflammasomes is a rapid form of secondary necrosis (referred to as 'GSDMD-independent secondary necrosis' in this manuscript), and that it depends on the caspase-1-dependent activation of either caspase-8 or -9, Bid cleavage, SMAC release and caspase-3 activity. Secondary necrosis describes the loss of membrane integrity of apoptotic cells or apoptotic bodies, and is thus appropriate since death in *Gsdmd*-deficient cells relies on initiator caspases as well as the executor caspase-3 and results in a loss of membrane integrity. Yet, it is remarkably different from regular apoptosis in the signaling pathways that underlies its induction, cellular morphology and the speed by which cells undergo death.

GSDMD-independent cell lysis is characterized by a rapid loss of mitochondrial potential and rapid activation of caspase-3, and an atypical apoptotic morphology. Indeed, cells undergoing this type of cell death show only initially signs of regular apoptotic blebs or apoptotic body formation, and quickly lose membrane integrity and start ballooning, similarly to pyroptotic cells. This morphology has in the past led to the speculation that caspase-1 might directly or indirectly cleave an alternative lytic cell death executor, such as another gasdermin family member. Indeed, recently Tsuchiya and colleagues proposed that caspase-3 processes GSDME in *Gsdmd*-deficient CL26 cells that harbor dimerizer-activated caspase-1 (Tsuchiya et al., 2019). Our data in primary mouse macrophages however show no involvement of GSDME in GSDMD-independent secondary necrosis (**Fig. 2E, Fig. S5A-B**), even though GSDME is detectable and processed (**Fig. S5B**). This discrepancy is most likely caused by differences in GSDME expression levels between different cell types. Recent results show that a number of cancer cell lines express sufficiently high levels of GSDME to cause pyroptosis upon treatment with apoptosis-inducing chemotherapy drugs (Wang et al., 2017b).It remains to be determined how much GSDME is expressed by CL-26 cells, a murine colorectal carcinoma cell line, compared to macrophages (Tsuchiya et al., 2019), but overwhelming evidence suggest that at least in macrophages, GSDME expression or activity appear to be insufficient to induce GSDME-dependent cell death after caspase-3 activation (Lee et al., 2018; Chen et al., 2019; Sarhan et al., 2018; Vince et al., 2018). Thus, other yet undefined factors drive the lysis of *Gsdmd*-deficient BMDMs.

Another striking difference between regular apoptosis and GSDMD-independent secondary necrosis is the signalling pathway underlying caspase-3 activation. Our data show that the main driver of this cell death is active caspase-1, and that it promotes cell death by cleaving several targets. The most critical of these targets appears to be Bid, which is converted by caspase-1 to tBid (independently of caspase-8) and which induces mitochondrial permeabilization and the release of cytochrome C, ATP and SMAC. Moreover, caspase-1 acts

as a kind of 'super-initiator' caspase by activating initiator caspases-8/-9. It is worth noting that caspase-8-activation is mostly driven by caspase-1 with a negligible contribution of direct caspase-8 activation at the ASC speck, as evident from much reduced caspase-8 cleavage in *Casp1/Casp11*-deficient compared to *Gsdmd*-deficient cells. This could potentially be driven by direct caspase-1-induced cleavage of caspase-8, or by caspase-1 somehow enhancing ASC-dependent caspase-8 activation. Caspase-9 activation however is downstream of Bid cleavage. The requirement for either caspase-8 and -9 appears to stem from the fact that caspase-1 fails to process caspase-3 efficiently, despite previous reports suggesting that caspase-1 cleaved caspase-3 directly (Taabazuing et al., 2017; Sagulenko et al., 2018). However, caspase-1 is efficient enough to activate Bid to induce SMAC release, to relieve inhibition by IAPs and allows full conversion to caspase-3 p17/p12.

Our findings are contradicting a recent report by the Suda lab, which proposed that cell death in *Gsdmd*-deficient cells is solely caused by the Bid-caspase-9-caspase-3 axis (Tsuchiya et al., 2019). The discrepancy is potentially related to cell line-intrinsic differences or to the method used to activate caspase-1. Tsuchiya et al. performed experiments in CL26-cells, which for example lack ASC, and thus lack ASC speck-induced activation of caspase-8 (Sagulenko et al., 2013; Vajjhala et al., 2015; Fu et al., 2016; Pierini et al., 2012), while we used immortalized macrophages, which recapitulate the behavior of primary BMDMs. Furthermore, they used a dimerizer-based system to activate caspase-1, which most likely induces higher levels of caspase-1 activity compared to physiological inflammasome triggers, and thus might explain why Tsuchiya and colleagues did not observe a role for caspase-8, which we however find necessary to amplify caspase-1 activity after treatment with established canonical inflammasome triggers. However, both studies agree that Bid cleavage is essential for cell death in *Gsdmd*-deficient cells, and that Bid is cleaved by caspase-1 independent of caspase-8.

Recent work has revealed a surprisingly high level of redundancy and crosstalk between the apoptotic, necroptotic and pyroptotic cell death pathways. Interestingly, in many cases these pathways or crosstalk are normally not detectable or only turned on when another pathway is inhibited. For example, deletion of caspase-8 or its autoprocessing sites are known to results in activation of RIP3/MLKL-dependent necroptosis, a pathway that can otherwise not be observed, and catalytic-dead caspase-8 results in activation of necroptosis as well pyroptosis (Kaiser et al., 2011; Kang et al., 2018; Oberst et al., 2011). It is assumed that this redundancy developed as a defense mechanism to guard against pathogen-induced inhibition of apoptosis, and accordingly viral inhibitors of the three major cell death pathways have been identified (Taxman et al., 2010; Nailwal and Chan, 2019; Li and Stollar, 2004), which highlights that necroptosis is not an artifact caused by lack of caspase-8 activity. Similarly, it could be speculated that the ability of caspase-1 to induce rapid secondary necrosis by activating

396 apoptotic caspases might have developed as a safeguard against viruses that inhibit GSDMD.
397 Indeed, recently, the pathogenic enterovirus 71, that is known to trigger the NLRP3-
398 inflammasome (Wang et al., 2017a), was shown to interfere with GSDMD activation. In
399 particular the viral protease 3C was shown to cleave GSDMD at Q193/194, interfering with N-
400 terminal fragment formation, oligomerization and GSDMD pore formation (Wang et al., 2015).
401 Furthermore, GSDMD-independent secondary necrosis appears to contribute to the clearance
402 of bacterial infection, as it could be shown that $Gsdmd^{-/-}$ mice are less susceptible to infection
403 with *Francisella novicida* compared to *Casp1-* or *Aim2*-deficient animals (Schneider et al.,
404 2017; Kanneganti et al., 2018b). Along the same lines, *Gsdmd*-deficient mice infected with *B.*
405 *thailandensis* show lower CFUs and lower IL-1β levels than *Casp1*/*Casp11*-deficient animals
406 (Wang et al., 2019). Similarly, it was reported that peritoneal IL-1β levels are higher in
407 *Salmonella typhimurium*-infected $Gsdmd^{-/-}$ mice than $Casp1^{-/-}$ controls (Monteleone et al.,
408 2018). These studies thus allow the conclusion that GSDMD-independent cell death is also
409 engaged *in vivo* and that it allows partial protection against intracellular bacterial pathogens.
410 Unexpectedly however, GSDMD-independent secondary necrosis does not appear to be
411 important in models of autoinflammatory diseases, since *Gsdmd*-deficiency rescues mice
412 expressing mutant NLRP3 or Pyrin, linked to Neonatal Onset Multisystem Inflammatory
413 Disease (NOMID) and Familial Mediterranean Fever (FMF) (Xiao et al., 2018; Kanneganti et
414 al., 2018a).

415 Considering that knock-out of GSDMD showed a big improvement in pro-inflammatory
416 symptoms associated with the autoinflammatory disease NOMID and FMF, and the
417 importance of the canonical inflammasome pathway in sterile inflammatory disease, research
418 has focused on the discovery of GSDMD specific inhibitors. To date several inhibitors have
419 been identified, though off target effects and specificity still need to be evaluated in more detail
420 (Rathkey et al., 2018; Sollberger et al., 2018; Rashidi et al., 2019). Furthermore, it is important
421 to consider that caspase-1 activity is unrestrained by these inhibitors, and that thus caspase-
422 1 might induce cell death and inflammation through the back-up pathway described in our
423 study.

424

425 MATERIAL AND METHODS

426 **Antibodies, chemicals and reagents.** Drugs: VX-765 (MedchemExpress), Caspase-3/7
427 inhibitor I (CAS 220509-74-0, Santa Cruz Biotechnology), Q-VD-Oph (Selleck Chemicals),
428 AZD5582 (Selleck Chemicals), 7-Cl-O-Nec1 (Abcam), GSK872 (Selleck Chemicals), K777
429 (Adipogen), PD 150606 (Tocris), Calpeptin (Selleck Chemicals), ABT-737 (Selleck
430 Chemicals), S63845 (Selleck Chemicals), Nigericin (InvivoGen).

431 Antibodies: GSDMD (Ab209845, Abcam), Casp-1 (Casper1, AG-20B-0042-C100, AdipoGen),
432 Tubulin (Ab40742, Abcam), IL-1β (AF-401-NA, R & D Systems), Caspase-3 (#9662, Cell
433 Signaling Technology), Caspase-7 (#9492, Cell Signaling Technology), Caspase-8 (#9429 and
434 4927, Cell Signaling Technology), Caspase-9 (#9508 and #9504, Cell Signaling Technology),
435 Bid (#2003, Cell Signaling Technology).

436 **Animal experiments.** All experiments were performed with approval from the veterinary office
437 of the Canton de Vaud and according to the guidelines from the Swiss animal protection law
438 (licence VD3257). C57BL/6J mice were purchased from Janvier Labs (France) and housed at
439 specific pathogen-free facility at the University of Lausanne. Mice lacking *Asc*, *Casp1*,
440 *Casp1/11*, *Gsdmd, Gsdme* or expressing mutant *Casp1^{C284A}* have been previously described
441 (Chen et al., 2019; Schneider et al., 2017; Mariathasan et al., 2004; Kayagaki et al., 2011). All
442 mice were either generated (*Gsdmd$^{-/-}$, Gsdme$^{-/-}$*) or back crossed (other lines) in the C56BL/6J
443 background.

444 **Cell culture and immortalization of macrophages.** Primary mouse macrophages (BMDMs)
445 were differentiated for 6 days and cultured for up to 9 days in DMEM (Gibco) supplemented
446 with 10% FCS (Bioconcept), 20% 3T3 supernatant (MCSF), 10% Hepes (Gibco) and 10% non-
447 essential amino acids (Gibco). Immortalization of macrophages was done as previously
448 described (Broz et al., 2010; Blasi et al., 1985). Immortalized macrophages (iBMDMs) were
449 cultured in DMEM complemented with 10% FCS (Bioconcept), 10% MCSF (3T3 supernatant),
450 10% Hepes (Amimed) and 10% non-essential amino acids (Life Technologies). To passage
451 the BMDM and iBMDMs cells were washed with PBS and left to detach at 4°C for 15 min and
452 scarped using cell scrapers (Sarstedt), spun down at 300 g for 5 min at 4°C and resuspended
453 in the appropriate amount of medium.

454

455 **Crispr genome editing in immortalized macrophages.** *Bid*-, *Casp9*-, *Casp-8*-, *Casp-*
456 *8/Casp9*-, *Casp1*-, *Casp3*-, *Casp7*- and *Casp3/7*-deficient immortalized BMDM (iBMDM) were
457 generated using the genome editing system Alt-R-CRISPR/Cas (IDT) according to the
458 manufacturer's protocol. Briefly, the gene-specific targeting crRNA (Bid:
459 TGGCTGTACTCGCCAAGAGC TGG Caspase-9: CACACGCACGGGCTCCAACT TGG,
460 Caspase-8: CTTCCTAGACTGCAACCGAG AGG, Caspase-1:
461 AATGAAGACTGCTACCTGGC AGG, Caspase-7: GATAAG TGGGCACTCGGTCC TGG,
462 Caspase-3: AATGTCATCTCGCTCTGGTA CGG or TGGGCCTGAAATACCAAGTC AGG)
463 was mixed with the universal RNA oligo tracrRNA to form a gRNA complex (crRNA:-
464 tracrRNA). The addition of the recombinant Cas9 nuclease V3 allowed the formation of an
465 RNP complex specific for targeting the desired genes. The tracrRNA only or RNP complexes
466 were subsequently reverse-transfected into either WT or *Gsdmd$^{-/-}$* immortalized iBMDM using

467   RNAiMax (Invitrogen). The bulk population was tested for successful gene mutation using the

468   T7 endonuclease digestion assay as follows: cells were lysed by the KAPA Biosystems Kit

469   according to the manufacturer's protocol, and genomic DNA flanking the guide RNA (crRNA)

470   binding site was amplified by PCR using gene-specific primers (Bid: fw:

471   CTGGACATTACTGGGGGCAG, rv: CTCGATAGCCCCTTGGTGTC, Caspase-9: fw:

472   CAAGCTCTCCAGACCTGACC, rv: GAGATCTGACGGGCACCATT, Caspase-8: fw:

473   GGGATGTTGGAGGAAGGCAA, rv: GGCACAGACTTTGAGGGGTT, Caspase-1: fw:

474   CAGACAAGATCCTGAGGGCA, rv: AGATGAGGATCCAGCGAGTAT, Caspase-7: fw:

475   TTGCCTGACCCAAG GTTTGT, rv: CCCAGCAACAGGAAAGCAAC; Caspase-3: fw: GTG

476   GGGGATATCGCTGTCAT, rv: TGTGTAAGGATGCGGACTGC). The amplified genomic DNA

477   was used to perform the heteroduplex analysis according to the manufacturer's protocol (IDT).

478   Single clones were derived from the bulk population by limiting dilution, and the absence of

479   protein expression in single clones was verified by immunoblotting and sequencing of genomic

480   regions, where required.

481   **siRNA knockdown.** 2.5×10^5 *Gsdmd*-deficient iBMDMs were seeded per well of a 6-well plate

482   and incubated overnight. For the siRNA transfection, medium was changed to OptiMEM and

483   siRNA transfection was done according to the manufacturer's protocol, transfecting 25 pmol

484   siRNA (non-targeting: siGENOME Non-targeting siRNA Control Pools [D-001206-14,

485   Dharmacon], caspase-3: Casp3 SMART POOL [M-043042-01, Dharmacon], caspase-7:

486   Casp7 SMART POOL [M-057362-01, Dharmacon])with 7.5 µl Lipofectamine RNAiMax

487   (Invitrogen) per well. Medium was exchanged for DMEM (10% FCS, 10% MCSF, 1% NeAA,

488   1% Hepes) after 6 hours. 48 hours post transfection cells were collected and reseeded in a

489   96-well plate at 3×10^4 cells/well. Cells were primed and treated as in cell death assays.

490   **Cell death assays.** The cells were seeded in 96-well plates (100ul/well) or 12-well plates (1

491   ml/well) at a density of 0.5×10^6 cells/ml overnight and primed the next day with 100 ng/mL

492   ultrapure LPS-B5 (055:B5, InvivoGen) for 4 hours. AIM2 inflammasome activation was

493   achieved by transfecting 0.4 ug poly(dA:dT) (InvivoGen) per 10^5 cells. In separate tubes

494   poly(dA:dT) and linear polyethylenimine (Polyscience, 1 ug per 10^5 cells) were mixed with

495   OptiMEM by vortexing and left for 3 min at room temperature. Then poly(dA:dT) and PEI were

496   mixed together, vortexed shortly and left for 15 min before adding a quarter of the total volume

497   on top of the cells. Transfection was facilitated by spinning cells for 5 min at 300 g at 37°C.

498   *Salmonella enterica* serovar Typhimurium SL1344 and *Francisella tularensis* subsp. *novicida*

499   U112 (*F. novicida*) infection were done in OptiMEM. For *S.* Typhimurium infection bacteria

500   were grown overnight and subcultured 1/40 for 3.5 hours in Luria low salt broth (LB low salt)

501   supplemented with appropriate antibiotics, whereas infection with *Francisella* were done from

502   the overnight culture grown in brain heart infusion (BHI) broth supplemented with 0.2% L-

503   cysteine (Sigma) and appropriate antibiotics. Bacteria were then added on top of the cells in

504 OptiMEM, spun at 300 g for 5 min and incubated at 37°C for the duration of the experiment or

505 extracellular bacterial growth supressed by addition of gentamycin at 30 min, 120 min post

506 infection for *S.* Typhimurium and *F. novicida* respectively. For the NLRP3 inflammasome

507 activation, LPS-B5 (055:B5, InvivoGen) priming was done in OptiMEM for 4 hours prior to

508 addition of 5 μM nigericin (Sigma) and incubated for indicated time. Similarly, cells were primed

509 with 100 ng/ml LPS LPS-B5 (055:B5, InvivoGen) for 4 hours in OptiMEM. LPS/FuGeneHD

510 complexes were prepared by mixing 100 μl Opti-MEM with 2 μg ultrapure LPS O111:B4

511 (InvivoGen) and 0.5 μl of FugeneHD (Sigma) per well to be transfected. The transfection

512 mixture was vortexed briefly, incubated for 10 minutes at room temperature and added

513 dropwise to the cells. Plates were centrifuged for 5 minutes at 200 g and 37 °C. Extrinsic

514 apoptosis was induced by adding 100ng/ml TNF-α and the SMAC mimetic AZD5582 at the

515 indicated concentration. Intrinsic apoptosis was induced by addition of the BH3 mimetic small

516 molecule inhibitor ABT-737 in combination with the Mcl-1 inhibitor S63845 at the indicated

517 concentrations.

518 **Cell death and cytokine release measurement.** Cell lysis was assessed by quantifying the

519 amount of lactate dehydrogenase in the cell supernatant using the LDH cytotoxicity kit (Takara)

520 according to the manufacturer's instructions. To measure cell permeabilization, propidium

521 iodide (Thermo Fisher Scientific) was added to the medium at 12.5 μg/ml and fluorescent

522 emission measured by Cytation5 (Biotek) over time. LDH and PI uptake were normalized to

523 untreated control and 100% lysis. Cytokine release into the supernatant in particular

524 interleukin-1β was measured by Elisa (ThermoFisher Scientifc) according to the

525 manufacturer's instructions.

526 **DNA fragmentation Assay.** DNA fragmentation during apoptosis and pyroptosis was

527 assessed by agarose gel electrophoresis as described before (Kasibhatla et al., 2006). In brief

528 *Gsdmd*$^{-/-}$ BMDMs were seeded in a 12-well plate and treated with apoptotic triggers or

529 transfected with poly(dA:dT) as described under cell death assays.

530 **Cell lysis and immunoblotting.** After treatment of cells, cell supernatant was collected and

531 1x sample buffer (Thermo Fisher) complemented with 66 nM Tris, 2% SDS was added to the

532 cell lysate. The proteins of the supernatant were precipitated on ice using and end

533 concentration of 4% TCA (wt./vol) for 30 min. Supernatant was then spun down at 20 000g for

534 20 min at 4°C washed with 100% Aceton and centrifuged at 20 000 g for 20 min at 4°C. The

535 protein pellet was air-dried and resuspended with the lysate. The samples were boiled for 10

536 min at 70°C and separated by a 10- or 12% SDS page gel. The transfer to the 0.2 uM PVDF

537 membranes was accomplished by Trans-Blot Turbo system. Membranes were blocked with

538 5% milk in TBS-T and incubates with the primary antibody for 2 hours at RT or overnight.

539 Membranes were washed 3 times with TBS-T and HRP-coupled antibodies added in 5% milk

in TBST-T for 1 hour. After washing, membranes were revealed by Fusion imager (VILBER) using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) or Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific).

Live cell imaging. BMDM or iBMDM were seeded 5×10^4/well in 8-well tissue culture treated μ-slides (iBidi) or 96-Well Cell Culture Microplates, *μClear®* (Greiner-Bio One) overnight and primed the next day with 100 ng/ml LPS 055:B5 for 4 hours. The AIM2 inflammasome was activated by transfection of poy(dA:dT) (see cell death assay). For time-lapse microscopy cells were incubated with CellTox Green (Promega) 1: 10 000 and AnnexinV (Biolegend) at at 500ng/ml or for mitochondrial health assessment MitotrackerGreen and MitotrackerCmXRos were added to OptiMEM at a final concentration of 125 nM. Images were taken every 5 min. or every 15min. respectively. Zeiss LSM800 point scanning confocal microscope equipped with 63× Plan-Apochromat NA 1.4 oil objective, Zeiss ESID detector module, LabTek heating/ CO_2 chamber and motorized scanning stage

Slice-SILAC. *Gsdmd-* and *Asc*-deficient iBMDMs were grown in SILAC DMEM (Thermo Scientific) medium supplemented with 10% dialyzed FBS, 200 mg/ml proline, 150 mg/ml heavy or light lysine and 50 mg/ml arginine respectively. Cells were passaged 5-6 times until 100% labelling was achieved. For the experiment cells were seeded at 5×10^5/well in 12-well plates overnight and primed the next day with 100 ng/ml LPS 055:B5 for 4h. Poly(dA:dT) transfection was then carried out as described under Cell Death Assays and plates incubated for 3h. Cell were scraped in OptiMEM and proteins precipitated by 4% TCA. The obtained protein pellet was then resuspended in FASP buffer (4% SDS, 0.1 M DTT, 100 mM Tris pH 7.5) heated for 5min. at 95°C, sonicated and cleared by 10min. centrifugation at 13 000 rpm. Downstream sample preparation including SDS gel preparation, mass spectrometry as well as data analysis have been described before (Di Micco et al., 2016).

Caspase-activity assay. Caspase-3/7 activity was either measured by luminescence using the Caspase-Glo® 3/7 (Promega) according to the manufacturer's protocol or by fluorescence. The caspase-activity assay was performed as follows using the fluorescent substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Sigma-Aldrich): Cells were lysed cells directly in the medium by adding 5x Lysis buffer (250 mM Hepes, 25 mM CHAPS, 25 mM DTT) and pipetting up and down. 30 μl of lysed cells was incubated with 30 ul of 2x Assay buffer (40 mM Hepes, 200 mM NaCl, 2mM EDTA, 0.2% CHAPS 20% Sucrose, 20 mM DTT) and 50 μM final concentration of substrate in black opaque OptiPlate-96 (PerkinElmer) and read at 400/505 at 37°C every 2 min. for 10 min.

Metabolic activity – ATP content. Metabolic activity was measured by TiterGlo (Promega) according to the manufacturer's protocol. In brief cells plus 25 μl supernatant were incubated

575　　with 25 μl TiterGlo shook for 2min at 600 rpm and incubated for 10 min. at room temperature

576　　before reading.

577　　**In vitro caspase-cleavage assay.** Active recombinant caspase-1 was purified as described

578　　before (Sborgi et al., 2016). For the *in vitro* cleavage Assay cell lysate from i*Gsdmd*$^{-/-}$/*Casp-*

579　　*3*$^{-/-}$/*Casp-7*$^{-/-}$ was prepared as described before (Boucher et al., 2012). Briefly, cells were lysed

580　　in ice-cold buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1% Igepal) and incubated on ice for

581　　30 min. Cellular proteins were recovered by centrifugation at 7,000 × g for 10 min and kept at

582　　−80 °C in 30 μL aliquots. Purified active caspase-1 (50 nM, 100 nM) was added to cell lysate

583　　and incubated for 2 h at 37°C. The mixture was then analysed by immunoblot.

584

593   AUTHORS CONTRIBUTIONS

594   RH designed, performed and analysed all experiments with the exception of the time-lapse
595   microscopy in SFig. 2, *in vitro* cleavage studies and experiments in SFig. 1A, Fig. 6G that were
596   performed by KS, DB, BD, and KWC respectively. MD and DH contributed to the generation
597   of CRISPR knockouts. RH and PB designed the study and wrote the paper.

598

599   CONFLICT OF INTEREST

600   The authors declare no commercial or financial conflict of interest.

601

REFERENCES

602   Aglietti, R.A., A. Estevez, A. Gupta, M.G. Ramirez, P.S. Liu, N. Kayagaki, C. Ciferri, V.M.
603   Dixit, and E.C. Dueber. 2016. GsdmD p30 elicited by caspase-11 during pyroptosis
604   forms pores in membranes. *Proc. Natl. Acad. Sci.* 113:7858–63.
605   doi:10.1073/pnas.1607769113.

606   Blasi, E., B.J. Mathieson, L. Varesio, J.L. Cleveland, P.A. Borchert, and U.R. Rapp. 1985.
607   Selective immortalization of murine macrophages from fresh bone marrow by a raf/myc
608   recombinant murine retrovirus. *Nature.* 318:667–670. doi:10.1038/318667a0.

609   Bock, F.J., and S.W.G. Tait. 2019. Mitochondria as multifaceted regulators of cell death. *Nat.*
610   *Rev. Mol. Cell Biol.* 21:85–100. doi:10.1038/s41580-019-0173-8.

611   Boucher, D., V. Blais, and J.-B. Denault. 2012. Caspase-7 uses an exosite to promote
612   poly(ADP ribose) polymerase 1 proteolysis. *Proc. Natl. Acad. Sci.*
613   doi:10.1073/pnas.1200934109.

614   Bratton, S.B., J. Lewis, M. Butterworth, C.S. Duckett, and G.M. Cohen. 2002. XIAP inhibition
615   of caspase-3 preserves its association with the Apaf-1 apoptosome and prevents CD95-
616   and Bax-induced apoptosis. *Cell Death Differ.* 9:881–892. doi:10.1038/sj.cdd.4401069.

617   Broz, P., and V.M. Dixit. 2016. Inflammasomes: mechanism of assembly, regulation and
618   signalling. *Nat. Rev. Immunol.* 16:407–20. doi:10.1038/nri.2016.58.

619   Broz, P., J. Von Moltke, J.W. Jones, R.E. Vance, and D.M. Monack. 2010. Differential
620   requirement for caspase-1 autoproteolysis in pathogen-induced cell death and cytokine
621   processing. *Cell Host Microbe*. 8:471–483. doi:10.1016/j.chom.2010.11.007.

622   Chen, K.W., B. Demarco, R. Heilig, K. Shkarina, A. Boettcher, C.J. Farady, P. Pelczar, and
623   P. Broz. 2019. Extrinsic and intrinsic apoptosis activate pannexin-1 to drive NLRP3
624   inflammasome assembly. *EMBO J.* 38:e101638. doi:10.15252/embj.2019101638.

625   Chen, M., Y. Xing, A. Lu, W. Fang, B. Sun, C. Chen, W. Liao, and G. Meng. 2015.
626   Internalized Cryptococcus neoformans Activates the Canonical Caspase-1 and the
627   Noncanonical Caspase-8 Inflammasomes . *J. Immunol.* 195:4962–4972.
628   doi:10.4049/jimmunol.1500865.

629   Cho, Y.S., S. Challa, D. Moquin, R. Genga, T.D. Ray, M. Guildford, and F.K.-M.M. Chan.
630   2009. Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates
631   Programmed Necrosis and Virus-Induced Inflammation. *Cell*. 137:1112–1123.
632   doi:10.1016/j.cell.2009.05.037.

633   Chwieralski, C.E., T. Welte, and F. Bühling. 2006. Cathepsin-regulated apoptosis. *Apoptosis.*
634   11:143–149. doi:10.1007/s10495-006-3486-y.

635   Cuda, C.M., A. V. Misharin, S. Khare, R. Saber, F.N. Tsai, A.M. Archer, P.J. Homan, G.K.
636   Haines, J. Hutcheson, A. Dorfleutner, G.R.S. Budinger, C. Stehlik, and H. Perlman.
637   2015. Conditional deletion of caspase-8 in macrophages alters macrophage activation
638   in a RIPK-dependent manner. *Arthritis Res. Ther.* 17:1–16. doi:10.1186/s13075-015-
639   0794-z.

640   Deveraux, Q.L., R. Takahashi, G.S. Salvesen, and J.C. Reed. 1997. X-linked IAP is a direct
641   inhibitor of cell-death proteases. *Nature.* 388:300–304. doi:10.1038/40901.

642   Ding, J., K. Wang, W. Liu, Y. She, Q. Sun, J. Shi, H. Sun, D. Wang, and F. Shao. 2016.
643   Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature.*
644   535:111–116. doi:10.1038/nature18590.

645   Du, C., M. Fang, Y. Li, L. Li, and X. Wang. 2000. Smac, a Mitochondrial Protein that
646   Promotes Cytochrome c–Dependent Caspase Activation by Eliminating IAP Inhibition.
647   *Cell.* 102:33–42. doi:10.1016/S0092-8674(00)00008-8.

648   Feoktistova, M., P. Geserick, B. Kellert, D.P. Dimitrova, C. Langlais, M. Hupe, K. Cain, M.
649   MacFarlane, G. Häcker, and M. Leverkus. 2011. CIAPs Block Ripoptosome Formation,
650   a RIP1/Caspase-8 Containing Intracellular Cell Death Complex Differentially Regulated
651   by cFLIP Isoforms. *Mol. Cell.* 43:449–463. doi:10.1016/j.molcel.2011.06.011.

652   Fu, T.M., Y. Li, A. Lu, Z. Li, P.R. Vajjhala, A.C. Cruz, D.B. Srivastava, F. DiMaio, P.A.
653   Penczek, R.M. Siegel, K.J. Stacey, E.H. Egelman, and H. Wu. 2016. Cryo-EM Structure
654   of Caspase-8 Tandem DED Filament Reveals Assembly and Regulation Mechanisms of
655   the Death-Inducing Signaling Complex. *Mol. Cell.* 64:236–250.
656   doi:10.1016/j.molcel.2016.09.009.

657   Hagar, J.A., D.A. Powell, Y. Aachoui, R.K. Ernst, and E.A. Miao. 2013. Cytoplasmic LPS
658   activates caspase-11: Implications in TLR4-independent endotoxic shock. *Science (80-.*
659   *).* 341:1250–1253. doi:10.1126/science.1240988.

660   He, S., L. Wang, L. Miao, T. Wang, F. Du, L. Zhao, and X. Wang. 2009. Receptor Interacting
661   Protein Kinase-3 Determines Cellular Necrotic Response to TNF-α. *Cell.* 137:1100–
662   1111. doi:10.1016/j.cell.2009.05.021.

663   He, W., H. Wan, L. Hu, P. Chen, X. Wang, Z. Huang, Z.-H. Yang, C.-Q. Zhong, and J. Han.
664   2015. Gasdermin D is an executor of pyroptosis and required for interleukin-1β
665   secretion. *Cell Res.* 25:1285–98. doi:10.1038/cr.2015.139.

666   Henry, C.M., and S.J. Martin. 2017. Caspase-8 Acts in a Non-enzymatic Role as a Scaffold
667   for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation.
668   *Mol. Cell*. 65:715-729.e5. doi:10.1016/j.molcel.2017.01.022.

669   Jost, P.J., S. Grabow, D. Gray, M.D. McKenzie, U. Nachbur, D.C.S. Huang, P. Bouillet, H.E.
670   Thomas, C. Borner, J. Silke, A. Strasser, and T. Kaufmann. 2009. XIAP discriminates
671   between type i and type II FAS-induced apoptosis. *Nature*. 460:1035–1039.
672   doi:10.1038/nature08229.

673   Kaiser, W.J., J.W. Upton, A.B. Long, D. Livingston-Rosanoff, L.P. Daley-Bauer, R. Hakem, T.
674   Caspary, and E.S. Mocarski. 2011. RIP3 mediates the embryonic lethality of caspase-8-
675   deficient mice. *Nature*. 471:368–373. doi:10.1038/nature09857.

676   Kang, T.-B., T. Ben-Moshe, E.E. Varfolomeev, Y. Pewzner-Jung, N. Yogev, A. Jurewicz, A.
677   Waisman, O. Brenner, R. Haffner, E. Gustafsson, P. Ramakrishnan, T. Lapidot, and D.
678   Wallach. 2004. Caspase-8 Serves Both Apoptotic and Nonapoptotic Roles. *J. Immunol.*
679   173:2976–2984. doi:10.4049/jimmunol.173.5.2976.

680   Kang, T.B., J.S. Jeong, S.H. Yang, A. Kovalenko, and D. Wallach. 2018. Caspase-8
681   deficiency in mouse embryos triggers chronic RIPK1-dependent activation of
682   inflammatory genes, independently of RIPK3. *Cell Death Differ.* 25:1107–1117.
683   doi:10.1038/s41418-018-0104-9.

684   Kanneganti, A., R.K.S. Malireddi, P.H. V Saavedra, L. Vande Walle, H. Van Gorp, H.
685   Kambara, H. Tillman, P. Vogel, H.R. Luo, R.J. Xavier, H. Chi, and M. Lamkanfi. 2018a.
686   GSDMD is critical for autoinflammatory pathology in a mouse model of Familial
687   Mediterranean Fever. *J. Exp. Med.* 215:1519–1529. doi:10.1084/jem.20172060.

688   Kanneganti, T.-D., Q. Zhu, M. Zheng, A. Balakrishnan, and R. Karki. 2018b. Francisella
689   novicida Protection against Activation and Is Required for Host Gasdermin D Promotes
690   AIM2 Inflammasome. doi:10.4049/jimmunol.1800788.

691   Kasibhatla, S., G.P. Amarante-Mendes, D. Finucane, T. Brunner, E. Bossy-Wetzel, and D.R.
692   Green. 2006. Analysis of DNA Fragmentation Using Agarose Gel Electrophoresis. *Cold
693   Spring Harb. Protoc.* 2006:pdb.prot4429. doi:10.1101/pdb.prot4429.

694   Kavanagh, E., J. Rodhe, M.A. Burguillos, J.L. Venero, and B. Joseph. 2014. Regulation of
695   caspase-3 processing by cIAP2 controls the switch between pro-inflammatory activation
696   and cell death in microglia. *Cell Death Dis.* 5:e1565–e1565.
697   doi:10.1038/cddis.2014.514.

698   Kayagaki, N., I.B. Stowe, B.L. Lee, K. O'Rourke, K. Anderson, S. Warming, T. Cuellar, B.
699   Haley, M. Roose-Girma, Q.T. Phung, P.S. Liu, J.R. Lill, H. Li, J. Wu, S. Kummerfeld, J.

700 Zhang, W.P. Lee, S.J. Snipas, G.S. Salvesen, L.X. Morris, L. Fitzgerald, Y. Zhang, E.M.
701 Bertram, C.C. Goodnow, and V.M. Dixit. 2015. Caspase-11 cleaves gasdermin D for
702 non-canonical inflammasome signalling. *Nature*. 526:666–671.
703 doi:10.1038/nature15541.

704 Kayagaki, N., S. Warming, M. Lamkanfi, L. Vande Walle, S. Louie, J. Dong, K. Newton, Y.
705 Qu, J. Liu, S. Heldens, J. Zhang, W.P. Lee, M. Roose-Girma, and V.M. Dixit. 2011. Non-
706 canonical inflammasome activation targets caspase-11. *Nature*. 479:117–121.
707 doi:10.1038/nature10558.

708 Kayagaki, N., M.T. Wong, I.B. Stowe, S.R. Ramani, L.C. Gonzalez, S. Akashi-takamura, K.
709 Miyake, J. Zhang, W.P. Lee, L.S. Forsberg, R.W. Carlson, and V.M. Dixit. 2013.
710 Independent of TLR4. *Science (80-.).* 130:1246–1249. doi:10.5061/dryad.bt51g.

711 Lamkanfi, M., and T.-D. Kanneganti. 2010. Caspase-7: a protease involved in apoptosis and
712 inflammation. *Int. J. Biochem. Cell Biol.* 42:21–4. doi:10.1016/j.biocel.2009.09.013.

713 Lamkanfi, M., T.-D. Kanneganti, P. Van Damme, T. Vanden Berghe, I. Vanoverberghe, J.
714 Vandekerckhove, P. Vandenabeele, K. Gevaert, and G. Núñez. 2008. Targeted
715 peptidecentric proteomics reveals caspase-7 as a substrate of the caspase-1
716 inflammasomes. *Mol. Cell. Proteomics*. 7:2350–63. doi:10.1074/mcp.M800132-
717 MCP200.

718 Lee, B.L., K.M. Mirrashidi, I.B. Stowe, S.K. Kummerfeld, C. Watanabe, B. Haley, T.L. Cuellar,
719 M. Reichelt, and N. Kayagaki. 2018. ASC- and caspase-8-dependent apoptotic pathway
720 diverges from the NLRC4 inflammasome in macrophages. *Sci. Rep.* 8:3788.
721 doi:10.1038/s41598-018-21998-3.

722 Li, H., H. Zhu, C. Xu, and J. Yuan. 1998. Cleavage of BID by Caspase 8 Mediates the
723 Mitochondrial Damage in the Fas Pathway of Apoptosis. *Cell*. 94:491–501.
724 doi:10.1016/S0092-8674(00)81590-1.

725 Li, M.L., and V. Stollar. 2004. Alphaviruses and apoptosis. *Int. Rev. Immunol.* 23:7–24.
726 doi:10.1080/08830180490265529.

727 Liu, X., Z. Zhang, J. Ruan, Y. Pan, V.G. Magupalli, H. Wu, and J. Lieberman. 2016.
728 Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores.
729 *Nature*. 535:153–158. doi:10.1038/nature18629.

730 Maelfait, J., E. Vercammen, S. Janssens, P. Schotte, M. Haegman, S. Magez, and R.
731 Beyaert. 2008. Stimulation of Toll-like receptor 3 and 4 induces interleukin-1beta
732 maturation by caspase-8. *J. Exp. Med.* 205:1967–73. doi:10.1084/jem.20071632.

733 Man, S.M., P. Tourlomousis, L. Hopkins, T.P. Monie, K.A. Fitzgerald, and C.E. Bryant. 2013.
734   Salmonella Infection Induces Recruitment of Caspase-8 to the Inflammasome To
735   Modulate IL-1β Production . *J. Immunol.* 191:5239–5246.
736   doi:10.4049/jimmunol.1301581.

737 Mariathasan, S., K. Hewton, D.M. Monack, D. Vucic, D.M. French, W.P. Lee, M. Roose-
738   Girma, S. Erickson, and V.M. Dixit. 2004. Differential activation of the inflammasome by
739   caspase-1 adaptors ASC and Ipaf. *Nature*. 430:213–218. doi:10.1038/nature02664.

740 Mascarenhas, D.P.A., D.M. Cerqueira, M.S.F. Pereira, F.V.S. Castanheira, T.D. Fernandes,
741   G.Z. Manin, L.D. Cunha, and D.S. Zamboni. 2017. Inhibition of caspase-1 or gasdermin-
742   D enable caspase-8 activation in the Naip5/NLRC4/ASC inflammasome. *PLOS Pathog.*
743   13:e1006502. doi:10.1371/journal.ppat.1006502.

744 Masters, S.L., A. Simon, I. Aksentijevich, and D.L. Kastner. 2009. Horror Autoinflammaticus :
745   The Molecular Pathophysiology of Autoinflammatory Disease. *Annu. Rev. Immunol.*
746   27:621–668. doi:10.1146/annurev.immunol.25.022106.141627.

747 Di Micco, A., G. Frera, J. Lugrin, Y. Jamilloux, E.-T. Hsu, A. Tardivel, A. De Gassart, L.
748   Zaffalon, B. Bujisic, S. Siegert, M. Quadroni, P. Broz, T. Henry, C.A. Hrycyna, and F.
749   Martinon. 2016. AIM2 inflammasome is activated by pharmacological disruption of
750   nuclear envelope integrity. *Proc. Natl. Acad. Sci. U. S. A.* 113:E4671-80.
751   doi:10.1073/pnas.1602419113.

752 Momeni, H.R. 2011. Role of calpain in apoptosis. *Cell J.* 13:65–72.

753 Monteleone, M., A.C. Stanley, K.W. Chen, M.J. Sweet, J.L. Stow, K.S. Correspondence, D.L.
754   Brown, J.S. Bezbradica, J.B. Von Pein, C.L. Holley, D. Boucher, M.R. Shakespear, R.
755   Kapetanovic, V. Rolfes, K. Schroder, M.J. Sweet, J.L. Stow, and K. Schroder. 2018.
756   Interleukin-1β Maturation Triggers Its Relocation to the Plasma Membrane for
757   Gasdermin-D-Dependent and -Independent Secretion. *Cell Rep.* 24:1425–1433.
758   doi:10.1016/j.celrep.2018.07.027.

759 Nailwal, H., and F.K.M. Chan. 2019. Necroptosis in anti-viral inflammation. *Cell Death Differ.*
760   26:4–13. doi:10.1038/s41418-018-0172-x.

761 Oberst, A., C.P. Dillon, R. Weinlich, L.L. McCormick, P. Fitzgerald, C. Pop, R. Hakem, G.S.
762   Salvesen, and D.R. Green. 2011. Catalytic activity of the caspase-8-FLIP L complex
763   inhibits RIPK3-dependent necrosis. *Nature*. 471:363–368. doi:10.1038/nature09852.

764 Ong, S.E., B. Blagoev, I. Kratchmarova, D.B. Kristensen, H. Steen, A. Pandey, and M. Mann.
765   2002. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and
766   accurate approach to expression proteomics. *Mol. Cell. Proteomics*. 1:376–386.

767   doi:10.1074/mcp.M200025-MCP200.

768 Van Opdenbosch, N., H. Van Gorp, M. Verdonckt, P.H.V. Saavedra, N.M. de Vasconcelos,
769   A. Gonçalves, L. Vande Walle, D. Demon, M. Matusiak, F. Van Hauwermeiren, J.
770   D'Hont, T. Hochepied, S. Krautwald, T.-D. Kanneganti, M. Lamkanfi, N. Van
771   Opdenbosch, H. Van Gorp, M. Verdonckt, H. V Pedro, V. Walle, D. Demon, M.
772   Matusiak, F. Van Hauwermeiren, D. Hont, T. Hochepied, S. Krautwald, and T.-D.
773   Kanneganti. 2017. Caspase-1 Engagement and TLR-Induced c-FLIP Expression
774   Suppress ASC/Caspase-8-Dependent Apoptosis by Inflammasome Sensors NLRP1b
775   and NLRC4. *Cell Rep.* 21:3427–3444. doi:10.1016/J.CELREP.2017.11.088.

776 Orning, P., D. Weng, K. Starheim, D. Ratner, Z. Best, B. Lee, A. Brooks, S. Xia, H. Wu, M.A.
777   Kelliher, S.B. Berger, P.J. Gough, J. Bertin, M.M. Proulx, J.D. Goguen, N. Kayagaki,
778   K.A. Fitzgerald, and E. Lien. 2018. Pathogen blockade of TAK1 triggers caspase-8–
779   dependent cleavage of gasdermin D and cell death. *Science (80-.).* 2818:eaau2818.
780   doi:10.1126/science.aau2818.

781 Pierini, R., C. Juruj, M. Perret, C.L. Jones, P. Mangeot, D.S. Weiss, and T. Henry. 2012.
782   AIM2/ASC triggers caspase-8-dependent apoptosis in Francisella-infected caspase-1-
783   deficient macrophages. *Cell Death Differ.* 19:1709–1721. doi:10.1038/cdd.2012.51.

784 Rashidi, M., D.S. Simpson, A. Hempel, D. Frank, E. Petrie, A. Vince, R. Feltham, J. Murphy,
785   S.M. Chatfield, G.S. Salvesen, J.M. Murphy, I.P. Wicks, and J.E. Vince. 2019. The
786   Pyroptotic Cell Death Effector Gasdermin D Is Activated by Gout-Associated Uric Acid
787   Crystals but Is Dispensable for Cell Death and IL-1β Release. *J. Immunol.* 203:736–
788   748. doi:10.4049/jimmunol.1900228.

789 Rathkey, J.K., J. Zhao, Z. Liu, Y. Chen, J. Yang, H.C. Kondolf, B.L. Benson, S.M.
790   Chirieleison, A.Y. Huang, G.R. Dubyak, T.S. Xiao, X. Li, and D.W. Abbott. 2018.
791   Chemical disruption of the pyroptotic pore-forming protein gasdermin D inhibits
792   inflammatory cell death and sepsis. *Sci. Immunol.* 3:eaat2738.
793   doi:10.1126/sciimmunol.aat2738.

794 Roy, N., Q.L. Deveraux, R. Takahashi, G.S. Salvesen, J.C. Reed, M. Robertson, T. Ghayur,
795   W.W. Wong, R. Kamen, and R. Weichselbaum. 1997. The c-IAP-1 and c-IAP-2 proteins
796   are direct inhibitors of specific caspases. *EMBO J.* 16:6914–25.
797   doi:10.1093/emboj/16.23.6914.

798 Sagulenko, V., S.J. Thygesen, D.P. Sester, A. Idris, J.A. Cridland, P.R. Vajjhala, T.L.
799   Roberts, K. Schroder, J.E. Vince, J.M. Hill, J. Silke, and K.J. Stacey. 2013. AIM2 and
800   NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC.
801   *Cell Death Differ.* 20:1149–1160. doi:10.1038/cdd.2013.37.

802 Sagulenko, V., N. Vitak, P.R. Vajjhala, J.E. Vince, and K.J. Stacey. 2018. Caspase-1 Is an
803   Apical Caspase Leading to Caspase-3 Cleavage in the AIM2 Inflammasome Response,
804   Independent of Caspase-8. *J. Mol. Biol.* 430:238–247. doi:10.1016/J.JMB.2017.10.028.

805 Sarhan, J., B.C. Liu, H.I. Muendlein, P. Li, R. Nilson, A.Y. Tang, A. Rongvaux, S.C. Bunnell,
806   F. Shao, D.R. Green, and A. Poltorak. 2018. Caspase-8 induces cleavage of gasdermin
807   D to elicit pyroptosis during Yersinia infection [Immunology and Inflammation]. *Proc.*
808   *Natl. Acad. Sci. U. S. A.* doi:10.1073/pnas.1809548115.

809 Sborgi, L., S. Rühl, E. Mulvihill, J. Pipercevic, R. Heilig, H. Stahlberg, C. Farady, D. Müller, P.
810   Broz, and S. Hiller. 2016. GSDMD membrane pore formation constitutes the mechanism
811   of pyroptotic cell death. *EMBO J.* 35:e201694696-13. doi:10.15252/embj.201694696.

812 Schneider, K.S., C.J. Groß, R.F. Dreier, B.S. Saller, R. Mishra, O. Gorka, R. Heilig, E.
813   Meunier, M.S. Dick, T. Ćiković, J. Sodenkamp, G. Médard, R. Naumann, J. Ruland, B.
814   Kuster, P. Broz, and O. Groß. 2017. The Inflammasome Drives GSDMD-Independent
815   Secondary Pyroptosis and IL-1 Release in the Absence of Caspase-1 Protease Activity.
816   *Cell Rep.* 21:3846–3859. doi:10.1016/J.CELREP.2017.12.018.

817 Scott, F.L., J.B. Denault, S.J. Riedl, H. Shin, M. Renatus, and G.S. Salvesen. 2005. XIAP
818   inhibits caspase-3 and -7 using two binding sites: Evolutionary conserved mechanism of
819   IAPs. *EMBO J.* 24:645–655. doi:10.1038/sj.emboj.7600544.

820 Shi, J., Y. Zhao, K. Wang, X. Shi, Y. Wang, H. Huang, Y. Zhuang, T. Cai, F. Wang, and F.
821   Shao. 2015. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell
822   death. *Nature.* 526:660–665. doi:10.1038/nature15514.

823 Shi, J., Y. Zhao, Y. Wang, W. Gao, J. Ding, P. Li, L. Hu, and F. Shao. 2014. Inflammatory
824   caspases are innate immune receptors for intracellular LPS. *Nature.* 514:187–192.
825   doi:10.1038/nature13683.

826 Sollberger, G., A. Choidas, G.L. Burn, P. Habenberger, R. Di Lucrezia, S. Kordes, S.
827   Menninger, J. Eickhoff, P. Nussbaumer, B. Klebl, R. Krüger, A. Herzig, and A.
828   Zychlinsky. 2018. Gasdermin D plays a vital role in the generation of neutrophil
829   extracellular traps. 3. Science Immunology. 6689 pp.

830 Stennicke, H.R., J.M. Jü rgensmeier, H. Shin, Q. Deveraux, B.B. Wolf, X. Yang, Q. Zhou, H.
831   Michael Ellerby, L.M. Ellerby, D. Bredesen, D.R. Green, J.C. Reed, C.J. Froelich, and
832   G.S. Salvesen. 1998. Pro-caspase-3 Is a Major Physiologic Target of Caspase-8*.

833 Taabazuing, C.Y., M.C. Okondo, and D.A. Bachovchin. 2017. Pyroptosis and Apoptosis
834   Pathways Engage in Bidirectional Crosstalk in Monocytes and Macrophages. *Cell*
835   *Chem. Biol.* 24:507-514.e4. doi:10.1016/J.CHEMBIOL.2017.03.009.

836   Takahashi, R., Q. Deveraux, I. Tamm, K. Welsh, N. Assa-Munt, G.S. Salvesen, and J.C.
837   Reed. 1998. A single BIR domain of XIAP sufficient for inhibiting caspases. *J. Biol.*
838   *Chem.* 273:7787–90. doi:10.1074/JBC.273.14.7787.

839   Taxman, D.J., M.T.H. Huang, and J.P.Y. Ting. 2010. Inflammasome inhibition as a
840   pathogenic stealth mechanism. *Cell Host Microbe*. 8:7–11.
841   doi:10.1016/j.chom.2010.06.005.

842   Tenev, T., K. Bianchi, M. Darding, M. Broemer, C. Langlais, F. Wallberg, A. Zachariou, J.
843   Lopez, M. MacFarlane, K. Cain, and P. Meier. 2011. The Ripoptosome, a Signaling
844   Platform that Assembles in Response to Genotoxic Stress and Loss of IAPs. *Mol. Cell*.
845   43:432–448. doi:10.1016/J.MOLCEL.2011.06.006.

846   Tsuchiya, K., S. Nakajima, S. Hosojima, D. Thi Nguyen, T. Hattori, T. Manh Le, O. Hori, M.R.
847   Mahib, Y. Yamaguchi, M. Miura, T. Kinoshita, H. Kushiyama, M. Sakurai, T. Shiroishi,
848   and T. Suda. 2019. Caspase-1 initiates apoptosis in the absence of gasdermin D. *Nat.*
849   *Commun.* 10:2091. doi:10.1038/s41467-019-09753-2.

850   Vajjhala, P.R., A. Lu, D.L. Brown, S.W. Pang, V. Sagulenko, D.P. Sester, S.O. Cridland, J.M.
851   Hill, K. Schroder, J.L. Stow, H. Wu, and K.J. Stacey. 2015. The Inflammasome Adaptor
852   ASC Induces Procaspase-8 Death Effector Domain Filaments. *J. Biol. Chem.*
853   290:29217–30. doi:10.1074/jbc.M115.687731.

854   Verhagen, A.M., P.G. Ekert, M. Pakusch, J. Silke, L.M. Connolly, G.E. Reid, R.L. Moritz, R.J.
855   Simpson, and D.L. Vaux. 2000. Identification of DIABLO, a mammalian protein that
856   promotes apoptosis by binding to and antagonizing IAP proteins. *Cell*. 102:43–53.
857   doi:10.1016/S0092-8674(00)00009-X.

858   Vince, J.E., D. De Nardo, W. Gao, A.J. Vince, C. Hall, K. McArthur, D. Simpson, S. Vijayaraj,
859   L.M. Lindqvist, P. Bouillet, M.A. Rizzacasa, S.M. Man, J. Silke, S.L. Masters, G.
860   Lessene, D.C.S. Huang, D.H.D. Gray, B.T. Kile, F. Shao, and K.E. Lawlor. 2018. The
861   Mitochondrial Apoptotic Effectors BAX/BAK Activate Caspase-3 and -7 to Trigger
862   NLRP3 Inflammasome and Caspase-8 Driven IL-1β Activation. *Cell Rep.* 25:2339-
863   2353.e4. doi:10.1016/j.celrep.2018.10.103.

864   Walsh, J.G., S.P. Cullen, C. Sheridan, A.U. Lüthi, C. Gerner, and S.J. Martin. 2008.
865   Executioner caspase-3 and caspase-7 are functionally distinct proteases. *Proc. Natl.*
866   *Acad. Sci. U. S. A.* 105:12815–12819. doi:10.1073/pnas.0707715105.

867   Wang, H., X. Lei, X. Xiao, C. Yang, W. Lu, Z. Huang, Q. Leng, Q. Jin, B. He, G. Meng, and J.
868   Wang. 2015. Reciprocal Regulation between Enterovirus 71 and the NLRP3
869   Inflammasome. *Cell Rep.* 12:42–48. doi:10.1016/j.celrep.2015.05.047.

870   Wang, J., K. Deobald, and F. Re. 2019. Bacteria through Pyroptosis and Direct Killing of
871   Gasdermin D Protects from Melioidosis. doi:10.4049/jimmunol.1900045.

872   Wang, W., F. Xiao, P. Wan, P. Pan, Y. Zhang, F. Liu, K. Wu, Y. Liu, and J. Wu. 2017a. EV71
873   3D Protein Binds with NLRP3 and Enhances the Assembly of Inflammasome Complex.
874   doi:10.1371/journal.ppat.1006123.

875   Wang, Y., W. Gao, X. Shi, J. Ding, W. Liu, H. He, K. Wang, and F. Shao. 2017b.
876   Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin.
877   *Nature*. 547:99–103. doi:10.1038/nature22393.

878   Wilkinson, J.C., A.S. Wilkinson, F.L. Scott, R.A. Csomos, G.S. Salvesen, and C.S. Duckettt.
879   2004. Neutralization of Smac/Diablo by inhibitors of apoptosis (IAPs): A caspase-
880   independent mechanism for apoptotic inhibition. *J. Biol. Chem.* 279:51082–51090.
881   doi:10.1074/jbc.M408655200.

882   Wu, G., J. Chai, T.L. Suber, J.W. Wu, C. Du, X. Wang, and Y. Shi. 2000. Structural basis of
883   IAP recognition by Smac/DIABLO. *Nature*. 408:1008–1012. doi:10.1038/35050012.

884   Xiao, J., C. Wang, J.-C. Yao, Y. Alippe, C. Xu, D. Kress, R. Civitelli, Y. Abu-Amer, T.-D.
885   Kanneganti, D.C. Link, and G. Mbalaviele. 2018. Gasdermin D mediates the
886   pathogenesis of neonatal-onset multisystem inflammatory disease in mice. *PLOS Biol.*
887   16:e3000047. doi:10.1371/journal.pbio.3000047.

888   Zhang, D.-W., J. Shao, J. Lin, N. Zhang, B.-J. Lu, S.-C. Lin, M.-Q. Dong, and J. Han. 2009.
889   RIP3, an Energy Metabolism Regulator That Switches TNF-Induced Cell Death from
890   Apoptosis to Necrosis. *Science (80-.).* 2.

891

FIGURE LEGENDS

Figure 1. Canonical inflammasome activation *Gsdmd*-deficient macrophages results in rapid secondary necrosis.

(A-B). LDH release, PI influx and IL-1β release from LPS-primed WT, *Asc$^{-/-}$, Casp1$^{-/-}$/Casp11$^{-/-}$* and *Gsdmd$^{-/-}$* primary or immortalized bone marrow derived macrophages (BMDMs, iBMDMs) after transfection of poly(dA:dT) in absence or presence of the indicated inhibitors. **(C-E)** DNA cleavage, PI influx and immunoblots showing caspase-3/-7 processing from LPS-primed *Gsdmd$^{-/-}$* BMDMs transfected with poly(dA:dT) or treated with 100 ng/ml TNF-α plus 10, 5 or 1 μM AZD5582 (extrinsic apoptosis) or 1 μM ABT-737 plus 10, 1 or 0.5 μM S63845 (intrinsic apoptosis). **(F)** Confocal images of LPS-primed *Gsdmd$^{-/-}$* BMDMs transfected with poly(dA:dT) or left untreated and stained with CellToxGreen (green). Scalebar 10 μM. Graphs show mean ± SD. Data and blot are representative of at least three independent experiments.

Figure 2. Caspase-3 drives GSDMD-independent secondary necrosis in inflammasome activated cells

(A-B). LDH release, caspase-3/-7 activity (DEVDase activity) and immunoblots showing caspase-3/-7 processing from LPS-primed WT, *Asc$^{-/-}$, Casp1$^{-/-}$/Casp11$^{-/-}$* and *Gsdmd$^{-/-}$* primary BMDMs after transfection of poly(dA:dT). **(C)** LDH release from LPS-primed WT, *Asc$^{-/-}$, Casp1$^{-/-}$/Casp11$^{-/-}$, Gsdmd$^{-/-}$, Gsdmd$^{-/-}$/Casp3$^{-/-}$, Gsdmd$^{-/-}$/Casp7$^{-/-}$* and *Gsdmd$^{-/-}$/Casp3$^{-/-}$/Casp7$^{-/-}$* iBMDMs after transfection of poly(dA:dT). **(D)** Confocal images of cells from C. Insets show membrane ballooning in dying cells at 3h post transfection. Scalebar 10 μm. **(E)** Quantification of LDH release in LPS primed WT, *Asc$^{-/-}$, Casp-1$^{-/-}$/Casp-11$^{-/-}$, Gsdmd$^{-/-}$, Gsdme$^{-/-}$, Gsdmd$^{-/-}$/Gsdme$^{-/-}$* BMDMs transfected with poly(dA:dT) for 4 hours. Graphs show mean ± SD. **p ≤ 0.01, ***p ≤ 0.001, "ns" = no significance (Unpaired t-test). Data and blot are representative of at least three independent experiments.

Figure 3: Caspase-1 is required for GSDMD-independent secondary necrosis

(A-C). Immunoblot showing Caspase-1 expression and caspase-3 processing, LDH release and caspase-3/-7 activity (DEVDase activity) from LPS-primed *Gsdmd$^{-/-}$* and *Gsdmd$^{-/-}$/Casp1$^{-/-}$* immortalized BMDMs after transfection of poly(dA:dT). **(D)** PI influx of WT, *Asc$^{-/-}$, Casp1$^{-/-}$/Casp11$^{-/-}$, Casp1$^{-/-}$, Casp1$^{C284A/C284A}$* and *Gsdmd$^{-/-}$* primary BMDMs after transfection of poly(dA:dT). Graphs show mean ± SD. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (Unpaired t-test). Data and blot are representative of at least three independent experiments.

Figure 4: Mitochondrial damage is caused by truncated Bid

(A) LDH release and TiterGlo measurements from LPS-primed WT, *Asc$^{-/-}$, Casp1$^{-/-}$/Casp11$^{-/-}$* and *Gsdmd$^{-/-}$* primary BMDMs after transfection with poly(dA:dT). **(B)** Schematic cleavage

profile of Bcl-2 family members generated from slice SILAC data. Bubble diameters are proportional to the number of quantified peptide matches, whereas the gradient color represents the H/L ratio, as indicated below. The green bubbles (negative log2H/L) represent protein isoforms reduced in *Gsdmd*$^{-/-}$ iBMDMs compared to *Asc*$^{-/-}$ iBMDMs at 3 hours post poly(dA:dT) transfection; the red bubbles (positive log2H/L) represent protein isoforms enriched in *Gsdmd*$^{-/-}$ iBMDMs compared to *Asc*$^{-/-}$ iBMDMs. **(C)** Immunoblots showing Bid processing from LPS-primed WT, *Asc*$^{-/-}$, *Casp1*$^{-/-}$/*Casp11*$^{-/-}$ and *Gsdmd*$^{-/-}$ primary BMDMs after transfection with poly(dA:dT). **(D)** LDH release from WT, *Asc*$^{-/-}$, *Casp1*$^{-/-}$/*Casp11*$^{-/-}$, *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Bid*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). Graphs show mean ± SD. *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001, "ns" = no significance (Unpaired t-test).Data and blots are representative of at least three independent experiments.

Figure 5: Caspase-1 drives Bid processing during GSDMD-independent secondary necrosis

(A) Immunoblots showing caspase-1, -7-, -3, -8 and -9 processing in WT, *Asc*$^{-/-}$, *Casp1*$^{-/-}$/*Casp11*$^{-/-}$ and *Gsdmd*$^{-/-}$ primary BMDMs after transfection with poly(dA:dT). **(B)** LDH release from WT, *Asc*$^{-/-}$, *Casp1*$^{-/-}$/*Casp11*$^{-/-}$, *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Casp8*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(C)** Immunoblots showing caspase-3 processing in *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Casp8*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(D)** Immunoblots showing Bid cleavage in *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Casp8*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(E)** Immunoblots showing Bid cleavage in *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Casp1*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(F)** *In vitro* cleavage assay showing processing of recombinant Bid by recombinant caspase-1. Graphs show mean ± SD. * "ns" = no significance (Unpaired t-test). Data and blot are representative of at least three independent experiments.

Figure 6: SMAC release and initiator caspases-8/-9 are required for GSDMD-independent secondary necrosis

(A) LDH release from WT, *Asc*$^{-/-}$, *Casp1*$^{-/-}$/*Casp11*$^{-/-}$, *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Casp9*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(B)** Immunoblots showing caspase-3 cleavage in *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Casp9*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(C)** LDH release from WT, *Asc*$^{-/-}$, *Casp1*$^{-/-}$/*Casp11*$^{-/-}$, *Gsdmd*$^{-/-}$, *Gsdmd*$^{-/-}$/*Casp8*$^{-/-}$, *Gsdmd*$^{-/-}$/*Casp9*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Casp8*$^{-/-}$/*Casp9*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(D)** Immunoblots showing caspase-3 cleavage in *Gsdmd*$^{-/-}$, *Gsdmd*$^{-/-}$/*Casp8*$^{-/-}$, *Gsdmd*$^{-/-}$/*Casp9*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Casp8*$^{-/-}$/*Casp9*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(E)** Immunoblots showing caspase-3 processing from *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$/*Bid*$^{-/-}$ iBMDMs after transfection with poly(dA:dT). **(F)** Schematic summary of the mechanism of caspase-3 cleavage and activation. **(G)** PI influx in untreated or poly(dA:dT)-transfected *Gsdmd*$^{-/-}$/*Bid*$^{-/-}$ iBMDMs in presence or

960   absence of the SMAC mimetic AZD5582. Graphs show mean ± SD. *p ≤ 0.05, **p ≤ 0.01,

961   (Unpaired t-test). Data and blot are representative of at least three independent experiments.

Figure 7: Model of cell death in *Gsdmd*-deficient myeloid cells after activation of
963   **caspase-1**

964   Model depicting the mechanism of canonical inflammasome activation in WT cells undergoing

965   caspase-1- and GSDMD-dependent pyroptosis and *Gsdmd*−/− cells undergoing caspase-1-

966   induced GSDMD-independent secondary necrosis.

SUPPLEMENTARY FIGURE LEGENDS

967   **Figure S1:**

968   **(A-B)** LDH release from LPS-primed WT, *Asc*−/−, *Casp1*−/−/*Casp11*−/− and *Gsdmd*−/− iBMDMs

969   after transfection of LPS (**A**) or infection with log-phase *S.* Typhimurium, treatment with

970   Nigericin, infection with *F. novicida* or **(B). (C)** PI influx from mock or poly(dA:dT) transfected

971   LPS-primed *Gsdmd*−/− iBMDMs and LDH release from mock or poly(dA:dT) transfected LPS-

972   primed WT, *Asc*−/−, *Casp1*−/−/*Casp11*−/− and *Gsdmd*−/− iBMDMs. **(D)** Immunoblots showing IL-

973   1β, caspase-1 and GSDMD processing in WT, *Asc*−/−, *Casp1*−/−/*Casp11*−/− and *Gsdmd*−/−

974   BMDMs after transfection of poly(dA:dT). Data and blot are representative of at least three

975   independent experiments.

976   **Figure S2:**

977   PI influx from poly(dA:dT) transfected LPS-primed *Gsdmd*−/− iBMDMs in the presence or

978   absence of the indicated inhibitors added to the cells 30 min. prior and during the experiment

979   at the following concentrations: 50, 25, 12.5, 6.25 µM VX765, 100, 50, 25, 12.5 µM Caspase-

980   3/7 specific inhibitor I, 30, 15, 7.5, 3.25 µM K777, 100, 50, 25, 12.5 µM, PD150606 and, 100,

981   50, 25, 12.5 µM Calpeptin, 60, 30, 15, 7.5 µM 7-Cl-O-Nec1, 100, 50, 25, 12.5 µM GSK872.

982   Data and blot are representative of at least three independent experiments.

983   **Figure S3:**

984   **(A-B).** Confocal microscopy images of poly(dA:dT) **(A)** or mock transfected **(B)** WT, *Asc*−/−,

985   *Casp1*−/−/*Casp11*−/− and *Gsdmd*−/− primary BMDMs. Cells were stained with CellToxGreen

986   (green) and AnnexinV (red). **(C)** shows selected cells from **(A).** Scalebar 10 µm.

987   **Figure S4:**

988   **(A)** LDH release and caspase-3/-7 activity (DEVDase activity) from LPS-primed WT, *Asc*−/−,

989   *Casp1*−/−/*Casp11*−/− and *Gsdmd*−/− primary BMDMs after transfection of *Salmonella*

Typhimurium at MOI 10. **(B)** Immunoblots for caspase-3 and -7 expression in lysates of *Gsdmd$^{-/-}$*, *Gsdmd$^{-/-}$/Casp3$^{-/-}$*, *Gsdmd$^{-/-}$/Casp7$^{-/-}$* and *Gsdmd$^{-/-}$/Casp3$^{-/-}$/Casp7$^{-/-}$* iBMDMs. **(C)** PI influx from LPS-primed WT, *Asc$^{-/-}$*, *Casp1$^{-/-}$/Casp11$^{-/-}$*, *Gsdmd$^{-/-}$*, pool of *Gsdmd$^{-/-}$/Casp3$^{-/-}$* clones (n=3), pool of *Gsdmd$^{-/-}$/Casp7$^{-/-}$* clones (n=5) and *Gsdmd$^{-/-}$/Casp3$^{-/-}$/Casp7$^{-/-}$* iBMDMs after transfection of poly(dA:dT). **(D)** Immunoblots showing caspase-3 and -7 expression and LDH release after transfection poly(dA:dT) transfection from LPS-primed *Gsdmd$^{-/-}$* iBMDMs transfected with control siRNA or siRNA targeting caspase-3, -7 or both. Graphs show mean ± SD. **p ≤ 0.01, ***p ≤ 0.001, (Unpaired t-test). Data and blot are representative of at least three independent experiments.

==Figure S5:==

(A) Quantification of PI uptake in LPS primed WT, *Asc$^{-/-}$*, *Casp-1$^{-/-}$/Casp-11$^{-/-}$*, *Gsdmd$^{-/-}$*, *Gsdme$^{-/-}$*, *Gsdmd$^{-/-}$/Gsdme$^{-/-}$* BMDMs transfected with poly(dA:dT) for 4 hours. **(B)** Immunoblot for GSDMD, GSDME, caspase-1 and tubulin on pooled supernatant and lysate samples collected at indicated times. Graph shows mean ± SD. Graph and blot are representative of at least three independent experiments.

Figure S6:

(A) Confocal microscopy images showing Mitotracker Green (Green) and Mitotracker Red (Red) and DIC, 30 min. and 60 min. post poly(dA:dT) transfection into LPS-primed *Gsdmd$^{-/-}$* and *Asc$^{-/-}$* iBMDMs. **(B-D)** LDH release and TiterGlo measurements at 0, 15, 30 and 45 min or as indicated from LPS-primed WT, *Asc$^{-/-}$*, *Casp1$^{-/-}$/Casp11$^{-/-}$* and *Gsdmd$^{-/-}$* primary BMDMs after transfection with poly(dA:dT) **(C)** or infection with log-phase *S.* Typhimurium at MOI 10 **(B,D)**. Scalebar 10 μm. Graphs show mean ± SD. Data and blot are representative of at least three independent experiments.

Figure S7:

(A) Immunoblots showing Bid expression in *Gsdmd$^{-/-}$* and *Gsdmd$^{-/-}$/Bid$^{-/-}$* iBMDMs. **(B-C).** Caspase-3/-7 activity (DEVDase activity) and PI influx in *Gsdmd$^{-/-}$* and pool of *Gsdmd$^{-/-}$/Bid$^{-/-}$* clones (n=5) iBMDMs after transfection with poly(dA:dT). **(D)** Confocal images of cells of *Gsdmd$^{-/-}$* and *Gsdmd$^{-/-}$/Bid$^{-/-}$* iBMDMs after transfection with poly(dA:dT) for 3 h. Insets show membrane ballooning in dying cells. Scalebar 10 μm. Graphs show mean ± SD. Data and blot are representative of at least three independent experiments.

Figure S8:

(A) Immunoblots showing caspase-1, -7-, -3, -8 and -9 processing in WT, *Asc$^{-/-}$*, *Casp1$^{-/-}$/Casp11$^{-/-}$* and *Gsdmd$^{-/-}$* primary BMDMs after transfection with poly(dA:dT). **(B)** Immunoblots showing caspase-8 expression in *Gsdmd$^{-/-}$* and *Gsdmd$^{-/-}$/Casp8$^{-/-}$* iBMDMs. **(C)** LDH release

1024   in LPS-primed *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$ /*Casp8*$^{-/-}$ iBMDMs after transfection with poly(dA:dT)

1025   in the presence of 100 ng/ml TNF−α, the SMAC mimetic AZD5582 (5 μM) or

1026   AZD5582/GSK872. **(D)** PI influx in *Gsdmd*$^{-/-}$ and pool of *Gsdmd*$^{-/-}$ /*Casp8*$^{-/-}$ clones (n=3)

1027   iBMDMs after transfection with poly(dA:dT). Graphs show mean ± SD. Data and blot are

1028   representative of at least three independent experiments.

1029   **Figure S9:**

1030   **(A)** Immunoblots showing Casp-9 expression in *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$ /*Casp9*$^{-/-}$ iBMDMs.

1031   **(B)** PI uptake of *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$ /*Casp9*$^{-/-}$ iBMDMs treated with 2 μM ABT737 and 2

1032   μM S63845. **(C)** PI uptake in LPS-primed and poly(dA:dT)-transfected *Gsdmd*$^{-/-}$ and pool of

1033   *Gsdmd*$^{-/-}$ /*Casp9*$^{-/-}$ clones (n=2) iBMDMs. **(D)** Immunoblots showing Casp-8 and Casp-9

1034   expression in *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$ /*Casp8*$^{-/-}$/*Casp9*$^{-/-}$ iBMDMs. **(E)** Immunoblots showing

1035   caspase-3 processing in *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$ /*Bid*$^{-/-}$ iBMDMs after transfection with

1036   poly(dA:dT). **(F)** Immunoblots showing caspase-3 processing in *Gsdmd*$^{-/-}$ and *Gsdmd*$^{-/-}$ /*Bid*$^-$

1037 $^{/-}$ iBMDMs after transfection with poly(dA:dT) and treatment with AZD5582. Graphs show mean

1038   ± SD. Data and blot are representative of at least three independent experiments.

Figure 1

Figure 2

Figure 3

A

	iGsdmd⁻/⁻	iGsdmd⁻/⁻ Casp1⁻/⁻

Time (h): 0 3 5 0 3 5

αCasp-1

αCasp-3 — FL

28 —

— p17

αTubulin

B

LDH (% of Maximum) vs Time (h)

iGsdmd⁻/⁻/Casp1⁻/⁻

iWT
iAsc⁻/⁻
iCasp1⁻/⁻/Casp11⁻/⁻
iGsdmd⁻/⁻

** ****

C

DEVDase activity (RFU/min) vs Time (h)

iGsdmd⁻/⁻   iGsdmd⁻/⁻/Casp1⁻/⁻

D

%PI+ cells vs Time (min)

WT
Asc⁻/⁻
Casp1⁻/⁻/Casp11⁻/⁻
Gsdmd⁻/⁻
Casp1^{C284A/C284A}
Casp1⁻/⁻

Figure 4

A

Aim2 - poly(dA:dT)
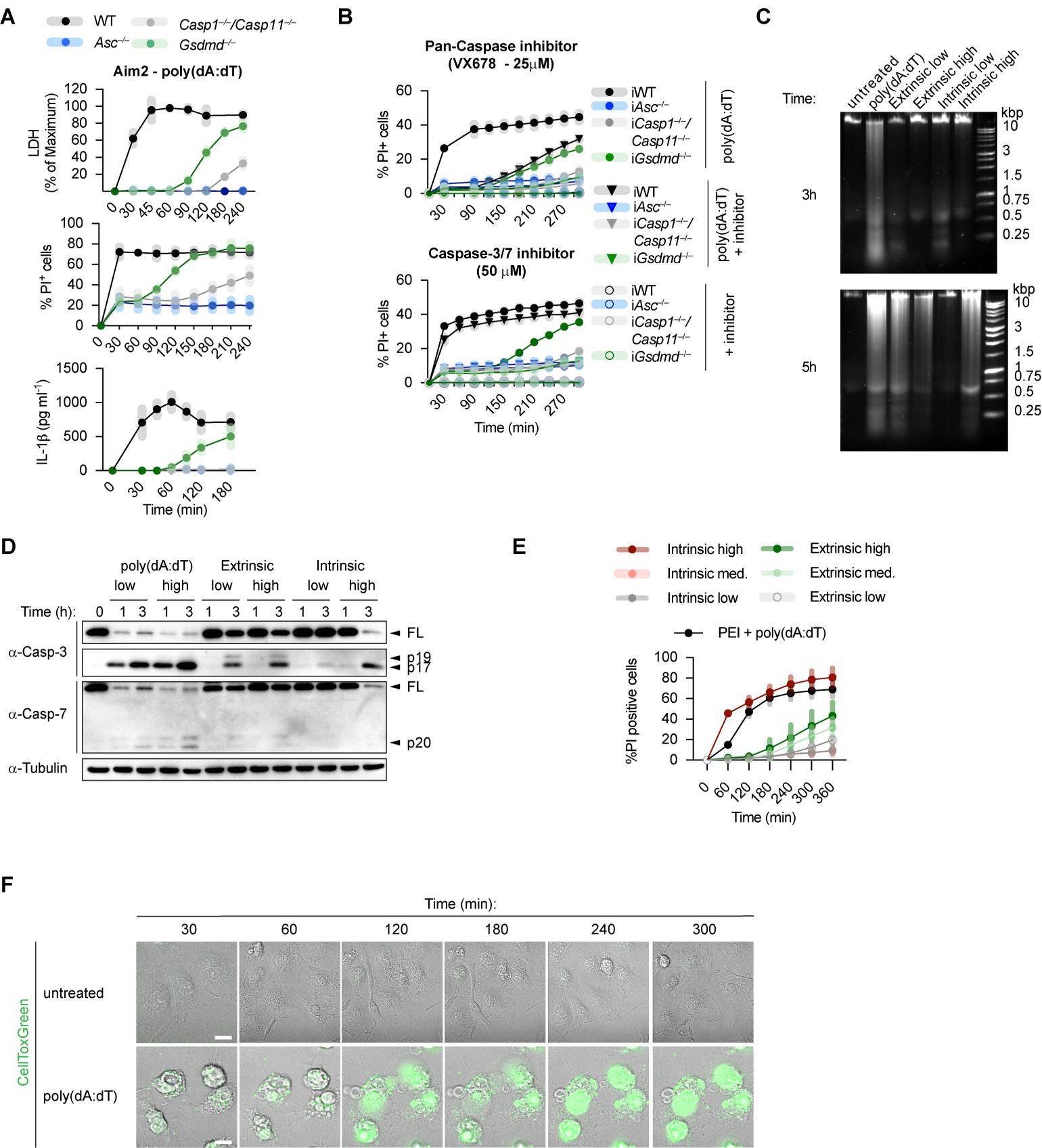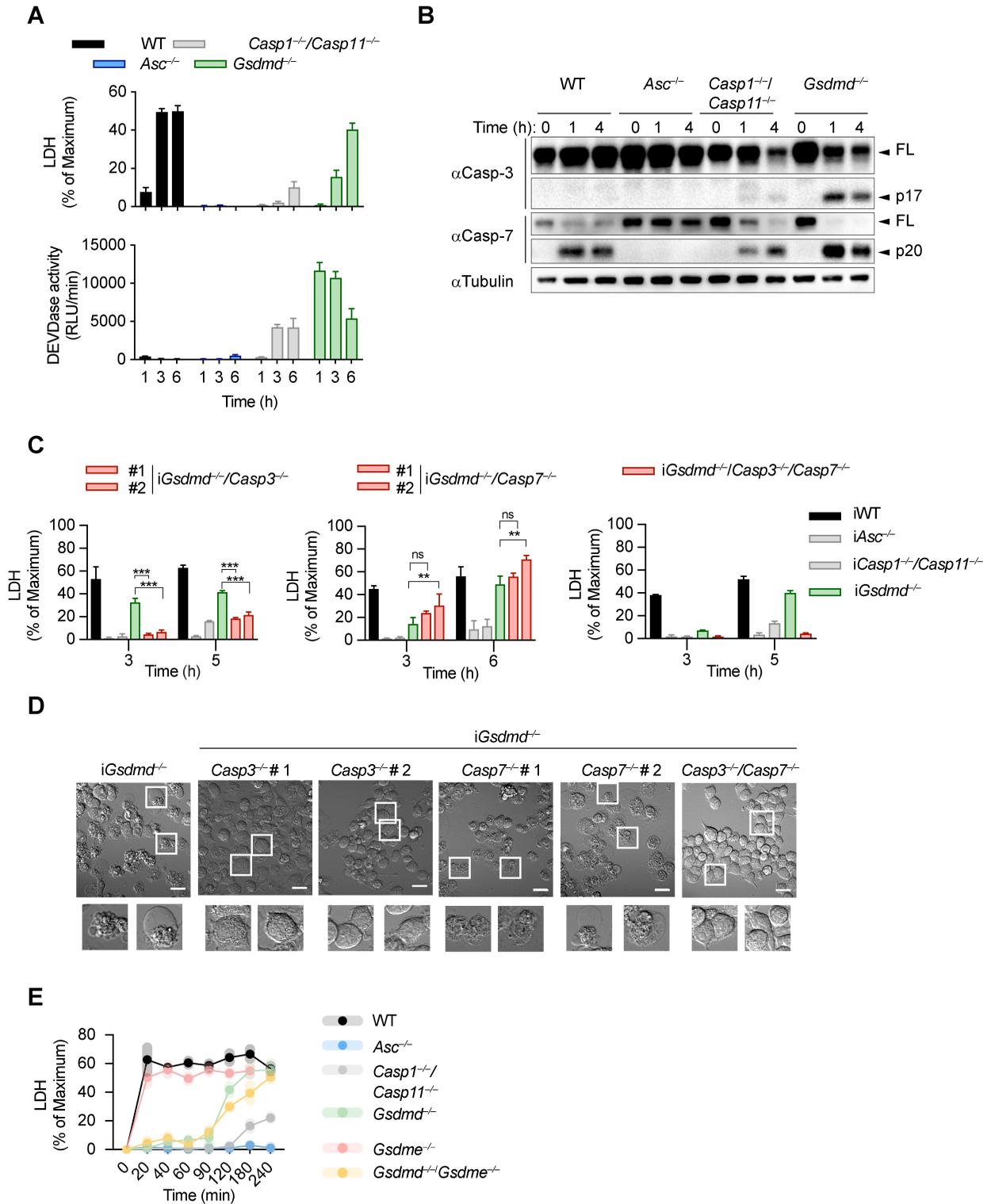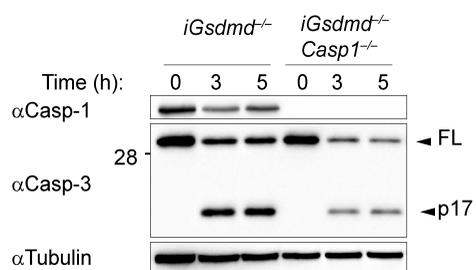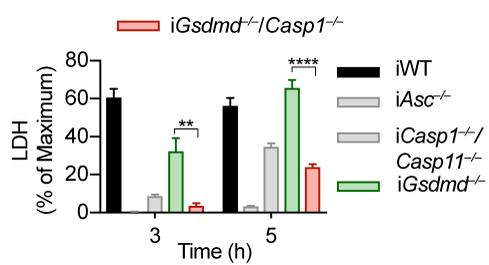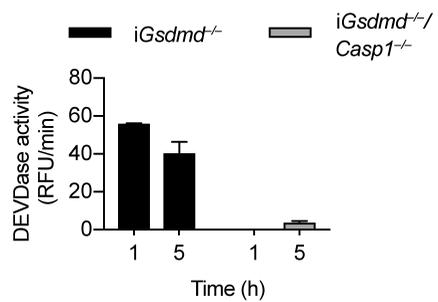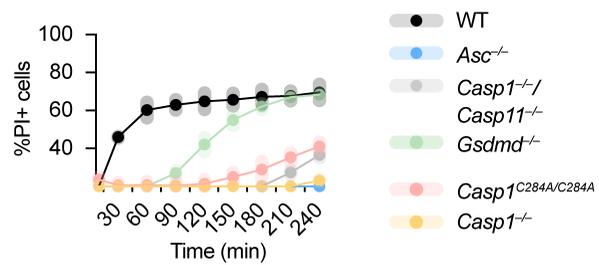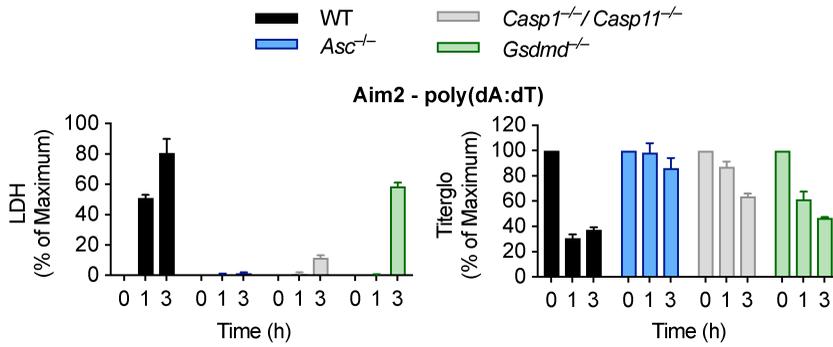
B
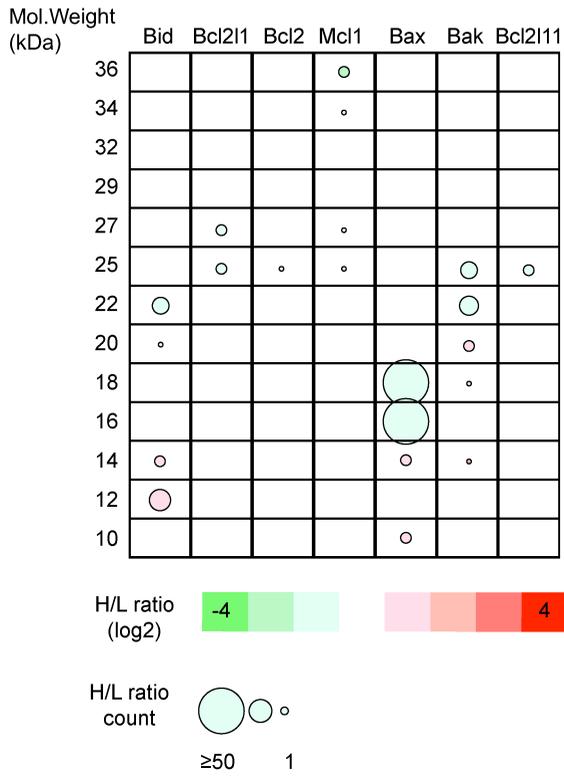
C
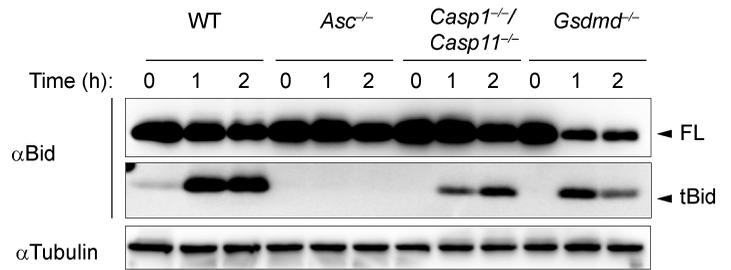
D
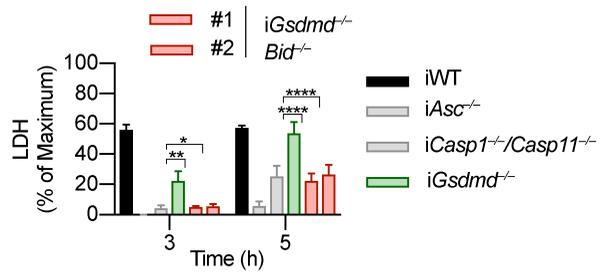
Figure 5

Figure 6

Figure 7