1 **Beyond inflammasomes: emerging function of gasdermins during apoptosis**
2 **and NETosis**
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4 Kaiwen W. Chen[1], Benjamin Demarco[1], Petr Broz[1*]
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6 [1]Department of Biochemistry, University of Lausanne, Switzerland
7
8 *Correspondence to Petr Broz
9 Department of Biochemistry, University of Lausanne, Switzerland.
10 Tel : + 41 21 692 5656
11 E-mail: petr.broz@unil.ch
12
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Abstract

Programmed cell death is key mechanism involved in several biological processes ranging from development and homeostasis to immunity, where it promotes the removal of stressed, damaged, malignant or infected cells. Abnormalities in the pathways leading to the initiation of cell death or the removal of dead cells, are consequently associated with a range of human diseases including, infection, autoinflammatory disease, neurodegenerative disease and cancer. Apoptosis, pyroptosis and NETosis are three well-studied modes of cell death that were traditionally believed to be independent of one another, however emerging studies indicate that there is extensive cross talk between these pathways, and that all three pathways can converge onto the activation of the same cell death effector – the pore-forming protein Gasdermin D (GSDMD). In this review, we highlight recent advances in gasdermin research, with a particular focus on the role of gasdermins in pyroptosis, NETosis and apoptosis, as well as cell-type specific consequences of gasdermin activation. In addition, we discuss controversies surrounding a related gasdermin family protein, Gasdermin E (GSDME) in mediating pyroptosis and secondary necrosis following apoptosis, chemotherapy and inflammasome activation.

Introduction

Pyroptosis is a form of necrotic cell death that has emerged as an important innate immune mechanism against intracellular pathogens. The existence of pyroptosis was first observed in the early 1990s when several laboratories documented that infection with *Shigella flexneri* or *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) triggered rapid cytotoxicity in murine macrophages (Monack et al., 1996, Zychlinsky et al., 1992). This peculiar form of pathogen-induced cell death features several characteristics of apoptosis such as DNA fragmentation and exposure of phosphotidylserine, in addition to hallmarks of necrosis such as rapid plasma membrane permeability (Brennan & Cookson, 2000). Subsequent studies revealed that these features of pathogen-infected cells were driven by inflammasomes, a large cytoplasmic, multiprotein complex that enables the activation of the proinflammatory protease, caspase-1 (Martinon et al., 2002). Thus in 2001 Cookson and Brennan coined the term ‘pyroptosis’ to distinguish this form of cell death from apoptosis and accidental necrosis (Cookson & Brennan, 2001). While an increasing number of pathogens were documented to induce macrophage pyroptosis, the mechanisms by which pyroptosis drives host defence *in vivo* was unclear, although it was assumed that killing the infected cell was important. This mechanism was confirmed *in vivo* from elegant studies by Miao and colleagues, where they demonstrate that macrophage pyroptosis attenuates intracellular pathogens and present them for neutrophil-mediated killing (Jorgensen et al., 2016, Miao et al., 2010).

Early studies by Fink and colleagues indicated that pyroptosis was a form of regulated necrosis that were driven by membrane pores of a 1.1-2.4 nm (Fink & Cookson, 2006). However, the molecular mechanisms of plasma membrane pore formation were unclear until 2015, when two landmark studies from the laboratories of Vishva Dixit and Feng Shao, and subsequently by Jiahua Han, identified Gasdermin D (GSDMD) as the essential pyroptosis mediator (He et al., 2015, Kayagaki et al., 2015, Shi et al., 2015). GSDMD consist of an N-terminal pyroptosis inducing domain (GSDMD^{NT} or p30) connected by a linker to a C-terminal regulatory domain (GSDMD^{CT}), which binds the N-terminus. Inflammasome-activated inflammatory caspases-1, -4 and -11 cleave GSDMD at a conserved site within the linker domain, thereby releasing the GSDMD^{NT} from an intramolecular inhibition by GSDMD^{CT}. This cleavage event allows GSDMD^{NT} to oligomerise in cellular membranes, assembling large pores with a diameter of

67 around 18 nm, and to cause pyroptosis (Aglietti et al., 2016, Ding et al., 2016, Liu et
68 al., 2016, Mulvihill et al., 2018, Ruan et al., 2018, Sborgi et al., 2016).
69
70 Emerging evidence suggest that GSDMD pores not only cause pyroptotic cell death,
71 but that they are also essential for other consequences of inflammasome or caspase-
72 1 activation, e.g. the release of mature IL-1 family cytokines, such as IL-1β and IL-18.
73 Unlike other cytokines, IL-1β and IL-18 lack a signal sequence and are therefore
74 secreted in an endoplasmic reticulum/Golgi-independent manner (Rubartelli et al.,
75 1990). Since inflammasome activation usually elicits near-concurrent secretion of
76 mature IL-1β and pyroptosis in macrophages, it is often proposed that IL-1β and IL-18
77 are passively released during cell lysis. In line with this model, *Gsdmd*-deficiency
78 severely abrogates IL-1β secretion upon canonical inflammasome activation
79 (Kayagaki et al., 2015, Shi et al., 2015); and single cell analysis of macrophages
80 revealed that IL-1β release coincides with the uptake of membrane-impermeable
81 nucleic acid dyes (e.g. SYTOX, propidium iodide), a widely used assay to measure
82 the loss of plasma membrane integrity (Liu et al., 2014, Polykratis et al., 2019). By
83 contrast, a number of studies reported that mature IL-1β can be secreted in the
84 absence of intracellular lactate dehydrogenase release, a commonly used assay to
85 quantify cell lysis in a bulk cell population (Chen et al., 2014, Gaidt et al., 2016, Kang
86 et al., 2013, Wolf et al., 2016, Zanoni et al., 2016). Since the standard lactate
87 dehydrogenase release assay may lack single-cell resolution, it remains plausible that
88 mature IL-1β are indeed released by a small fraction of lysed cells upon inflammasome
89 activation. However, several lines of evidence support the notion that that cell lysis is
90 not an absolute requirement for IL-1β secretion. For example, ectopic expression of
91 mature IL-1β in primary macrophages is sufficient to induce its secretion in the
92 absence of inflammasome activation (Monteleone et al., 2018); and single cell
93 analysis of live, viable murine embryonic fibroblast revealed considerable IL-1β
94 secretion after caspase-1 or -8 activation (Conos et al., 2016). Consistent with these
95 observations, a number of recent studies demonstrated that sublytic GSDMD pores
96 (18 nm) are indeed large enough for the release of mature IL-1β (Evavold et al., 2018,
97 Heilig et al., 2018) or entry of nucleic acid dyes (DiPeso et al., 2017, Russo et al.,
98 2016), indicating that GSDMD can act as a conduit for IL-1β release in the absence of
99 cell lysis. Studies carried out by us on ESCRT-III-dependent membrane repair have

100 further strengthened the notion that cells can tolerate a certain number of GSDMD
101 membrane pores (Ruhl et al., 2018). The model that emerges from these studies
102 implies that caspase activation proceeds from a sub-lytic phase in which cells feature
103 transient assembly of GSDMD pores to a lytic phase where GSDMD pores cause a
104 complete breakdown of membrane integrity. Whether cells transit from the sub-lytic to
105 the lytic phase depends on the strength of the activating signal, level of GSDMD
106 expression and activation, cell type and the activity of membrane repair mechanism.
107 Furthermore, recent findings indicate that while $GSDMD^{NT}$ is sufficient to assemble
108 pores *in vitro* or when overexpressed, its activity might be regulated by additional
109 mechanisms under physiological conditions. For example, it has been proposed that
110 complete GSDMD-dependent cell lysis requires SARM1-dependent depolarization of
111 mitochondria in macrophages (Carty et al., 2019), indicating that mitochondrial
112 damage is critical for the transition into the lytic-phase of GSDMD activation in this cell
113 type.
114
115 Altogether these new findings highlight that more research is necessary to understand
116 how GSDMD expression and activity is regulated on a translational and post-
117 translational level, and which cellular membranes/organelles need to be targeted by
118 $GSDMD^{NT}$ to induce pyroptotic cell death or to exert its lysis-independent functions. In
119 the following we however focus on an emerging host of studies that have begun to
120 uncover cell-type-specific and/or inflammasome-independent functions of GSDMD,
121 and on the enigmatic role of GSDME, another member of the gasdermin family, in cell
122 death.
123
124 **GSDMD function in neutrophils**
125
126 ***Neutrophils resist pyroptosis upon canonical inflammasome activation***
127 Neutrophils express a repertoire of pattern recognition receptors (PRR) and are
128 recruited in large quantity to a site of infection or inflammation, therefore are excellent
129 candidates to drive inflammasome-dependent responses *in vivo* (Thomas & Schroder,
130 2013). However, earlier studies overlooked possible functions for neutrophil
131 inflammasomes, after observing that neutrophils contributed to IL-1β processing
132 through caspase-1-independent mechanisms in a mouse model of acute arthritis
133 (K/BxN serum transfer) or upon FAS (CD95) ligation (Guma et al., 2009, Joosten et

134 al., 2009, Miwa et al., 1998). In addition, two earlier studies proposed that neutrophils
135 are unlikely to signal via inflammasomes during *Salmonella* Typhimurium or
136 *Burkholderia pseudomallei* infection because these cells do not express NLRC4, an
137 inflammasome-forming PRR that senses bacterial virulence factors (Ceballos-Olvera
138 et al., 2011, Miao et al., 2010). Subsequent studies have now challenged these
139 findings, as multiple groups readily detect expression of inflammasome-forming PRRs
140 including NLRC4, NLRP3 and AIM2, and other components of the inflammasome
141 signalling complex including the adaptor protein ASC, and the protease zymogen,
142 caspase-1 in murine and human neutrophils (Bakele et al., 2014, Chen et al., 2016,
143 Chen et al., 2014, Karmakar et al., 2015, Karmakar et al., 2016, Mankan et al., 2012).
144 In agreement with this, exposure of neutrophils to the NLRC4 agonist *Salmonella*
145 Typhimurium or the AIM2 agonist cytosolic double-stranded DNA triggered caspase-
146 1 activation and caspase-1-dependent IL-1β processing (Chen et al., 2014). Although
147 *Nlrp3* mRNA is basally expressed at much higher levels in neutrophils than
148 macrophages (Chen et al., 2014), only soluble NLRP3 agonists such as ATP or the
149 bacterial toxin nigericin, but not particulate or crystalline NLRP3 agonists (e.g. silica
150 or monosodium urate crystals) are able to activate the neutrophil NLRP3
151 inflammasome (Chen et al., 2016). This highlights that inflammasome signalling is
152 specialised even between the two closely related myeloid cell lineage. In agreement
153 with this, while caspase-1 activation triggers rapid macrophage pyroptosis (Kayagaki
154 et al., 2015, Shi et al., 2015), canonical inflammasome (e.g. NLRC4, NLRP3, AIM2)
155 activation in neutrophils selectively triggers caspase-1-dependent IL-1β processing
156 without concomitant pyroptotic cell death (Chen et al., 2014, Chen et al., 2018b,
157 Karmakar et al., 2015, Karmakar et al., 2016). Although neutrophils are relatively
158 short-lived cells and murine neutrophils have a half-life of 18 h in circulation (5.4 days
159 in humans) (Pillay et al., 2010), exposure of neutrophils to cytokines (e.g. GM-CSF,
160 IL-1β, IFN-γ) and pathogen-derived products (e.g. LPS) can significantly increase their
161 lifespan up to 96 h, indicating that neutrophils can significantly prolong their lifespan
162 during infection (Colotta et al., 1992). This unique ability of neutrophils to resist
163 pyroptosis enables the recruited neutrophils to maintain their lifespan to clear the
164 microbial insult or cellular debris; and sustain IL-1β release to recruit, activate and
165 prolong the lifespan of neutrophils at a site of infection (Chen et al., 2014, Karmakar
166 et al., 2015, Karmakar et al., 2016). While inflammasomes are important for host

167 defence, gain-of-function mutations in inflammasomes can also drive a variety of
168 hereditary inflammatory disease (e.g. Muckle-Wells Syndrome, macrophage
169 activating syndrome) (Agostini et al., 2004, Canna et al., 2014, Romberg et al., 2014).
170 These diseases are currently attributed to inflammasome dysfunction in monocytes
171 and macrophages, in which IL-1β/18 production is rapidly curtailed by pyroptotic cell
172 death. Intriguingly, IL-1β production and inflammation are not self-limiting in these
173 diseases, suggesting that the cellular source of IL-1β in these diseases may indeed
174 be derived from other cell types. Since neutrophils express majority of the
175 inflammasome signalling components, and that neutrophil IL-1β production proceeds
176 in the absence of pyroptosis, it would be of interest to examine the contribution of
177 neutrophil-derived IL-1β in human inflammatory disease in future studies.
178
179 The mechanisms by which neutrophils resist caspase-1-dependent pyroptosis is likely
180 to be controlled by careful fine tuning of the expression of specific pyroptotic
181 machineries in these cells. Although GSDMD is expressed at comparable levels
182 between neutrophils and macrophages (Chen et al., 2018b, Heilig et al., 2018),
183 neutrophils express relatively low level of ASC and caspase-1, therefore, neutrophil
184 inflammasomes assemble with a smaller ASC 'speck' with reduced caspase-1 activity
185 (Boucher et al., 2018, Chen et al., 2018a, Chen et al., 2018b). Since caspase-1
186 cleaves pro-IL-1β better than it cleaves GSDMD (Chen et al., 2018b), this specific fine-
187 tuning of caspase-1 activity in neutrophils ensures that caspase-1 only generates
188 sublytic GSDMD pores to enable IL-1β secretion without concomitant cell lysis (Chen
189 et al., 2018b) **(Figure 1)**. However, it is possible that additional mechanisms exist to
190 restrict caspase-1-driven pyroptosis in neutrophils. For example, neutrophils may
191 repair plasma membrane GSDMD pores via ESCRT-III repair mechanisms as
192 reported for macrophages and HeLa cells (Ruhl et al., 2018). However, this hypothesis
193 would be ambitious to demonstrate since it is technically challenging to manipulate
194 primary neutrophils. SARM1 is a TIR-containing protein that is involved in TLR
195 signalling. A recent study revealed a surprising role for SARM in driving optimal
196 macrophage pyroptosis (Carty et al., 2019). Interestingly, *Sarm1*-deficient
197 macrophages appears to be phenotypically similar to neutrophils, as both cell types
198 release IL-1β in the absence of pyroptosis upon canonical inflammasome activation
199 (Carty et al., 2019, Chen et al., 2014). Neutrophils were already documented to
200 suppress TLR4-TRIF signalling to repress RIPK3-dependent cell death (Chen et al.,

201 2018a), therefore, it is conceivable that neutrophils likewise suppress SARM1
202 expression to subvert caspase-1-dependent pyroptosis. Further studies should
203 characterise the expression of SARM1 in neutrophils, and whether overexpression of
204 SARM1 triggers neutrophil caspase-1-dependent pyroptosis.
205
206 ***Caspase-11 and neutrophil elastase cleave GSDMD to elicit NETosis***
207 Although caspase-1 activation does not trigger pyroptosis in neutrophils, activation of
208 the caspase-11 (non-canonical) inflammasome by cytosolic LPS or cytosolic Gram-
209 negative bacteria triggered robust GSDMD cleavage and cell lysis in neutrophils,
210 indicating that these cells are not intrinsically resistant to GSDMD pores (Chen et al.,
211 2018b). Surprisingly, pyroptotic neutrophils appeared morphologically distinct from
212 caspase-1 or -11-activated macrophages. Instead, caspase-11 and GSDMD
213 activation triggered classical hallmarks of NETosis, including nuclear delobulation,
214 histone citrullination, DNA extrusion, and rupture of nuclear, granule and plasma
215 membrane **(Figure 1)**. Strikingly, neutrophil elastase, myeloperoxidase and PAD4,
216 three key enzymes involved in classical NETosis are dispensable for caspase-11-
217 dependent NET extrusion, indicating that caspase-11 and GSDMD may directly
218 induce these hallmarks of NETosis (Chen et al., 2018b). In support of this, the
219 combination of recombinant GSDMD and caspase-11 is sufficient to trigger neutrophil
220 nuclear membrane rupture, chromatin relaxation and histone H3 degradation in a cell-
221 free system. Further, application of exogenous Dnase I to neutralise caspase-11 and
222 GSDMD-driven NETs impairs *in vivo* host defence against a cytosolic mutant of
223 *Salmonella* (*ΔsifA*), revealing a previously undescribed host protective function of
224 NETs against cytoplasmic infection (Chen et al., 2018b). Given that cell type-specific
225 signalling has such a profound impact on the phenotypical outcome of GSDMD-
226 induced cell death, it will be very interesting to investigate the consequences of
227 GSDMD activation in other granulocytes, as well as non-immune cells.
228
229 ***Neutrophil elastase cleaves GSDMD to trigger neutrophil cell death and NETs***
230 Although GSDMD was initially identified as a substrate of inflammatory caspases, two
231 recent studies documented that GSDMD is also processed the serine proteases,
232 neutrophil elastase in neutrophils **(Figure 2)** (Kambara et al., 2018, Sollberger et al.,
233 2018). Although neutrophil elastase and cleave GSDMD several amino acids

234 upstream of the canonical caspase cleavage site, this did not affect the ability of
235 GSDMD N-terminal fragment to oligomerise and induce lytic cell death upon
236 overexpression in HEK 293T cells, in line with the observation that the membrane
237 insertion and lytic properties of GSDMD N-terminal fragment lies within the first 243
238 amino acid (Shi et al., 2015). However, despite these observations, conclusion from
239 the both studies were vastly different. In one study, neutrophil elastase-dependent
240 GSDMD cleavage was proposed to trigger cell death in aging neutrophils.
241 Consequently, when challenged intraperitoneally with *E. coli* K12, *Gsdmd*-deficient
242 mice accumulated more neutrophils at a site of infection and were more resistant to
243 infection than wild type animals (Kambara et al., 2018). However, whether GSDMD
244 promotes spontaneous neutrophil cell death is controversial, as other studies
245 documented similar rate of spontaneous neutrophil death in wild type versus *Gsdmd*-
246 deficient neutrophils (Burgener et al., 2019, Chen et al., 2018b). In agreement with
247 macrophage studies that the $GSDMD^{NT}$ fragment triggers proinflammatory cell death,
248 a second study reported that activation of GSDMD by neutrophil elastase drive
249 neutrophil cell lysis and NET extrusion, a well-appreciated antimicrobial defence
250 mechanism (Sollberger et al., 2018). Therefore, it appears that GSDMD activity in
251 neutrophils can either promote or dampen host defence. The signalling mechanisms
252 that dictate these differences have not been investigated in detail, however, it is
253 tempting to speculate that the signal strength and cellular location of neutrophil
254 elastase is a key regulator. It is well documented that a high concentration of neutrophil
255 elastase translocates from azurophilic granules to the nucleus at the early stages of
256 NETosis, and that nuclear membrane damage precedes cellular rupture (Metzler et
257 al., 2014, Papayannopoulos et al., 2010, Sollberger et al., 2018). In this scenario, it is
258 likely that the close proximity of cleaved GSDMD preferentially disrupts the nuclear
259 membrane to initiate the hallmarks of NETosis. In contrast, it is conceivable that a
260 much lower intensity of neutrophil elastase 'escapes' from azurophilic granules into
261 the cytosol in aged neutrophils, which cleaves a low but steady amount of GSDMD to
262 trigger neutrophil death without accompanying NETosis. Since GSDMD drives a
263 variety of inflammatory disease and is thus an attractive pharmacological target,
264 additional studies are clearly required to further characterise the function of GSDMD
265 in neutrophils during inflammatory disease and infection.
266

267 **GSDMD function during apoptosis**

268

269 **Emerging evidences of apoptosis-induced inflammation**

270 Apoptosis is a form of programmed cell death that is important for embryonic
271 development, removal of auto-reactive lymphocytes and clearance of damaged or
272 superfluous cells. In contrast to pyroptosis, apoptotic cell death is generally regarded
273 as an immunologically silent process. This is achieved by several mechanisms,
274 including sequential breakdown of the dying cell into small membrane-bound apoptotic
275 bodies, the release of 'find-me' and 'eat-me' signals to promote efferocytosis of dying
276 cells and caspase-mediated cleavage of innate immune sensors and proinflammatory
277 cytokines (Luthi et al., 2009, Ning et al., 2019, Poon et al., 2014). However, despite
278 these observations, *in vitro* studies revealed that genetic or pharmacological inhibition
279 of endogenous apoptosis inhibitors such as the mammalian inhibitor of apoptosis
280 proteins (IAPs) cIAP1, 2 and XIAP, or kinases such as transforming growth factor beta-
281 activated kinase 1 (TAK1) and IκB kinase β (IKKβ), sensitise myeloid cells including
282 macrophages, dendritic cells and neutrophils to caspase-8 activation, cell lysis and
283 NLRP3 inflammasome activation (Chen et al., 2018a, Dondelinger et al., 2015, Lawlor
284 et al., 2017, Lawlor et al., 2015, Vince et al., 2012, Wicki et al., 2016, Yabal et al.,
285 2014). In agreement with these *in vitro* studies, global loss of *Map3k7* (TAK1), IKKβ,
286 or *Birc2* (cIAP1) in combination with *Birc3* (cIAP2) or *Birc4* (XIAP) similarly drives
287 excessive inflammation that results in embryonic lethality (Moulin et al., 2012, Sato et
288 al., 2005, Shim et al., 2005, Tanaka et al., 1999).

289

290 ***Direct cleavage of GSDMD by caspase-8 promotes cell lysis and inflammation***

291 While the studies above clearly implicate an important function for caspase-8 in driving
292 inflammation and even embryonic lethality, the molecular mechanisms by which
293 caspase-8 promotes cell lysis and NLRP3 activation remains unsolved. By using
294 pharmacological inhibitors of TAK1 or IAPs (e.g. SMAC-mimetics), we and others
295 recently demonstrate that the pyroptotic effector GSDMD plays a major role in this
296 process (Chen et al., 2019b, Orning et al., 2018, Sanjo et al., 2019, Sarhan et al.,
297 2018). Unexpectedly, under these conditions, GSDMD is processed into the lytic p30
298 fragment via two pathways. The first pathway involves direct cleavage of GSDMD by
299 caspase-8 at position D276, similar to canonical caspase cleavage site described for
300 caspase-1 and -11. However, caspase-8 is 30-fold less efficient than caspase-1 in

301 processing GSDMD, and caspase-8-dependent GSDMD cleavage is only observed
302 under conditions of strong caspase-8 activation (Chen et al., 2019b). This likely
303 explains why early studies failed to observe GSDMD processing into the active p30
304 fragment by recombinant caspase-8 (Shi et al., 2015). The second pathway leading to
305 GSDMD activation occurs via potassium efflux and activation of the NLRP3
306 inflammasome (Conos et al., 2017), however, the mechanisms by which caspase-8
307 drives NLRP3 activation is still a matter of debate and is discussed in greater details
308 in the subsequent paragraphs.
309
310 The finding that caspase-8 triggers direct GSDMD activation is exciting and raises
311 several important questions. For example, what is the physiological function of
312 caspase-8-dependent GSDMD activation? Numerous pathogens are equipped with
313 virulence factors that inhibit host NF-κB signalling pathways. This could in turn
314 promote caspase-8 activation and induce GSDMD cleavage and pyroptosis, as
315 recently reported for *Yersinia* infection (Orning et al., 2018, Sarhan et al., 2018).
316 However, as pyroptosis is best known as an innate immune mechanism to restrict
317 intracellular pathogen infection, how GSDMD activation can promote host defence
318 against *Yersinia*, a predominantly extracellular pathogen is unclear, and has not been
319 formally demonstrated. RIPK1/caspase-8-dependent apoptosis can promote the
320 release of alarmins and activate neighbouring immune cells for cytokine production
321 and anti-*Yersinia* defence (Peterson et al., 2017), yet whether GSDMD is also required
322 in this scenario is unclear and warrants further investigation. Likewise, it would be of
323 interest to investigate whether the caspase-8-GSDMD axis induces NET extrusion to
324 combat extracellular pathogens, or whether this signalling axis is exploited by *Yersinia*
325 to promote pathogen dissemination *in vivo.*
326
327 Another important question that arises from the discovery that caspase-8 cleaves
328 GSDMD is the molecular mechanisms by which apoptosis remains immunologically
329 silent during tissue homeostasis. Several lines of evidence suggest that executioner
330 caspases play a key role in regulating the level of GSDMD activity in apoptotic cells,
331 as previous studies documented that caspase-3, and a lesser extent caspase-7,
332 cleave GSDMD at position D88 (D87 in humans) to disrupt its pore-forming ability
333 (Rogers et al., 2017, Taabazuing et al., 2017). In keeping with this, *Gsdmd*$^{D88A/\mathrm{D88A}}$

knock-in macrophages harbouring a caspase-3/7-uncleavable mutation accumulated GSDMD^{NT} pores, resulting in enhanced pyroptosis compared to wild type macrophages (Chen et al., 2019b). However, naïve *Gsdmd*$^{D88A/D88A}$ mice appear phenotypically similar to wild type littermates (Chen et al., 2019b); thus whether GSDMD inactivation is required to suppress pyroptosis during physiological conditions *in vivo* has not been formally demonstrated.

Aberrant caspase-8 activity has been implicated in a variety of inflammatory diseases, and in some cases can even drive lethality. For example, caspase-8 drives lethal dermatitis in the absence of linear ubiquitin chain assembly complex (LUBAC) (Taraborrelli et al., 2018), and caspase-8 activity triggers embryonic lethality observed in *Birc2*$^{-/-}$ *Birc3*$^{-/-}$ mice (Zhang et al., 2019). Furthermore, caspase-8-dependent intestinal damage is a key driver for septic shock in mice (Mandal et al., 2018). Since the caspase-8 is emerging a key mediator of cell death and inflammation, it would be of great interest to investigate whether caspase-8-dependent GSDMD activation is sufficient to drive pathogenesis of these diseases in the near future.

GSDMD and pannexin-1 control NLRP3 activation in apoptotic cells

Although apoptosis was traditionally considered an immunologically silent form of cell death, an increasing number of studies documented that apoptotic caspase-8 promotes assembly of the NLRP3 inflammasome (Chen et al., 2019a, Chen et al., 2019b, Lawlor et al., 2017, Lawlor et al., 2015, Vince et al., 2012, Wicki et al., 2016). The existence of this signalling axis was first demonstrated by Vince and colleagues, who reported that loss of IAPs sensitized macrophages and dendritic cells to caspase-8-dependent cell death and NLRP3 activation upon TNF or TLR ligation (Vince et al., 2012). Although this signalling axis is implicated in a variety of physiological conditions, including the pathogenesis of X-linked lymphoproliferative syndrome type 2 in humans (Lawlor et al., 2017, Yabal et al., 2014), and during influenza or *Yersinia* infection (Kuriakose et al., 2016, Orning et al., 2018), the exact mechanism by which apoptotic caspases activate NLRP3 is still a matter of debate and might involve several pathways. Orning *et al.* recently proposed that caspase-8-driven GSDMD pores triggers NLRP3 assembly (Orning et al., 2018), analogous to the noncanonical inflammasome pathway, where caspase-11-driven GSDMD pores promote

367 membrane damage, potassium efflux and NLRP3 inflammasome activation **(Figure**
368 **3)** (Kayagaki et al., 2015, Ruhl & Broz, 2015, Shi et al., 2015). In contrast, our study
369 revealed that GSDMD is dispensable for caspase-1 activation during TNF-induced
370 caspase-8 activation. Instead, we demonstrate that caspase-8-dependent NLRP3
371 activation requires the channel-forming transmembrane glycoprotein, pannexin-1. For
372 this, caspase-8 promotes downstream executor caspase-3/7 activation, which cleave
373 and activate pannexin-1 channel activity, membrane permeability and NLRP3
374 inflammasome activation **(Figure 3)** (Chen et al., 2019a, Chen et al., 2019b). Further
375 support for the importance of pannexin-1 in driving NLRP3 activation during apoptosis
376 comes from the fact that caspase-3/7 and pannexin-1 is also required also for NLRP3
377 activation upon caspase-9-dependent intrinsic apoptosis, which unlike caspase-8,
378 does not have the ability to cleave GSDMD (Chen et al., 2019a, Chen et al., 2019b,
379 Vince et al., 2018).
380
381 The reasons for this discrepancy are unclear, however, it is tempting to speculate that
382 the cellular activity of executor caspase-3/7 critically controls the amount of GSDMD
383 pores and pannexin-1 activation in a given cell, and that dictates which pathway is
384 preferentially activated. For example, a given cell with high caspase-3/7 activity would
385 inactivate GSDMD pores and favour NLRP3 activation via pannexin-1 channels. On
386 the other hand, cells with low caspase-3/7 activity would favour NLRP3 activation via
387 GSDMD pores but not pannexin-1 channels. Given that executor caspase-3/7 activity
388 is often suppressed in transformed cells and that many cancer chemotherapies induce
389 tumour cell death through caspase-8, future studies should further characterize this
390 pathway in the context of cancer chemotherapy, and whether modulating this
391 signalling axis can promote tumour clearance.
392
393 ***GSDME activation by caspase-3 promotes pyroptosis in some but not all cells***
394 The discovery that cleavage of GSDMD at the linker region by inflammatory caspases
395 unleashes the pore-forming function of $GSDMD^{NT}$ has significantly enhanced the
396 field's understanding of gasdermin family proteins. Indeed, recent studies found that
397 GSDME features a caspase-3 cleavage motif in its linker region. Similar to GSDMD,
398 cleavage of GSDME by caspases-3/-7 liberates the N-terminal pyroptosis-inducing
399 domain ($GSDME^{NT}$) from its autoinhibitory C-terminal regulatory domain to trigger
400 membrane pores and pyroptosis (Rogers et al., 2017, Wang et al., 2017). Interestingly,

cleavage of GSDME by caspase-3 does not necessarily destine the cell to undergo pyroptosis. In this regard, immune cells appear to be the most resistant to GSDME pores. Indeed, despite evidence of GSDME processing into the active GSDME^{NT} fragment, a number of studies documented that GSDME is dispensable for pyroptosis or secondary necrosis upon extrinsic or intrinsic apoptosis in primary and immortalised murine macrophages, THP-1 monocytes and Jurkat T cells (Chen et al., 2019b, Lee et al., 2018, Tixeira et al., 2018, Vince et al., 2018). A simple explanation for this phenomenon is that GSDME pores need to surpass a critical threshold to initiate pyroptosis. In support of this, cancer cell lines that express high levels of GSDME are extremely susceptible to pyroptosis after exposure of apoptosis-inducing therapies such as cisplatin, doxorubicin and etoposide, while the same treatment triggers apoptosis in GSDME-deficient or low expressing cells (Wang et al., 2017). Although emerging studies demonstrate that MLKL-driven necrotic cell death promotes anti-tumour immunity (Brumatti et al., 2016, Snyder et al., 2019), whether GSDME-driven pyroptosis restricts tumour growth *in vivo* is still unclear and remains an open question. For example, a study reported that GSDME expression suppresses melanoma cell growth a murine xenograft model, whereas other studies documented that *Gsdme* deficiency had no impact on tumour formation during intestinal cancer (Croes et al., 2019, Zhou et al., 2018). Further studies are required to clarify the importance of GSDME during tumorigenesis.

Conclusion and outlook

Since the discovery of the GSDMD as executor of pyroptosis in 2015, it has taken centre stage in other cell death pathways as well, highlighting that inflammasomes are only one possible signalling pathway that can activate the protein. It is thus conceivable that other proteases, be it from the host or from pathogenic microorganisms, could also activate GSDMD or the other family members, as shown for caspase-3/-7 and GSDME. However, proteolysis may not be the only mechanism of gasdermin activation, as point mutations in the GSDM^{CT}, result in activation without the removal of the C-terminal domain (Shi et al., 2015). It is thus clear that additional work will be necessary to better understand the activation and regulation mechanism that control this new family of cell death executors. Furthermore, given the importance of gasdermin-induced death in causing tissue damage and inflammation, additional

434 efforts should be made to develop specific gasdermin inhibitors and to explore the
435 possibility of therapeutical targeting of the gasdermin family.
436
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442
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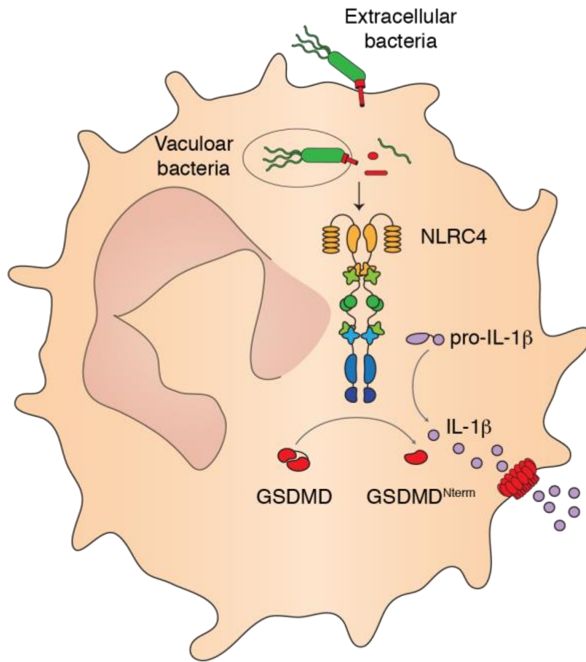
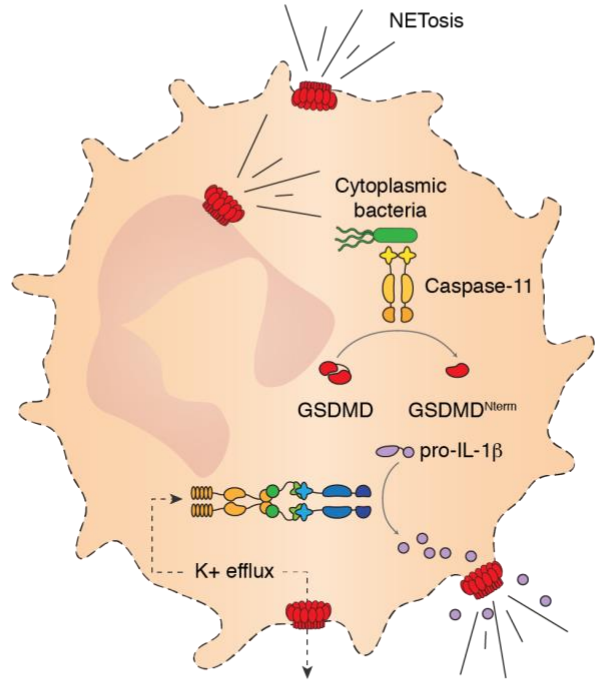
746 **Figure 1. Canonical and non-canonical inflammasome activation in neutrophils.**
747 Neutrophils express several inflammasome-forming PRR including NLRC4, NLRP3,
748 AIM2 and caspase-11. Activation of canonical inflammasome selectively triggers IL-
749 1β maturation without accompanying cell lysis. IL-1β secretion in living neutrophils
750 require the pore-forming protein GSDMD. Upon cytoplasmic Gram-negative bacterial
751 infection, caspase-11 triggers robust GSDMD cleavage. $GSDMD^{NT}$ targets plasma
752 membrane and nuclear membrane to elicit neutrophil extracellular traps (NETs).
753 Caspase-11-driven GSDMD pores promotes potassium efflux and activation of the
754 NLRP3 inflammasome.
755
756 **Figure 2. GSDMD promotes spontaneous neutrophil cell death and NET**
757 **extrusion.** In aging neutrophils, release of neutrophil elastase (NE) from specific
758 neutrophil granules cleave and activate GSDMD, resulting in neutrophil cell death.
759 Upon treatment with classical NETosis activators (e.g. PMA), reactive oxygen species
760 (ROS) promote the release of NE from the granules to cytosol in an ill-defined manner.
761 NE cleaves and activate GSDMD, leading to nuclear and plasma membrane rupture
762 and neutrophil cell lysis by NETosis.
763
764 **Figure 3. GSDMD is a novel effector protein in the extrinsic apoptosis pathway.**
765 In TNF-stimulated cells, loss or inhibition of IAP and TAK1 function promotes assembly
766 of a caspase-8-activating platform called TNF Complex IIb (also commonly referred
767 as the ripoptosome). Active caspase-8 cleaves GSDMD at D276, leading to
768 pyroptosis. Caspase-8-driven GSDMD activation, or caspase-3/7-dependent
769 pannexin-1 activation promotes potassium efflux and NLRP3 assembly. NLRP3-
770 dependent caspase-1 activation cleaves GSDMD to further drive pyroptosis.
771 Probenecid, spironolactone and trovafloxacin are pannexin-1 channel inhibitors.
772
773 Fig. 1
774





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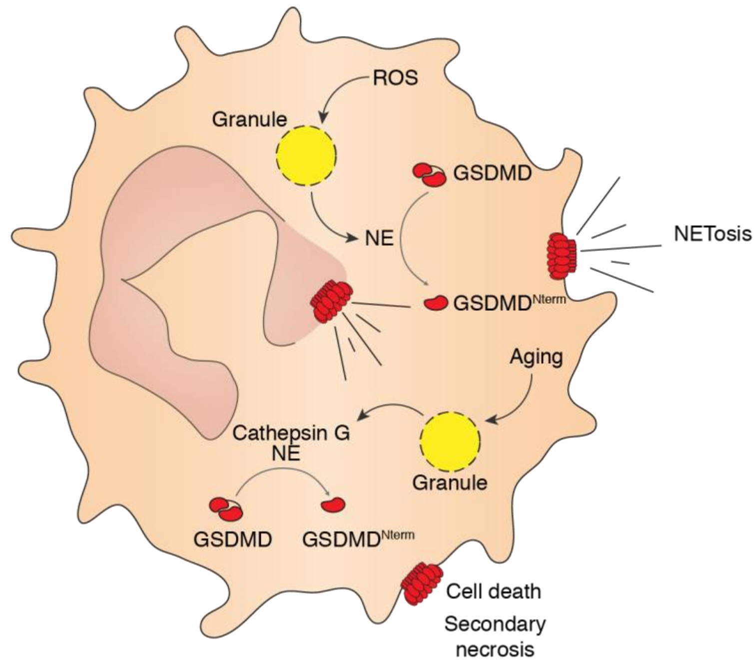
778 Fig. 2



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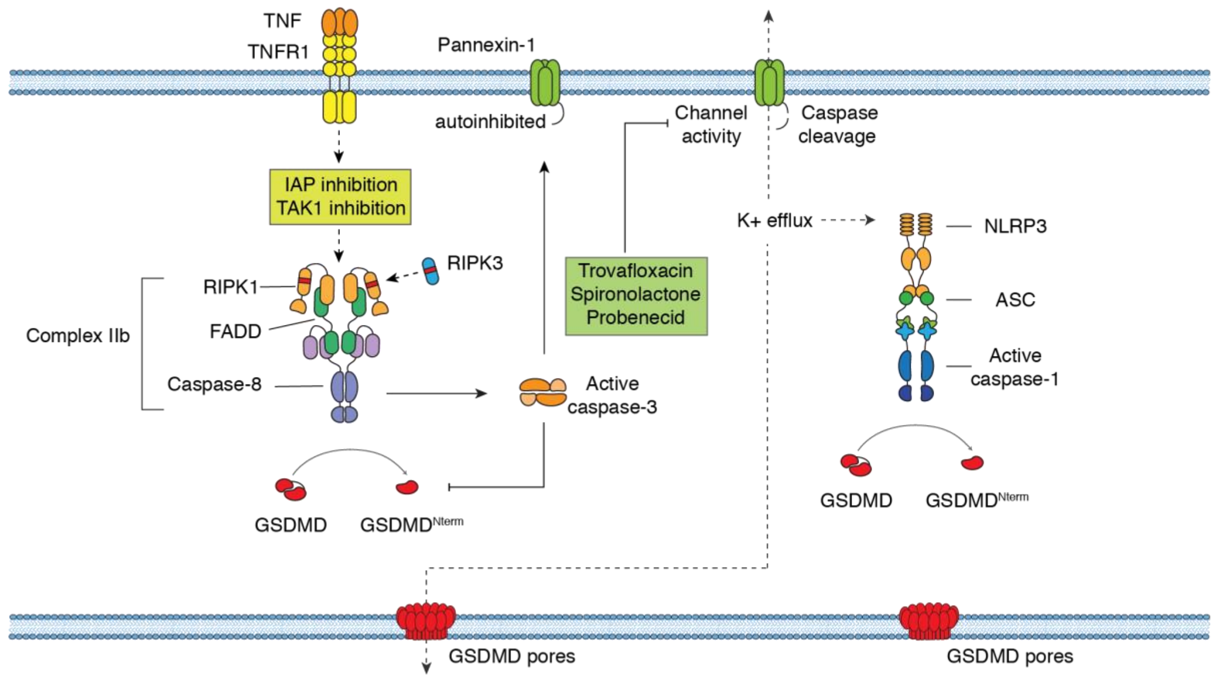
781 Fig. 3



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