Beyond inflammasomes: emerging function of gasdermins during apoptosis and NETosis

Kaiwen W. Chen¹, Benjamin Demarco¹, Petr Broz¹

¹Department of Biochemistry, University of Lausanne, Switzerland

*Correspondence to Petr Broz
Department of Biochemistry, University of Lausanne, Switzerland.
Tel : + 41 21 692 5656
E-mail: petr.broz@unil.ch
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 (GSDMDNT or p30) connected by a linker to a C-terminal regulatory domain (GSDMDCT), 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 GSDMDNT from an intramolecular inhibition by GSDMDCT. This cleavage event allows GSDMDNT to oligomerise in cellular membranes, assembling large pores with a diameter of
around 18 nm, and to cause pyroptosis (Aglietti et al., 2016, Ding et al., 2016, Liu et al., 2016, Mulvihill et al., 2018, Ruan et al., 2018, Sborgi et al., 2016).

Emerging evidence suggest that GSDMD pores not only cause pyroptotic cell death, but that they are also essential for other consequences of inflammasome or caspase-1 activation, e.g. the release of mature IL-1 family cytokines, such as IL-1β and IL-18. Unlike other cytokines, IL-1β and IL-18 lack a signal sequence and are therefore secreted in an endoplasmic reticulum/Golgi-independent manner (Rubartelli et al., 1990). Since inflammasome activation usually elicits near-concurrent secretion of mature IL-1β and pyroptosis in macrophages, it is often proposed that IL-1β and IL-18 are passively released during cell lysis. In line with this model, Gsdmd-deficiency severely abrogates IL-1β secretion upon canonical inflammasome activation (Kayagaki et al., 2015, Shi et al., 2015); and single cell analysis of macrophages revealed that IL-1β release coincides with the uptake of membrane-impermeable nucleic acid dyes (e.g. SYTOX, propidium iodide), a widely used assay to measure the loss of plasma membrane integrity (Liu et al., 2014, Polykratis et al., 2019). By contrast, a number of studies reported that mature IL-1β can be secreted in the absence of intracellular lactate dehydrogenase release, a commonly used assay to quantify cell lysis in a bulk cell population (Chen et al., 2014, Gaidt et al., 2016, Kang et al., 2013, Wolf et al., 2016, Zanoni et al., 2016). Since the standard lactate dehydrogenase release assay may lack single-cell resolution, it remains plausible that mature IL-1β are indeed released by a small fraction of lysed cells upon inflammasome activation. However, several lines of evidence support the notion that cell lysis is not an absolute requirement for IL-1β secretion. For example, ectopic expression of mature IL-1β in primary macrophages is sufficient to induce its secretion in the absence of inflammasome activation (Monteleone et al., 2018); and single cell analysis of live, viable murine embryonic fibroblast revealed considerable IL-1β secretion after caspase-1 or -8 activation (Conos et al., 2016). Consistent with these observations, a number of recent studies demonstrated that sublytic GSDMD pores (18 nm) are indeed large enough for the release of mature IL-1β (Evavold et al., 2018, Heilig et al., 2018) or entry of nucleic acid dyes (DiPeso et al., 2017, Russo et al., 2016), indicating that GSDMD can act as a conduit for IL-1β release in the absence of cell lysis. Studies carried out by us on ESCRT-III-dependent membrane repair have
further strengthened the notion that cells can tolerate a certain number of GSDMD membrane pores (Ruhl et al., 2018). The model that emerges from these studies implies that caspase activation proceeds from a sub-lytic phase in which cells feature transient assembly of GSDMD pores to a lytic phase where GSDMD pores cause a complete breakdown of membrane integrity. Whether cells transit from the sub-lytic to the lytic phase depends on the strength of the activating signal, level of GSDMD expression and activation, cell type and the activity of membrane repair mechanism. Furthermore, recent findings indicate that while GSDMDNT is sufficient to assemble pores in vitro or when overexpressed, its activity might be regulated by additional mechanisms under physiological conditions. For example, it has been proposed that complete GSDMD-dependent cell lysis requires SARM1-dependent depolarization of mitochondria in macrophages (Carty et al., 2019), indicating that mitochondrial damage is critical for the transition into the lytic-phase of GSDMD activation in this cell type.

Altogether these new findings highlight that more research is necessary to understand how GSDMD expression and activity is regulated on a translational and post-translational level, and which cellular membranes/organelles need to be targeted by GSDMDNT to induce pyroptotic cell death or to exert its lysis-independent functions. In the following we however focus on an emerging host of studies that have begun to uncover cell-type-specific and/or inflammasome-independent functions of GSDMD, and on the enigmatic role of GSDME, another member of the gasdermin family, in cell death.

GSDMD function in neutrophils

Neutrophils resist pyroptosis upon canonical inflammasome activation

Neutrophils express a repertoire of pattern recognition receptors (PRR) and are recruited in large quantity to a site of infection or inflammation, therefore are excellent candidates to drive inflammasome-dependent responses in vivo (Thomas & Schroder, 2013). However, earlier studies overlooked possible functions for neutrophil inflammasomes, after observing that neutrophils contributed to IL-1β processing through caspase-1-independent mechanisms in a mouse model of acute arthritis (K/BxN serum transfer) or upon FAS (CD95) ligation (Guma et al., 2009, Joosten et
al., 2009, Miwa et al., 1998). In addition, two earlier studies proposed that neutrophils are unlikely to signal via inflammasomes during *Salmonella* Typhimurium or *Burkholderia pseudomallei* infection because these cells do not express NLRC4, an inflammasome-forming PRR that senses bacterial virulence factors (Ceballos-Olvera et al., 2011, Miao et al., 2010). Subsequent studies have now challenged these findings, as multiple groups readily detect expression of inflammasome-forming PRRs including NLRC4, NLRP3 and AIM2, and other components of the inflammasome signalling complex including the adaptor protein ASC, and the protease zymogen, caspase-1 in murine and human neutrophils (Bakele et al., 2014, Chen et al., 2016, Chen et al., 2014, Karmakar et al., 2015, Karmakar et al., 2016, Mankan et al., 2012).

In agreement with this, exposure of neutrophils to the NLRC4 agonist *Salmonella* Typhimurium or the AIM2 agonist cytosolic double-stranded DNA triggered caspase-1 activation and caspase-1-dependent IL-1β processing (Chen et al., 2014). Although *Nlrp3* mRNA is basally expressed at much higher levels in neutrophils than macrophages (Chen et al., 2014), only soluble NLRP3 agonists such as ATP or the bacterial toxin nigericin, but not particulate or crystalline NLRP3 agonists (e.g. silica or monosodium urate crystals) are able to activate the neutrophil NLRP3 inflammasome (Chen et al., 2016). This highlights that inflammasome signalling is specialised even between the two closely related myeloid cell lineage. In agreement with this, while caspase-1 activation triggers rapid macrophage pyroptosis (Kayagaki et al., 2015, Shi et al., 2015), canonical inflammasome (e.g. NLRC4, NLRP3, AIM2) activation in neutrophils selectively triggers caspase-1-dependent IL-1β processing without concomitant pyroptotic cell death (Chen et al., 2014, Chen et al., 2018b, Karmakar et al., 2015, Karmakar et al., 2016). Although neutrophils are relatively short-lived cells and murine neutrophils have a half-life of 18 h in circulation (5.4 days in humans) (Pillay et al., 2010), exposure of neutrophils to cytokines (e.g. GM-CSF, IL-1β, IFN-γ) and pathogen-derived products (e.g. LPS) can significantly increase their lifespan up to 96 h, indicating that neutrophils can significantly prolong their lifespan during infection (Colotta et al., 1992). This unique ability of neutrophils to resist pyroptosis enables the recruited neutrophils to maintain their lifespan to clear the microbial insult or cellular debris; and sustain IL-1β release to recruit, activate and prolong the lifespan of neutrophils at a site of infection (Chen et al., 2014, Karmakar et al., 2015, Karmakar et al., 2016). While inflammasomes are important for host
defence, gain-of-function mutations in inflammasomes can also drive a variety of hereditary inflammatory disease (e.g. Muckle-Wells Syndrome, macrophage activating syndrome) (Agostini et al., 2004, Canna et al., 2014, Romberg et al., 2014). These diseases are currently attributed to inflammasome dysfunction in monocytes and macrophages, in which IL-1β/18 production is rapidly curtailed by pyroptotic cell death. Intriguingly, IL-1β production and inflammation are not self-limiting in these diseases, suggesting that the cellular source of IL-1β in these diseases may indeed be derived from other cell types. Since neutrophils express majority of the inflammasome signalling components, and that neutrophil IL-1β production proceeds in the absence of pyroptosis, it would be of interest to examine the contribution of neutrophil-derived IL-1β in human inflammatory disease in future studies.

The mechanisms by which neutrophils resist caspase-1-dependent pyroptosis is likely to be controlled by careful fine tuning of the expression of specific pyroptotic machineries in these cells. Although GSDMD is expressed at comparable levels between neutrophils and macrophages (Chen et al., 2018b, Heilig et al., 2018), neutrophils express relatively low level of ASC and caspase-1, therefore, neutrophil inflammasomes assemble with a smaller ASC ‘speck’ with reduced caspase-1 activity (Boucher et al., 2018, Chen et al., 2018a, Chen et al., 2018b). Since caspase-1 cleaves pro-IL-1β better than it cleaves GSDMD (Chen et al., 2018b), this specific fine-tuning of caspase-1 activity in neutrophils ensures that caspase-1 only generates sublytic GSDMD pores to enable IL-1β secretion without concomitant cell lysis (Chen et al., 2018b) (Figure 1). However, it is possible that additional mechanisms exist to restrict caspase-1-driven pyroptosis in neutrophils. For example, neutrophils may repair plasma membrane GSDMD pores via ESCRT-III repair mechanisms as reported for macrophages and HeLa cells (Ruhl et al., 2018). However, this hypothesis would be ambitious to demonstrate since it is technically challenging to manipulate primary neutrophils. SARM1 is a TIR-containing protein that is involved in TLR signalling. A recent study revealed a surprising role for SARM in driving optimal macrophage pyroptosis (Carty et al., 2019). Interestingly, Sarm1-deficient macrophages appears to be phenotypically similar to neutrophils, as both cell types release IL-1β in the absence of pyroptosis upon canonical inflammasome activation (Carty et al., 2019, Chen et al., 2014). Neutrophils were already documented to suppress TLR4-TRIF signalling to repress RIPK3-dependent cell death (Chen et al.,
2018a), therefore, it is conceivable that neutrophils likewise suppress SARM1 expression to subvert caspase-1-dependent pyroptosis. Further studies should characterise the expression of SARM1 in neutrophils, and whether overexpression of SARM1 triggers neutrophil caspase-1-dependent pyroptosis.

Caspase-11 and neutrophil elastase cleave GSDMD to elicit NETosis

Although caspase-1 activation does not trigger pyroptosis in neutrophils, activation of the caspase-11 (non-canonical) inflammasome by cytosolic LPS or cytosolic Gram-negative bacteria triggered robust GSDMD cleavage and cell lysis in neutrophils, indicating that these cells are not intrinsically resistant to GSDMD pores (Chen et al., 2018b). Surprisingly, pyroptotic neutrophils appeared morphologically distinct from caspase-1 or -11-activated macrophages. Instead, caspase-11 and GSDMD activation triggered classical hallmarks of NETosis, including nuclear delobulation, histone citrullination, DNA extrusion, and rupture of nuclear, granule and plasma membrane (Figure 1). Strikingly, neutrophil elastase, myeloperoxidase and PAD4, three key enzymes involved in classical NETosis are dispensable for caspase-11-dependent NET extrusion, indicating that caspase-11 and GSDMD may directly induce these hallmarks of NETosis (Chen et al., 2018b). In support of this, the combination of recombinant GSDMD and caspase-11 is sufficient to trigger neutrophil nuclear membrane rupture, chromatin relaxation and histone H3 degradation in a cell-free system. Further, application of exogenous Dnase I to neutralise caspase-11 and GSDMD-driven NETs impairs in vivo host defence against a cytosolic mutant of Salmonella (ΔsifA), revealing a previously undescribed host protective function of NETs against cytoplasmic infection (Chen et al., 2018b). Given that cell type-specific signalling has such a profound impact on the phenotypical outcome of GSDMD-induced cell death, it will be very interesting to investigate the consequences of GSDMD activation in other granulocytes, as well as non-immune cells.

Neutrophil elastase cleaves GSDMD to trigger neutrophil cell death and NETs

Although GSDMD was initially identified as a substrate of inflammatory caspases, two recent studies documented that GSDMD is also processed the serine proteases, neutrophil elastase in neutrophils (Figure 2) (Kambara et al., 2018, Sollberger et al., 2018). Although neutrophil elastase and cleave GSDMD several amino acids
upstream of the canonical caspase cleavage site, this did not affect the ability of GSDMD N-terminal fragment to oligomerise and induce lytic cell death upon overexpression in HEK 293T cells, in line with the observation that the membrane insertion and lytic properties of GSDMD N-terminal fragment lies within the first 243 amino acid (Shi et al., 2015). However, despite these observations, conclusion from the both studies were vastly different. In one study, neutrophil elastase-dependent GSDMD cleavage was proposed to trigger cell death in aging neutrophils. Consequently, when challenged intraperitoneally with *E. coli* K12, *Gsdmd*-deficient mice accumulated more neutrophils at a site of infection and were more resistant to infection than wild type animals (Kambara et al., 2018). However, whether GSDMD promotes spontaneous neutrophil cell death is controversial, as other studies documented similar rate of spontaneous neutrophil death in wild type versus *Gsdmd*-deficient neutrophils (Burgener et al., 2019, Chen et al., 2018b). In agreement with macrophage studies that the GSDMDNT fragment triggers proinflammatory cell death, a second study reported that activation of GSDMD by neutrophil elastase drive neutrophil cell lysis and NET extrusion, a well-appreciated antimicrobial defence mechanism (Sollberger et al., 2018). Therefore, it appears that GSDMD activity in neutrophils can either promote or dampen host defence. The signalling mechanisms that dictate these differences have not been investigated in detail, however, it is tempting to speculate that the signal strength and cellular location of neutrophil elastase is a key regulator. It is well documented that a high concentration of neutrophil elastase translocates from azurophilic granules to the nucleus at the early stages of NETosis, and that nuclear membrane damage precedes cellular rupture (Metzler et al., 2014, Papayannopoulos et al., 2010, Sollberger et al., 2018). In this scenario, it is likely that the close proximity of cleaved GSDMD preferentially disrupts the nuclear membrane to initiate the hallmarks of NETosis. In contrast, it is conceivable that a much lower intensity of neutrophil elastase ‘escapes’ from azurophilic granules into the cytosol in aged neutrophils, which cleaves a low but steady amount of GSDMD to trigger neutrophil death without accompanying NETosis. Since GSDMD drives a variety of inflammatory disease and is thus an attractive pharmacological target, additional studies are clearly required to further characterise the function of GSDMD in neutrophils during inflammatory disease and infection.

**GSDMD function during apoptosis**
Emerging evidence of apoptosis-induced inflammation

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

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

While the studies above clearly implicate an important function for caspase-8 in driving inflammation and even embryonic lethality, the molecular mechanisms by which caspase-8 promotes cell lysis and NLRP3 activation remains unsolved. By using pharmacological inhibitors of TAK1 or IAPs (e.g. SMAC-mimetics), we and others recently demonstrate that the pyroptotic effector GSDMD plays a major role in this process (Chen et al., 2019b, Orning et al., 2018, Sanjo et al., 2019, Sarhan et al., 2018). Unexpectedly, under these conditions, GSDMD is processed into the lytic p30 fragment via two pathways. The first pathway involves direct cleavage of GSDMD by caspase-8 at position D276, similar to canonical caspase cleavage site described for caspase-1 and -11. However, caspase-8 is 30-fold less efficient than caspase-1 in
processing GSDMD, and caspase-8-dependent GSDMD cleavage is only observed under conditions of strong caspase-8 activation (Chen et al., 2019b). This likely explains why early studies failed to observe GSDMD processing into the active p30 fragment by recombinant caspase-8 (Shi et al., 2015). The second pathway leading to GSDMD activation occurs via potassium efflux and activation of the NLRP3 inflammasome (Conos et al., 2017), however, the mechanisms by which caspase-8 drives NLRP3 activation is still a matter of debate and is discussed in greater details in the subsequent paragraphs.

The finding that caspase-8 triggers direct GSDMD activation is exciting and raises several important questions. For example, what is the physiological function of caspase-8-dependent GSDMD activation? Numerous pathogens are equipped with virulence factors that inhibit host NF-κB signalling pathways. This could in turn promote caspase-8 activation and induce GSDMD cleavage and pyroptosis, as recently reported for *Yersinia* infection (Orning et al., 2018, Sarhan et al., 2018). However, as pyroptosis is best known as an innate immune mechanism to restrict intracellular pathogen infection, how GSDMD activation can promote host defence against *Yersinia*, a predominantly extracellular pathogen is unclear, and has not been formally demonstrated. RIPK1/caspase-8-dependent apoptosis can promote the release of alarmins and activate neighbouring immune cells for cytokine production and anti-*Yersinia* defence (Peterson et al., 2017), yet whether GSDMD is also required in this scenario is unclear and warrants further investigation. Likewise, it would be of interest to investigate whether the caspase-8-GSDMD axis induces NET extrusion to combat extracellular pathogens, or whether this signalling axis is exploited by *Yersinia* to promote pathogen dissemination *in vivo*.

Another important question that arises from the discovery that caspase-8 cleaves GSDMD is the molecular mechanisms by which apoptosis remains immunologically silent during tissue homeostasis. Several lines of evidence suggest that executioner caspases play a key role in regulating the level of GSDMD activity in apoptotic cells, as previous studies documented that caspase-3, and a lesser extent caspase-7, cleave GSDMD at position D88 (D87 in humans) to disrupt its pore-forming ability (Rogers et al., 2017, Taabazuing et al., 2017). In keeping with this, *Gsdmd*^D88A/D88A^
knock-in macrophages harbouring a caspase-3/7-uncleavable mutation accumulated GSDMD\textsuperscript{NT} pores, resulting in enhanced pyroptosis compared to wild type macrophages (Chen et al., 2019b). However, naïve Gsdmd\textsuperscript{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 \textit{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 \textit{Birc2}\textsuperscript{−/−} \textit{Birc3}\textsuperscript{−/−} 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.

\textbf{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 \textit{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 \textit{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...
membrane damage, potassium efflux and NLRP3 inflammasome activation (Figure 3) (Kayagaki et al., 2015, Ruhl & Broz, 2015, Shi et al., 2015). In contrast, our study revealed that GSDMD is dispensable for caspase-1 activation during TNF-induced caspase-8 activation. Instead, we demonstrate that caspase-8-dependent NLRP3 activation requires the channel-forming transmembrane glycoprotein, pannexin-1. For this, caspase-8 promotes downstream executor caspase-3/7 activation, which cleave and activate pannexin-1 channel activity, membrane permeability and NLRP3 inflammasome activation (Figure 3) (Chen et al., 2019a, Chen et al., 2019b). Further support for the importance of pannexin-1 in driving NLRP3 activation during apoptosis comes from the fact that caspase-3/7 and pannexin-1 is also required also for NLRP3 activation upon caspase-9-dependent intrinsic apoptosis, which unlike caspase-8, does not have the ability to cleave GSDMD (Chen et al., 2019a, Chen et al., 2019b, Vince et al., 2018).

The reasons for this discrepancy are unclear, however, it is tempting to speculate that the cellular activity of executor caspase-3/7 critically controls the amount of GSDMD pores and pannexin-1 activation in a given cell, and that dictates which pathway is preferentially activated. For example, a given cell with high caspase-3/7 activity would inactivate GSDMD pores and favour NLRP3 activation via pannexin-1 channels. On the other hand, cells with low caspase-3/7 activity would favour NLRP3 activation via GSDMD pores but not pannexin-1 channels. Given that executor caspase-3/7 activity is often suppressed in transformed cells and that many cancer chemotherapies induce tumour cell death through caspase-8, future studies should further characterize this pathway in the context of cancer chemotherapy, and whether modulating this signalling axis can promote tumour clearance.

**GSDME activation by caspase-3 promotes pyroptosis in some but not all cells**

The discovery that cleavage of GSDMD at the linker region by inflammatory caspases unleashes the pore-forming function of GSDMD\textsubscript{NT} has significantly enhanced the field’s understanding of gasdermin family proteins. Indeed, recent studies found that GSDME features a caspase-3 cleavage motif in its linker region. Similar to GSDMD, cleavage of GSDME by caspases-3/-7 liberates the N-terminal pyroptosis-inducing domain (GSDME\textsubscript{NT}) from its autoinhibitory C-terminal regulatory domain to trigger 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\textsuperscript{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 \textit{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 \textit{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.

\textbf{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\textsuperscript{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
efforts should be made to develop specific gasdermin inhibitors and to explore the
possibility of therapeutical targeting of the gasdermin family.

Acknowledgement
This work was supported by a Swiss Government Excellence (ESKAS) postdoctoral
fellowship and a Marie Sklodowska-Curie Actions Individual Fellowship (MSCA-IF-
2018-838252) to K.W.C, and a European Research Council Grant (ERC2017-CoG-
770988-InflamCellDeath) to P.B.

Reference
Aglietti RA, Estevez A, Gupta A, Ramirez MG, Liu PS, Kayagaki N, Ciferri C, Dixit VM,
Dueber EC (2016) GsdmD p30 elicited by caspase-11 during pyroptosis forms pores
in membranes. Proc Natl Acad Sci U S A 113: 7858-63
NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-
Wells autoinflammatory disorder. Immunity 20: 319-25
Bakele M, Joos M, Burdi S, Allgaier N, Poschel S, Fehrenbacher B, Schaller M,
289: 5320-9
Boucher D, Monteleone M, Coll RC, Chen KW, Ross CM, Teo JL, Gomez GA, Holley
self-cleavage is an intrinsic mechanism to terminate inflammasome activity. J Exp Med
215: 827-840
Brennan MA, Cookson BT (2000) Salmonella induces macrophage death by caspase-
1-dependent necrosis. Mol Microbiol 38: 31-40
Brumatti G, Ma C, Lalouei N, Nguyen NY, Navarro M, Tanzer MC, Richmond J, Ghisi
M, Salmon JM, Silke N, Pomilio G, Glaser SP, de Valle E, Gugasyan R, Gurthridge
caspase-8 inhibitor emricasan combines with the SMAC mimic birinapant to induce
necroptosis and treat acute myeloid leukemia. Sci Transl Med 8: 339ra69
Cathepsin G Inhibition by Serpinb1 and Serpinb6 Prevents Programmed Necrosis in
Neutrophils and Monocytes and Reduces GSDMD-Driven Inflammation. Cell Rep 27:
3646-3656 e5
Canna SW, de Jesus AA, Gouni S, Brooks SR, Marrero B, Liu Y, DiMattia MA, Zaal
KJ, Sanchez GA, Kim H, Chapelle D, Plass N, Huang Y, Villarino AV, Biancotto A,
Fleisher TA, Duncan JA, O'Shea JJ, Benseler S, Grom A et al. (2014) An activating
NLRC4 inflammasome mutation causes autoinflammation with recurrent macrophage
activation syndrome. Nat Genet 46: 1140-6
Carty M, Kearney J, Shanahan KA, Hams E, Sugisawa R, Connolly D, Doran CG,
Survival and Cytokine Release after Inflammasome Activation Is Regulated by the
Toll-IL-1R Protein SARM. Immunity 50: 1412-1424 e6
Ceballos-Olvera I, Sahoo M, Miller MA, Del Barrio L, Re F (2011) Inflammasome-dependent pyroptosis and IL-18 protect against Burkholderia pseudomallei lung infection while IL-1beta is deleterious. PLoS Pathog 7: e1002452


Chen KW, Demarco B, Broz P (2019a) Panneexin-1 promotes NLRP3 activation during apoptosis but is dispensable for canonical or Non-canonical inflammasome activation. Eur J Immunol


Neutrophils express several inflammasome-forming PRR including NLRC4, NLRP3, AIM2 and caspase-11. Activation of canonical inflammasome selectively triggers IL-1β maturation without accompanying cell lysis. IL-1β secretion in living neutrophils require the pore-forming protein GSDMD. Upon cytoplasmic Gram-negative bacterial infection, caspase-11 triggers robust GSDMD cleavage. GSDMDNT targets plasma membrane and nuclear membrane to elicit neutrophil extracellular traps (NETs). Caspase-11-driven GSDMD pores promotes potassium efflux and activation of the NLRP3 inflammasome.

In aging neutrophils, release of neutrophil elastase (NE) from specific neutrophil granules cleave and activate GSDMD, resulting in neutrophil cell death. Upon treatment with classical NETosis activators (e.g. PMA), reactive oxygen species (ROS) promote the release of NE from the granules to cytosol in an ill-defined manner. NE cleaves and activate GSDMD, leading to nuclear and plasma membrane rupture and neutrophil cell lysis by NETosis.

In TNF-stimulated cells, loss or inhibition of IAP and TAK1 function promotes assembly of a caspase-8-activating platform called TNF Complex IIb (also commonly referred as the ripoptosome). Active caspase-8 cleaves GSDMD at D276, leading to pyroptosis. Caspase-8-driven GSDMD activation, or caspase-3/7-dependent pannexin-1 activation promotes potassium efflux and NLRP3 assembly. NLRP3-dependent caspase-1 activation cleaves GSDMD to further drive pyroptosis. Probenecid, spironolactone and trovafloxacin are pannexin-1 channel inhibitors.

Fig. 1
Fig. 2

Fig. 3