

REVIEW

# Gasdermins and their role in immunity and inflammation

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The gasdermins are a family of pore-forming proteins recently implicated in the immune response. One of these proteins, gasdermin D (GSDMD), has been identified as the executioner of pyroptosis, an inflammatory form of lytic cell death that is induced upon formation of caspase-1-activating inflammasomes. The related proteins GSDME and GSDMA have also been implicated in autoimmune diseases and certain cancers. Most gasdermin proteins are believed to have pore-forming capabilities. The best-studied member, GSDMD, controls the release of the proinflammatory cytokines IL-1 $\beta$  and IL-18 and pyroptotic cell death. Because of its potential as a driver of inflammation in septic shock and autoimmune diseases, GSDMD represents an attractive drug target. In this review, we discuss the gasdermin proteins with particular emphasis on GSDMD and its mechanism of action and biological significance.

## Introduction

Innate immunity plays a crucial role in protecting the host from microbial infection. Programmed cell death is a key pillar of this defense. Many pathogens infect cells and exploit intracellular niches to facilitate their replication and spread. In turn, host cells have evolved mechanisms to detect and counteract these efforts. Certain signals however, are perceived as being so dangerous that the cell commits to programmed cell death. This commitment results in cell lysis and the release of intracellular inflammatory signals, so-called danger signals. Programmed cell death removes the bacterial intracellular niche and alerts neighboring cells to the presence of infection. One form of programmed cell death is called pyroptosis and was first described in 1992 as a form of apoptosis occurring during infection with the Gram-negative bacterium *Shigella flexneri* (Zychlinsky et al., 1992). This cell death process was coined “pyroptosis” due to its inflammatory nature and classified as a distinct form of cell death (Cookson and Brennan, 2001).

## Pyroptosis

Pyroptosis is characterized by pore formation in the plasma membrane, swelling, and rupture of the cell and is activated in response to diverse microbial ligands. Examples include bacterial flagellin, bacterial secretion systems or toxins, and LPS or DNA that gains access to the cell cytosol (Broz and Dixit, 2016). These are strong danger signals requiring the cell to respond

appropriately. The importance of pyroptosis in controlling several viral and bacterial infections has been clearly demonstrated (Miao et al., 2010; Aachoui et al., 2013; Maltez et al., 2015; Zhu et al., 2017, 2018; Banerjee et al., 2018; Cerqueira et al., 2018; Gonçalves et al., 2019; Wang et al., 2019). Pyroptosis is a protective host-defense measure, as it also controls the release of inflammatory cytokines and danger signals and removes the replicative niche of a pathogen. In addition, pyroptosis functions to trap intracellular bacteria within the cellular debris of the pyroptotic cell (Jorgensen et al., 2017). These pore-induced intracellular traps can then recruit neutrophils and initiate further immunological responses (Jorgensen et al., 2016). However, as with all aspects of the immune response, pyroptosis can also have deleterious outcomes. For example, during endotoxic or septic shock, excessive pyroptosis leads to an overwhelming inflammatory response, resulting in tissue and organ damage (van der Poll and Opal, 2008).

## Inflammasomes

The major pathway leading to pyroptosis in cells involves the inflammasome. Inflammasomes are large multiprotein complexes typically comprising a pattern recognition receptor or initiator protein such as NLRP3 or AIM2, an adaptor protein called ASC, and an executioner caspase, which is typically caspase-1. The initiator can detect microbial ligands directly or indirectly, while caspase-1 drives the activation of pyroptosis.

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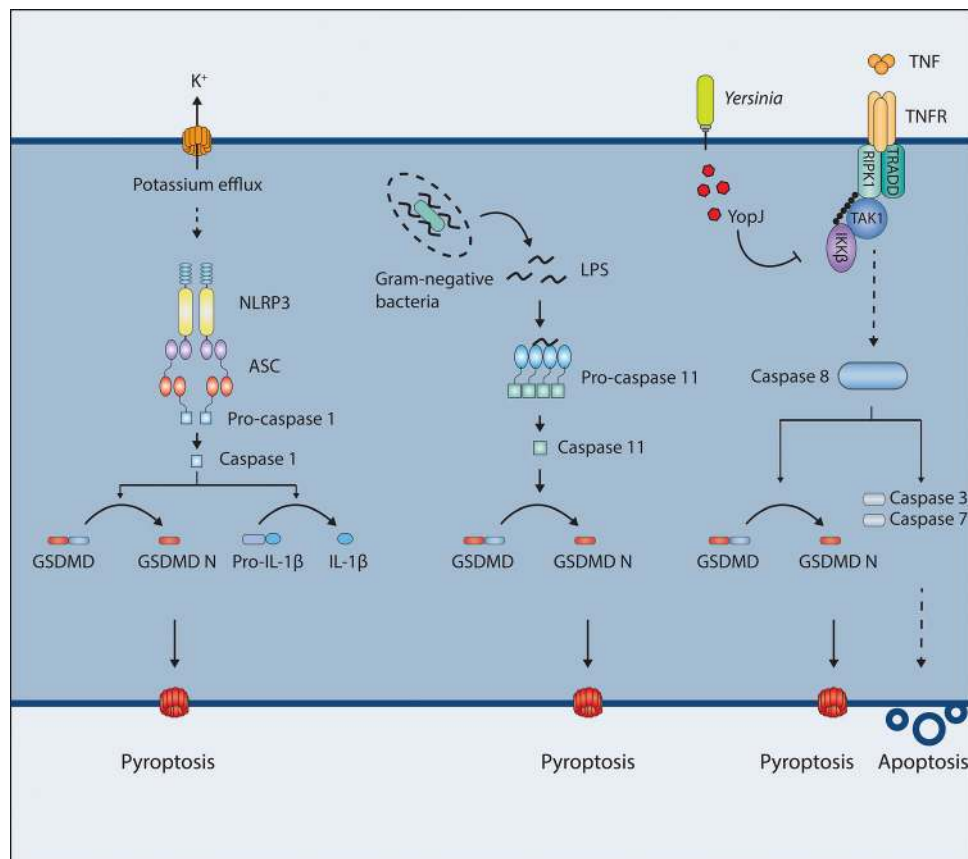


Figure 1. **Different paths to pyroptosis.** Caspase-1, caspase-8, and caspase-11 all process GSDMD and lead to pyroptosis. Caspase-1 is activated downstream of inflammasomes such as the NLRP3 inflammasome triggered by potassium efflux. Caspase-11 is activated by intracellular LPS from Gram-negative bacteria or by oxPAPCs. Caspase-8 is activated during extrinsic apoptosis, for instance during TAK1 or I $\kappa$ B kinase complex (IKK) inhibition or under certain conditions of intrinsic apoptosis.

Caspases are activated by dimerization, leading to autocleavage and generation of catalytically active protein complexes (Yang et al., 1998; Boucher et al., 2018; Lee et al., 2018b). Active caspase-1, in turn, leads to proteolytic processing of substrate proteins, which include the proinflammatory cytokines IL-1 $\beta$  and IL-18 as well as gasdermin D (GSDMD). There are cases, however, where pyroptosis is activated independently of caspase-1. For instance, noncanonical inflammasome activation, mediated by caspase-11, is a pathway that converges on GSDMD independently of NLR sensor and adaptor proteins. Activation of caspase-11 (or caspase-4 and -5 in humans) occurs following recognition of LPS (Kayagaki et al., 2011, 2013; Hagar et al., 2013; Shi et al., 2014) or host-derived oxidized phospholipids (oxPAPCs; Zanoni et al., 2016). A third mode of pyroptotic cell death has recently been described. During certain types of extrinsic and intrinsic apoptosis, activated caspase-8 can induce a lytic form of cell death with features of both pyroptosis and apoptosis (Orning et al., 2018; Sarhan et al., 2018; Chen et al., 2019). Fig. 1 describes these mechanisms, highlighting the key pathways controlling pyroptosis.

#### Gasdermins, the pore-forming effectors

In 2015, GSDMD was identified as the executioner of pyroptosis (Kayagaki et al., 2015; Shi et al., 2015a). Two groups demonstrated

that caspase-1 and caspase-11 cleaved and activated GSDMD, leading to pore formation and pyroptosis. GSDMD is part of a larger family of proteins consisting of GSDMA, GSDMB, GSDMC, GSDMD, Gasdermin E (GSDME, also referred to as DFNA5 [deafness, autosomal dominant 5]), and DFNB59 (Pejvakin; GSDMA1-3, GSDMC1-4, GSDMD, GSDME, and DFNB59 in mice). While several gasdermins have been associated with human diseases through genetic linkages, the precise function and mechanism of activation of most of these proteins remain largely unknown. Interestingly, all except DFNB59 adopt a similar architecture consisting of a pore-forming N-terminal domain and a C-terminal regulatory domain, where cleavage and separation of the two domains is thought to be required for activation. In line with this, the N-terminal domains of GSDMA, GSDMB, GSDMC, GSDMD, and GSDME have all been shown to form pores in artificial membranes (Ding et al., 2016; Rogers et al., 2017).

#### GSDMD

GSDMD is the best-understood member of the gasdermin family. The protein consists of an N-terminal pore-forming domain and a C-terminal inhibitory domain, with a cleavage site in the linker between the two domains at position D276 in mouse and D275 in human. This cleavage site is targeted by caspase-1 activated downstream of inflammasome complexes, including AIM2

sensing of viral DNA, NLRC4 sensing of bacterial flagellin, or NLRP3 sensing of cellular disturbances and plasma membrane disruption (Broz and Dixit, 2016). Additionally, LPS-induced caspase-11 activation leads to robust GSDMD cleavage. Further, in response to *Yersinia spp.* infection, where the activity of TAK1 and IKK (I $\kappa$ B kinase complex) kinases are blocked, a caspase-8 pathway is activated, leading to cleavage and activation of GSDMD at this same site. Of these three caspases, caspase-1 appears to be the strongest driver of GSDMD cleavage and caspase-8 the weakest, perhaps acting more as a backup measure in situations where other caspases are impaired (Ramirez et al., 2018). Finally, in addition to the caspases above, both the neutrophil specific elastase (ELANE) and cathepsin G have been shown to cleave GSDMD upstream of the canonical caspase cleavage site (Kambara et al., 2018; Burgener et al., 2019).

The crystal structure of mouse and human GSDMD was solved in 2019 (Liu et al., 2019). The C-terminal domain functions as an intrinsic inhibitor of the molecule. Once GSDMD is cleaved, the N-terminal domain is liberated and free to oligomerize and insert into the plasma membrane, resulting in pore formation. It is believed that pore formation results in loss of osmotic homeostasis, swelling of the cell, and death (Kayagaki et al., 2015; Shi et al., 2015a; Ding et al., 2016; Liu et al., 2016). However, newer studies raise some questions about the validity of this model (Chen et al., 2016; Davis et al., 2019; de Vasconcelos et al., 2019). Nevertheless, expression of the N-terminal domain by itself is sufficient to trigger pyroptosis. The N-terminal domain binds to phospholipids such as cardiolipin, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate, and with weaker affinity to phosphatidic acid and phosphatidyl serine (Liu et al., 2016). These lipids are present on bacteria, on the mitochondria, and in the plasma membrane. Because of the presence of cardiolipin on bacteria, it has been suggested that GSDMD can also directly lyse bacteria (Ding et al., 2016; Liu et al., 2016; Zhu et al., 2018). The *in vivo* relevance of these findings is unclear, and whether this mechanism comes into play during infection remains to be seen. Binding of the N-terminal GSDMD domain causes oligomerization of protomers resulting in pores in the membrane. Of note, because of the presence of phospholipids on the inner leaflet of healthy cells, N-terminal GSDMD only causes pore formation and lysis from within. GSDMD can also be cleaved by caspase-3 in the N-terminal domain at amino acid D88 upstream of the caspase cleavage site (Taabazuing et al., 2017; Chen et al., 2019). This caspase-3 cleavage was shown to decrease pyroptosis by inhibiting oligomerization and pore formation of the N-terminal GSDMD domain.

### GSDME

GSDME (DFNA5) has also been shown to form lytic membrane pores. GSDME is cleaved and activated by caspase-3 and is thought to contribute to secondary necrosis in response to apoptotic stimuli (Rogers et al., 2017), a condition that is poorly understood. In the absence of GSDME, cells appeared to disassemble into small apoptotic bodies instead of lysing. It was recently shown that GSDME also targets the mitochondrial membrane, with concomitant release of cytochrome c and apoptosome formation (Rogers et al., 2019). Another study

showed that under conditions where caspase-1 was absent or nonfunctional, cells proceeded to undergo pyroptosis even in the absence of GSDMD activation (Schneider et al., 2017). Upon further investigation, this appears to be dependent on caspase-8. It is possible that GSDME, through activation of caspase-3, is responsible for this death. Both of the above studies were conducted in nonimmune cells, however, and it is still not clear if GSDME plays any role in innate immune cells such as macrophages (Lee et al., 2018a).

The name DFNA5 stems from the observation that certain mutations in GSDME result in hearing loss (Van Laer et al., 1998). Most of these mutations affect the inhibitory C-terminal domain of GSDME (Gregan et al., 2003) and could elicit spontaneous pore formation and pyroptosis. GSDME is also a candidate tumor suppressor. It is a transcriptional target of p53 and is silenced in different cancers (Masuda et al., 2006; Akino et al., 2007; Kim et al., 2008). Loss of GSDME has been shown to abrogate the effectiveness of some chemotherapeutic drugs (Lage et al., 2001; Wang et al., 2017).

### GSDMA

In 2018, the crystal structure of the mouse GSDMA3 pore was solved (Ruan et al., 2018), providing critical insight into GSDM pore formation. Although the C-terminal domain of GSDMD, as well as the full-length GSDMA3, had previously been solved (Ding et al., 2016; Kuang et al., 2017; Liu et al., 2018) and later the full-length mouse and human GSDMD (Liu et al., 2019), this was the first crystal structure giving insights into the molecular basis of pore formation. It revealed that GSDMA forms pores consisting of 27–28 protomers with an internal diameter of ~180 Å. The pore is formed by two  $\beta$ -hairpins from each N-terminal protomer oligomerizing into an antiparallel  $\beta$ -barrel that inserts into the membrane.

GSDMA is expressed in epithelial cells and has been linked to autoimmune diseases and cancer (Saeki and Sasaki, 2012). GSDMA is frequently silenced in gastric cancers (Saeki et al., 2007). Other conditions involve epidermal hyperplasia, hyperkeratosis, and hair loss in mice and occur upon mutation of the gene encoding GSDMA3 (Porter et al., 2002; Runkel et al., 2004). It has been suggested to be involved in alopecia, as eight different alopecia-causing mutations have been mapped to GSDMA3 (Tanaka et al., 2013). Robust inflammatory phenotypes in the skin have been observed in GSDMA3 mutant mice (Ruge et al., 2011; Zhou et al., 2012). It is therefore reasonable to speculate that one potential role of GSDMA is triggering pyroptosis in the skin, possibly as a host-defense strategy. It was also proposed that GSDMA could induce autophagy by targeting the mitochondria (Shi et al., 2015b). The N-terminal GSDMA domain enhances autophagy, and these events are reversed through expression of the C-terminal regulatory domain. Genome-wide association studies have also revealed polymorphisms in GSDMB associated with early childhood asthma (Moffatt et al., 2010; Kang et al., 2012; Zhao et al., 2015).

### GSDMC

There is little known about GSDMC. It is not associated with any known human disease, although its restricted expression to the

esophagus, stomach, and large and small intestine suggests it might play a role in intestinal immunity and inflammation. Its expression is also differentially regulated in certain cancers such as colorectal cancer (Miguchi et al., 2016), metastatic melanoma (Watabe et al., 2001), and esophageal cancer (Saeki et al., 2009). The N-terminus of gasdermin C has been shown to induce cytotoxicity, but the mechanisms controlling its activation are unknown (Ding et al., 2016).

#### DFNB59

As mentioned, most gasdermins adopt a similar architecture. There is one exception to this: DNFB59, which has a truncated C-terminal domain. DNFB59 has not been shown to induce pore formation but instead has other proposed roles. It is broadly expressed and has been associated with deafness through its expression in inner ear hair cells (Delmaghani et al., 2006; Schwander et al., 2007). A recent article suggested the involvement of DNFB59 in selective degradation of peroxisomes (pexophagy) during exposure to loud noise (Defourny et al., 2019). DNFB59 was shown to bind the microtubule-associated protein 1 lightchain  $\beta$  through a predicted chaperone domain of DNFB59. Mice lacking DNFB59 exhibited hearing loss during noise overstimulation, and the authors claim this was caused by defective pexophagy.

#### Pore formation as a means of cytokine release and cell death

Upon activation of caspase-1 or 11, the proinflammatory cytokines IL-1 $\beta$  and IL-18 are processed from precursor zymogenic forms to biologically active cytokines. The pro-forms of these cytokines are generated through transcriptional mechanisms typically downstream of TLRs or cytokine receptors that signal via NF- $\kappa$ B. The release of IL-1 $\beta$  and IL-18 is critical for mounting an adequate immune response to many pathogens (Miao et al., 2010; Vladimer et al., 2013). Unlike most cytokines released through conventional means, the IL-1 family of cytokines lack a leader sequence and therefore are not released through typical protein secretion pathways. Until recently, the mechanisms controlling the release of these cytokines, referred to as unconventional protein secretion, were unknown. The discovery of GSDMD shed new light on these mechanisms. The release of IL-1 $\beta$  and IL-18 (and likely other IL-1 family members) was found to be strongly dependent on GSDMD (Kayagaki et al., 2015; Shi et al., 2015a). It was proposed that IL-1 $\beta$  maturation promotes the association of the cytokine adjacent to the plasma membrane based on charge interactions (Monteleone et al., 2018). This happens as the isoelectric charge of the protein changes after being cleaved by caspase-1. The positively charged mature IL-1 $\beta$  associates with negatively charged PIP2-enriched plasma membrane ruffles. GSDMD pores in these ruffles then facilitate the release of IL-1 $\beta$ . A major function of pore formation therefore is to control the release of these cytokines. In addition, GSDMD also leads to rupture of the cell membrane and lysis of the cell, followed by the release of more cytokines and alarmins. As mentioned previously the crystal structure of the N-terminal GSDMA3 pore provided clearer understanding of how gasdermin pores form. In this study it was reported that 27 or 28 individual N-terminal protomers oligomerized into a ring-shaped

$\beta$ -barrel that inserted into the membrane (Ruan et al., 2018). The pores were shown to have an inner diameter of 18 nm. Studies focusing on GSDMD have reported 16 protomer-shaped pores with inner diameters of 12–14 nm (Aglietti et al., 2016; Ding et al., 2016; Liu et al., 2016; Sborgi et al., 2016); however, these studies have not examined the crystal structure of GSDMD. In contrast, studies by Sborgi et al. (2016) and Mulvihill et al. (2018) showed that GSDMD pores could instead be averaging  $\sim$ 20 nm, closer to the diameter of the GSDMA3 pore.

GSDMD pores are often described in the literature as inducing an osmotic imbalance resulting in influx of water, cell swelling, and rupture. However, this view is not uniformly accepted (Chen et al., 2016; Davis et al., 2019; de Vasconcelos et al., 2019). If the pores were large enough to allow the free flow of ions and proteins across the membrane, this would prevent the formation of an osmotic gradient, limit the flux of water, and prevent swelling and rupture. However, even if the flow of small proteins can counteract osmotic differences across the membrane, the pores would still exclude larger proteins and may serve as a bottleneck and contribute to an osmotic gradient. Nevertheless, expression of N-terminal GSDMD alone still leads to rapid induction of pyroptosis and cell rupture. Davis et al. (2019) observed that swelling was due to cleavage and loss of intermediate filaments. Interestingly, they showed that cells lacking caspase-1 or GSDMD still swelled even though their membrane was impermeable to dyes. In the case of nigericin treatment, the swelling appeared to depend on NLRP3 and ASC and led to calpain-dependent cleavage of vimentin and loss of intermediate filaments.

#### Hyperactivation and membrane repair

It has recently been shown by several groups that the cleavage of GSDMD and formation of pores does not uniformly lead to loss of plasma membrane integrity and cell rupture (Zanoni et al., 2016; Evavold et al., 2018; Heilig et al., 2018). The first observation revealed that in dendritic cells, the activation of caspase-11 by oxPAPCs resulted in the release of IL-1 $\beta$ , via mechanisms similar to that observed when cytosolic LPS ligates caspase-11. However, in contrast to the LPS-induced caspase-11 response, oxPAPC-induced cells did not die. This mode of activation led to a “hyperactive” state, as significant amounts of IL-1 $\beta$  were released from cells that maintained their membrane integrity. This was also verified in other cell types such as macrophages and neutrophils, with the IL-1 $\beta$  release being GSDMD dependent (Chen et al., 2014; Evavold et al., 2018; Heilig et al., 2018). Additionally, in contrast to their murine counterparts, human peripheral blood mononuclear cells trigger IL-1 $\beta$  secretion in response to LPS alone. This release appears independent of pyroptosis but instead is dependent on the alternative inflammasome driven by TLR4-RIPK1-CASP8 upstream of NLRP3 (Gaidt et al., 2016).

Exactly how these cells release IL-1 $\beta$  through GSDMD pores yet fail to progress to pyroptosis remains unclear. One possible explanation relates to the density of GSDMD pores. If the amount of cleaved GSDMD is below a certain threshold, the cells could maintain viability and still release IL-1 $\beta$ . In oxPAPC-activated DCs in the hyperactive state, perhaps there was an insufficient density of pores present to lead to cell swelling and

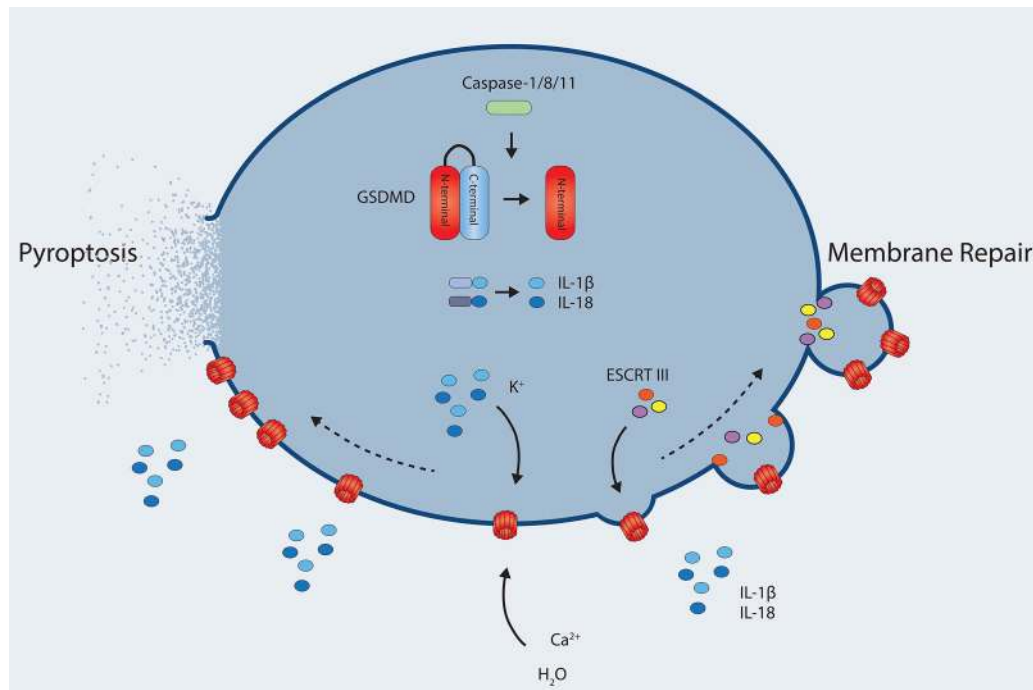


Figure 2. **GSDMD activation does not always lead to cell death.** GSDMD pores can lead to pyroptosis or a state of hyperactivation through ESCRT-mediated membrane repair.

rupture. This could be coupled to inflammasome size, quantity of caspase-1 present, and the kinetics of caspase-1 cleavage (Boucher et al., 2018). However, since the amount of mature IL-1 $\beta$  appears to be considerable, another explanation could be that sufficient amounts of GSDMD does get cleaved but that the N-terminal GSDMD is prevented from inserting into the plasma membrane and forming pores. Alternatively, under these conditions, the membrane is repaired to prevent rupture more readily. In support of the former case, Mulvihill et al. (2018) showed that the phospholipid composition of the plasma membrane affects pore formation, with phosphatidylinositide increasing and cholesterol decreasing insertion of GSDMD into the membrane. Altering the plasma membrane composition could potentially limit the number of GSDMD pores present. In support of the latter case, it was recently demonstrated that the ESCRT-III (endosomal sorting complexes required for transport III) repair machinery protects cells from undergoing pyroptosis following GSDMD activation by removing GSDMD pores and possibly preventing osmotic lysis of the perforated cell (Rühl et al., 2018). The authors showed that this was triggered by calcium flux, suggesting that changes in ionic concentrations after pore formation could reverse pyroptosis through engagement of the ESCRT machinery (Fig. 2). Similar findings were found during necroptosis, where ESCRT-III limits mixed-lineage kinase domain-like pseudokinase (MLKL)-induced cell death through shedding of MLKL pores (Gong et al., 2017). Most recently, a new study shed further light on the switch between hyperactivation and pyroptosis through the identification of the Toll-IL-1R domain-containing protein SARM (sterile  $\alpha$  and HEAT armadillo motif-containing protein) as a key regulator of these events (Carty et al., 2019). SARM is conserved from

nematode to mammals and was previously shown to be required during fungal infection in *Caenorhabditis elegans*. Deletion of SARM in mammalian macrophages enhanced inflammasome assembly, IL-1 $\beta$  processing, and GSDMD cleavage, yet reduced pyroptosis in bone marrow-derived macrophages (BMDMs). SARM mediates this effect via two mechanisms. Through its Toll-IL-1R domain, SARM binds NLRP3 and ASC and subsequently inhibits ASC oligomerization, while at the same time colocalizing to the mitochondria to induce mitochondrial depolarization and blockade of pyroptosis. Exactly how this latter mechanism impacts pyroptosis is still unclear.

Interestingly, it has also been shown that uric acid crystals activate GSDMD cleavage; however, GSDMD is dispensable for IL-1 $\beta$  release and cell death, suggesting that downstream of caspase-1, GSDMD-independent processes are engaged (Rashidi et al., 2019). The addition of glycine to the extracellular medium can also inhibit pyroptosis of BMDMs (Brennan and Cookson, 2000; Fink and Cookson, 2006). Glycine prevents the release of LDH; however, this does not seem to affect pore formation itself as visualized by uptake of PI and release of IL-1 $\beta$  (Evavold et al., 2018). The effect of glycine on cells could appear also to vary from cell to cell, as it was shown by Davis et al. (2019) to have no effect on THP-1 cells.

### Structural insights on GSDMD

The original finding by Kayagaki et al. (2015), one of the two studies that identified GSDMD, was based on a genetic approach that induced an I105N mutation in mouse GSDMD. Even though this mutation, along with its human counterpart I104N, did not affect GSDMD cleavage or liposome binding, it blocked pyroptosis and IL-1 $\beta$  secretion (Aglietti et al., 2016). At high

concentrations of  $\geq 5 \mu\text{M}$  in a calcium-loaded liposome release assay, it was shown that the I104N GSDMD mutation could induce pore formation and  $\text{Ca}^{2+}$  release, but at lower concentrations, it was severely impeded in doing so. From the crystal structures of GSDMA3, it was shown that the GSDMA3-corresponding residue of I104 is located in one of the two  $\beta$ -hairpins that insert into the plasma membrane (Ruan et al., 2018).

GSDMD is cleaved by caspases-1, -8, and -11 at residue D276 in the mouse protein and D275 in the human protein. This cleavage is essential to release the N-terminal domain from the inhibitory grip of the C-terminus, and mutations at this site result in noncleavable and nonfunctional GSDMD (Shi et al., 2015a). Other residues of interest include those that allow the C-terminus to interfere and inhibit the activation of the N-terminal domain. Mutation of three amino acids in the C-terminal domain modeled to interact with the N-terminus (L290, Y373, and A377) results in autoactivation of human GSDMD and cell death (Ding et al., 2016). The same was shown for the mouse counterparts (L292, Y376, and A380; Rathkey et al., 2017; Liu et al., 2018). The crystal structure of GSDMD provided further clarity into this. It showed that the residues F50 and W51 in the  $\beta$ 1- $\beta$ 2 loop of the mouse N-terminal domain (F49 and W50 in human GSDMD) inserted into a hydrophobic pocket consisting of L292, E295, Y376, A380, S470, and A474 in the C-terminal domain (Liu et al., 2019). Mutations of the residues in the hydrophobic pocket compromised autoinhibition and led to membrane permeabilization. In addition to the  $\beta$ 1- $\beta$ 2 loop, the  $\alpha$ 1 helix is also masked by the C-terminal domain, and mutations of key residues in both the  $\beta$ 1- $\beta$ 2 loop and the  $\alpha$ 1 helix demonstrated the importance of these motifs in pyroptosis (Fig. 3). The importance of the  $\alpha$ 1 helix in membrane binding had also been described by Ruan et al. (2018) when they solved the crystal structure for the GSDMA3 pore. The authors mutated three residues of the human GSDMD  $\alpha$ 1 helix (R7, R10, and R11) and observed diminished liposome leakage and pore formation. Taken together, these findings could explain the reduced efficacy of pyroptosis after caspase-3 cleavage at site D87/88 (human/mouse GSDMD; Taabazuung et al., 2017; Chen et al., 2019), as both the  $\beta$ 1- $\beta$ 2 loop and the  $\alpha$ 1 helix are located within this region.

Additional attempts to understand the mechanism of action for GSDMD came from Liu et al. (2016), who argued that positively charged residues are required for insertion into the negatively charged plasma membrane and recognized a set of conserved positively charged residues across several species. Mutation of these residues identified R138, K146, R152, and R154 as essential for membrane insertion and pyroptosis induction.

Finally, it was also proposed that oligomerization of GSDMD induces disulfide bonds between the different N-terminal promoters (Liu et al., 2016). The critical residue here was suggested to be C191 in human GSDMD (C192 in mouse GSDMD), as mutations in this amino acid reduced oligomerization and blocked pyroptosis (Liu et al., 2016; Rathkey et al., 2017; Hu et al., 2018). Interestingly, however, in the crystal structure of both GSDMA3 and GSDMD, no cysteine-cysteine disulfide bonds were observed. Modeling of the GSDMD pore based on the GSDMD

structure and GSDMA3 pore structure has identified three possible interfaces important for oligomerization (Ruan et al., 2018; Liu et al., 2019). Mutations of residues in these interfaces blocked oligomerization and pyroptosis. Interestingly, both C191 and I104 are located in the third interface, suggesting that these residues are important for N-terminal-N-terminal interaction, and drugs or mutations targeting these residues would block pyroptosis by inhibiting this oligomerization step.

### GSDMD function in other cell types

The majority of work to date has focused on macrophages. Recently, several groups have also defined important roles for GSDMD in neutrophils. In contrast to macrophages, neutrophils do not readily undergo pyroptosis after inflammasome activation (Chen et al., 2014; Karmakar et al., 2015; Martín-Sánchez et al., 2016). In these cells, GSDMD is cleaved not only by caspases but also by cathepsin G and the neutrophil-specific serine protease, ELANE. Burgener et al. (2019) showed that cathepsin G is normally inhibited by the cytosolic protease inhibitors Serpinb1a and Serpinb6a. In Serpinb1a and Serpinb6a knockout mice, cathepsin G is free to cleave GSDMD at L274, leading to GSDMD-dependent inflammation. These studies also suggested that cathepsin G inhibitors could block ELANE-induced GSDMD cleavage. Interestingly, cathepsin G-dependent cleavage of GSDMD did not induce pyroptosis or NETosis of neutrophils but rather programmed necrosis. Additionally, in human neutrophils, ELANE was shown to cleave GSDMD at C268 (in human GSDMD), a site seven residues upstream of the caspase cleavage site, generating a pore-forming N-terminal fragment (Kambara et al., 2018). ELANE is released into the cytosol following lysosomal membrane permeabilization, leading to GSDMD cleavage and neutrophil death. GSDMD-deficient mice were also protected from intraperitoneal infection with *Escherichia coli*, showing a decreased bacterial burden 36 h after infection as well as less bacterial dissemination in the spleen, liver, and kidneys. The authors propose that this is due to delayed neutrophil death in the absence of GSDMD; however, GSDMD-deficient mice are less susceptible to septic shock in general, and it is hard to delineate the precise contribution of neutrophils in this in vivo scenario.

More recently, Sollberger et al. (2018) demonstrated that ELANE-dependent cleavage of GSDMD was required for formation of neutrophil extracellular traps (NETs). NETs are large web-like structures composed of DNA and proteins that are released from neutrophils to defend against microbial infection. Another study identified a caspase-11-driven GSDMD pathway important in NET formation. This pathway was independent of ELANE and unleashed when cytosolic LPS activated caspase-11 (Chen et al., 2018). Thus, NET formation can be triggered through different pathways, both of which require GSDMD, (Fig. 4). In the caspase-11-dependent pathway, GSDMD is required for permeabilization of the nuclear membrane in addition to the plasma membrane. The authors suggested that caspase-11 is also required for histone degradation and chromatin decondensation normally mediated by ELANE. Furthermore, mice deficient in caspase-11 or GSDMD displayed higher bacterial burdens after peritoneal injection with *Salmonella*

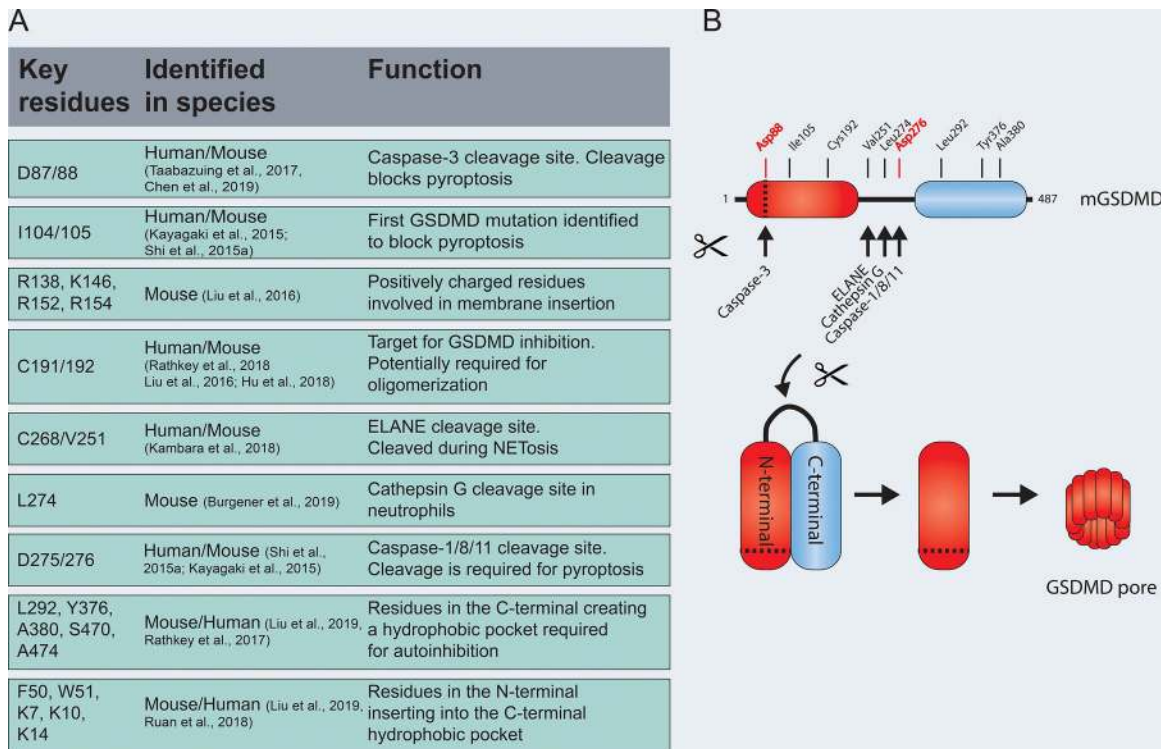


Figure 3. **Schematic of mouse GSDMD along with key residues.** GSDMD is cleaved by caspases at D88 and D276 and by neutrophil ELANE at V251 and cathepsin G at Leu274. Two drug screens have identified C192 as a potential target site for inhibition of pyroptosis.

Typhimurium. DNaseI treatment 4 h after infection to degrade extracellular DNA resulted in increased splenic bacterial burden in WT but not in *Gsdmd*<sup>-/-</sup> or *Casp11*<sup>-/-</sup> mice, demonstrating the importance of NETs to in vivo *Salmonella* infection. Thus, GSDMD can form pores both in the plasma membrane and in the nuclear envelope. A recent study also found that GSDMD targets mitochondria, leading to cytochrome c release and apoptosome formation, linking GSDMD to intrinsic apoptosis as well (de Vasconcelos et al., 2019; Rogers et al., 2019). Along those lines, it is also possible to hypothesize that GSDMD could target intracellular vesicles, leading to release of proteins and ions into the cytosol with downstream signaling effects.

GSDMD is expressed in a wide range of tissues and cell types and is regulated by the transcription factor IRF2 (Kayagaki et al., 2019). It is highly expressed in gut epithelial cells, and both caspase-1 and -11 have previously been shown to play an important role in controlling intestinal pathogens (Saeki et al., 2000; Saeki and Sasaki, 2012; Knodler et al., 2014; Sellin et al., 2014). In intestinal epithelial cells (IECs), GSDMD contributes to NLRP9-dependent pyroptosis when mice are infected with rotavirus (Zhu et al., 2017) and NLRC4-dependent pyroptosis in response to *Salmonella* infection (Rauch et al., 2017). Interestingly, even though IECs from GSDMD-deficient mice did not undergo pyroptosis after FlaTox-challenge, a method for delivery of the NLRC4 activator flagellin, these mice still succumbed to the challenge. This was accompanied by the observation that the cells underwent NLRC4-dependent but caspase-1- and GSDMD-independent IEC expulsion, leading to intestinal pathology. In the absence of caspase-1, this expulsion was driven

by caspase-8, as shown by resistance to FlaTox challenge in *Casp1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Ripk3*<sup>-/-</sup> mice.

### GSDMD and control of sterile and nonsterile inflammation

GSDMD controls both cytokine release and cell death and therefore not surprisingly plays an important role in controlling microbial infection. As mentioned above, GSDMD is central for controlling rotavirus and *Salmonella* infection. Further reports have shown that GSDMD is also required for restricting infections caused by *Brucella abortus* (Cerqueira et al., 2018), *Legionella pneumophila* (Gonçalves et al., 2019), *Burkholderia thailandensis* (Wang et al., 2019), and *Francisella novicida* (Banerjee et al., 2018; Zhu et al., 2018) infection. Additionally, the direct antimicrobial effect of GSDMD on bacterial membranes expands the range of mechanisms to limit microbial growth and spread (Ding et al., 2016; Liu et al., 2016; Zhu et al., 2018).

Like many other mediators of host defense, emerging evidence suggests that GSDMD activation is a double-edged sword. A classic example of this relates to septic shock, where mice lacking caspase-11 or GSDMD are protected from septic shock (Hagar et al., 2013; Kayagaki et al., 2013, 2015; Kang et al., 2018). Excessive activation of pyroptosis leads to increased release of damage-associated molecular patterns or danger signals. Given the potential of these signals to further enhance inflammatory responses, it is not surprising that GSDMD can have deleterious consequences.

GSDMD also contributes to inflammation in noninfectious conditions. GSDMD is a driver of the autoinflammatory pathology observed in familial Mediterranean fever (FMF), an

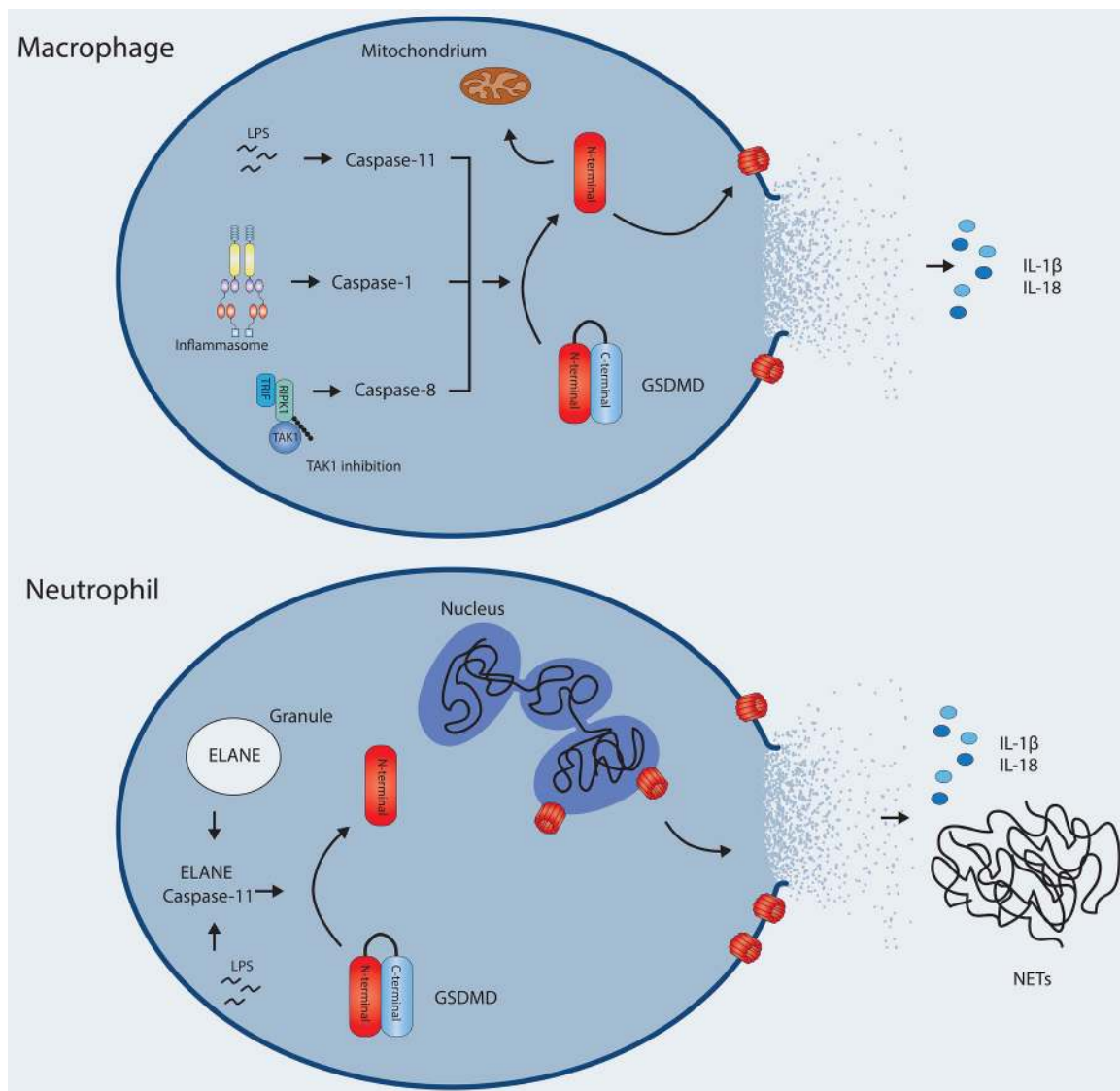


Figure 4. **GSDMD has a variety of functions in different cells.** GSDMD activation in macrophages often leads to pyroptosis when targeting the plasma membrane, but it can also target the mitochondria and lead to apoptosis. In neutrophils, GSDMD does not appear to mediate pyroptosis but rather NETosis by targeting the nucleus in addition to the plasma membrane.

autoimmune disease driven by mutations in the gene *Mefv* (Kanneganti et al., 2018). *Mefv* encodes the protein Pypin, and missense mutations in this gene can lead to activation of the Pypin inflammasome and release of IL-1 $\beta$  and IL-18 and concomitant autoinflammation. In a mouse model of FME, mice containing the FME-associated *Mefv*<sup>V726A/V726A</sup> mutation displayed autoinflammation, runted appearance, and elevated cytokine production, all of which were rescued in mice lacking GSDMD. Deletion of GSDMD in these mice led to normal growth, fully rescued inflammation, and decreased levels of systemic IL-1 $\beta$  secretion. BMDMs from mice harboring the *Mefv*<sup>V726A/V726A</sup> mutation displayed elevated IL-1 $\beta$  secretion upon *Clostridium difficile* infection, which was also strongly decreased in *Mefv*<sup>V726A/V726A</sup> *Gsdmd*<sup>-/-</sup> cells.

GSDMD also contributes to sterile inflammation in liver disease (Khanova et al., 2018; Xu et al., 2018). In a mouse model

of alcoholic hepatitis, it was shown that GSDMD and caspase-11 were both activated and that expression of constitutively active GSDMD worsened hepatocellular death and leukocyte inflammation (Khanova et al., 2018). Similarly, samples from nonalcoholic steatohepatitis patients displayed GSDMD-driven pyroptosis and GSDMD deletion-mitigated development of steatohepatitis in mice (Xu et al., 2018). Nonalcoholic fatty liver disease is a major cause of hepatocellular carcinoma and results in large numbers of liver transplants in the developed world. In contrast, however, it was reported that GSDMD protects against liver damage during acetaminophen overdose. *Gsdmd*<sup>-/-</sup> mice displayed increased liver damage, reportedly due to elevated levels of caspase-8 and necroptosis (Yang et al., 2019).

NLRP3 has been associated with a plethora of different inflammatory diseases. Its role is highlighted in patients with gain-of-function mutations in NLRP3 who develop



cryopyrin-associated periodic syndromes. This is a broad definition for autoinflammatory systemic diseases ranging in severity from familial cold autoinflammatory syndrome to Muckle–Wells syndrome and the most severe manifestation, neonatal-onset multisystem inflammatory disorder (Mangan et al., 2018). As GSDMD is activated downstream of NLRP3 and required for the release of IL-1 $\beta$  and IL-18, it is likely to be involved in many of the diseases associated with NLRP3 and IL-1 $\beta$ . In line with this, GSDMD was recently reported to contribute to the inflammation in mice with cryopyrin-associated periodic syndrome mutations. In a mouse model of NLRP3 gain-of-function mutations, deletion of GSDMD ameliorated the inflammatory symptoms (Xiao et al., 2018).

Finally, GSDMD is also linked to experimental autoimmune encephalomyelitis (EAE) in mice, an animal model of multiple sclerosis (MS). MS is a chronic inflammatory disease of the central nervous system (CNS) that results in demyelination and chronic neuroinflammation. While other inflammasome components such as NLRP3 and IL-1 $\beta$  have been previously implicated in the pathogenesis of MS and EAE, the precise role of GSDMD was not examined (Gris et al., 2010; Inoue et al., 2012). In a recent study, Li et al. (2019) identified an essential role for myeloid cell GSDMD in initiating EAE. Interestingly, myeloid cell–conditional KO mice were protected from EAE as a result of impaired immune cell infiltration to the CNS, T cell activation, and demyelination. IL-1 $\beta$  and IL-18 released from pyroptotic myeloid cells promoted myeloid cell infiltration to the CNS and T cell differentiation to drive EAE. In addition to the genetic approaches used, the authors also demonstrated the clinical utility of a recently described GSDMD inhibitor, disulfiram, in this model (Hu et al., 2018). Indeed, mice administered disulfiram were protected from the onset and pathological symptoms of EAE, broadening the potential for targeting GSDMD to treat MS and potentially additional inflammatory diseases.

### Therapeutic targeting of GSDMD

Infection is a growing problem in the world, with millions of patients dying each year. Given the increase in antibiotic-resistant bacteria, this trend is only expected to continue (Dellinger et al., 2013; Fleischmann et al., 2016; Laxminarayan et al., 2016; Rhodes et al., 2017). As gasdermin-induced pore formation likely plays a pivotal role in sepsis and numerous other disease conditions, the identification of drugs targeting GSDMD and other gasdermin family members is of great interest (Liu and Lieberman, 2017; Shi et al., 2017; Pandeya et al., 2019). Several studies have identified small-molecule inhibitors that target GSDMD and block pyroptosis (Hu et al., 2018; Rathkey et al., 2018; Sollberger et al., 2018). Two of these interact with the C191/192 residue. Necrosulfonamide (NSA) was identified as a potent inhibitor of oligomerization of the GSDMD N-terminal domain through binding to the C191 amino acid (Rathkey et al., 2018). Interestingly, NSA was originally discovered as a strong inhibitor of MLKL-induced pore formation and death (Sun et al., 2012). Conversely, Rashidi et al. (2019) tested NSA in response to uric acid crystals and found that it rather targets ASC oligomerization upstream of GSDMD, as well as affecting pro-IL-1 $\beta$  expression upon LPS stimulation. In a second study, Hu et al.

(2018) discovered disulfiram and Bay 11-7082 to be inhibitors of GSDMD oligomerization, also targeting the same C191/192 residue. Disulfiram, a drug in use to treat alcohol addiction, and Bay 11-7082, an inhibitor of NF- $\kappa$ B, were both found using a fluorogenic liposome leakage assay. From these two studies, both disulfiram and NSA reduced mortality in an LPS-induced mouse model of septic shock. Disulfiram was also found to ameliorate disease progression in EAE as described above (Li et al., 2019). Both Bay 11-7082 and disulfiram also inhibited other proteins in the pyroptotic pathway, an indicator of the lack of specificity often accompanying cysteine-modifying molecules. NSA is likely to also share this feature (Pandeya et al., 2019). This lack of specificity warrants caution, especially for in vivo use of these molecules. However, they do represent a first step in identifying inhibitors of GSDMD and validate the potential benefit these drugs might have in treating septic shock and other conditions. Another study also identified LDC7559 as an inhibitor of GSDMD N-terminal activity but did not identify the mechanism of action of this drug (Sollberger et al., 2018). Screening a large library of small-molecule compounds that blocked neutrophil NETosis, they identified LDC7559 as a member of a class of molecules with a pyrazolo-oxazepine scaffold.

### Conclusions

Pyroptosis, mediated in large part by GSDMD, is an essential component of innate immunity that can have both beneficial and detrimental roles depending on the conditions. During bacterial infection, GSDMD is required for pyroptosis, serves as a conduit to release the IL-1 family of cytokines and other danger signals and facilitates the removal of the bacterial replication niche. However, GSDMD can also exacerbate inflammation, resulting in conditions of autoinflammation and septic shock. Interestingly, in the case of autoinflammation, some but not all inflammasome-driven diseases can be treated with IL-1 $\beta$ -blocking molecules, possibly due to the contribution of IL-18, other danger signals, and cell death. In certain disease models of autoinflammation, mice lacking GSDMD display complete protection. These findings have positioned this protein as a target of great interest for drug development. Inhibitors of GSDMD have shown promise in proof-of-principle studies with protection in mouse models of septic shock and EAE. Future studies will likely identify improved specific drugs targeting GSDMD that could be used to ameliorate GSDMD-mediated diseases.

GSDMD is part of a larger family of genes, many of which are linked to human disease. Studies have predicted most of the other gasdermin members to also have pore-forming potential. These other gasdermins are widely expressed in different tissues yet are understudied. Are they also used to defend against infection and control the release of immune-regulatory molecules? Their association with diseases ranging from different skin conditions to loss of hearing and cancer are intriguing and represent an exciting field of future study. As for GSDMD, reports of its importance in biology and disease are rapidly increasing, demonstrating both protective and detrimental roles. Better understanding of the gasdermin family, which is likely to come given the interest in this area, may lead to improved treatments for inflammatory diseases that impact human health.

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