

Review Article

# Molecular and structural aspects of gasdermin family pores and insights into gasdermin-elicited programmed cell death

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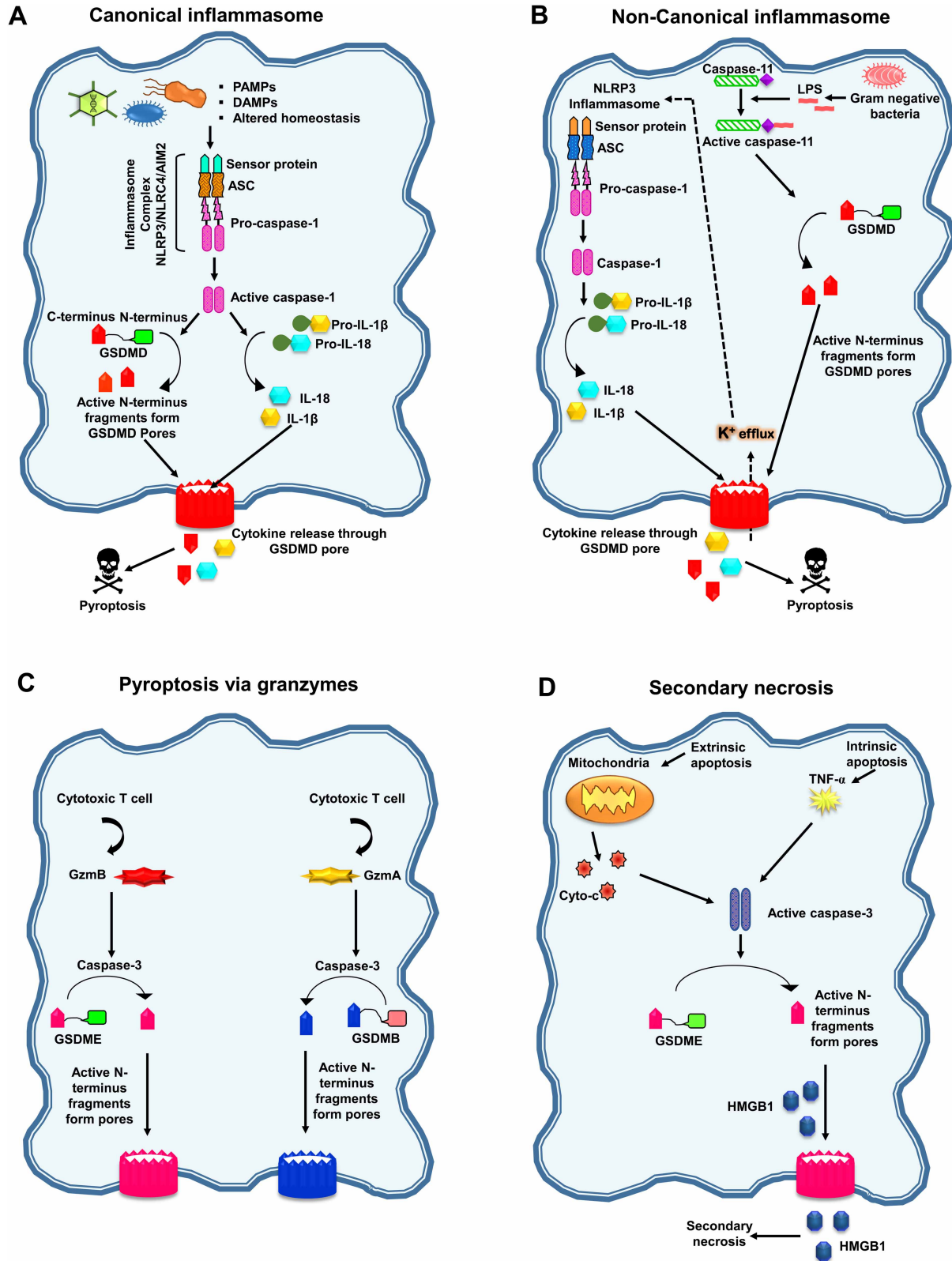
Pyroptosis is a highly inflammatory and lytic type of programmed cell death (PCD) commenced by inflammasomes, which sense perturbations in the cytosolic environment. Recently, several ground-breaking studies have linked a family of pore-forming proteins known as gasdermins (GSDMs) to pyroptosis. The human genome encodes six GSDM proteins which have a characteristic feature of forming pores in the plasma membrane resulting in the disruption of cellular homeostasis and subsequent induction of cell death. GSDMs have an N-terminal cytotoxic domain and an auto-inhibitory C-terminal domain linked together through a flexible hinge region whose proteolytic cleavage by various enzymes releases the N-terminal fragment that can insert itself into the inner leaflet of the plasma membrane by binding to acidic lipids leading to pore formation. Emerging studies have disclosed the involvement of GSDMs in various modalities of PCD highlighting their role in diverse cellular and pathological processes. Recently, the cryo-EM structures of the GSDMA3 and GSDMD pores were resolved which have provided valuable insights into the pore formation process of GSDMs. Here, we discuss the current knowledge regarding the role of GSDMs in PCD, structural and molecular aspects of autoinhibition, and pore formation mechanism followed by a summary of functional consequences of gasdermin-induced membrane permeabilization.

## Introduction

Inflammasomes act as sensors of the innate immune system and function at its principal subcellular sites by detecting pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) and driving immediate inflammatory immune responses [1–3]. Inflammasomes are made up of a sensor protein such as NLR containing protein that detects the danger signals and recruits adaptors such as ASC. ASC ultimately incorporates effector proteins into the assembly for example caspase-1 via homotypic death domain interactions which results in higher-order assembly of inflammasomes yielding activated caspases [4–6]. The NLR/ALR-containing inflammasome are often termed as canonical inflammasomes, for example, NLRP3 inflammasome. On the other hand, oxidized lipids or lipopolysaccharides (LPS) from Gram-negative bacteria can activate murine caspase-11 (caspase-4/5 in humans), forming the non-canonical inflammasome (Figure 1A,B) [7,8]. Human caspase-1, -4, -5, and -12 and, mouse caspase-1, -11, and -12 are collectively referred to as inflammatory caspases. Direct downstream effects of activated inflammasomes include maturation of inflammatory cytokines such as IL-1 $\beta$  and IL-18, and subsequent pyroptosis [9]. Pyroptosis is a lytic and inflammatory form of PCD which was initially described in 2000 [10,11]. The characteristic morphological features of

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**Figure 1. GSDMs can be activated by diverse range of enzymes leading to the induction of various forms of programmed cell death.** Part 1 of 2

(A) Diverse microbial and cellular stress signals are detected by the sensor proteins of the canonical inflammasomes resulting in the recruitment of adaptor protein ASC and procaspase-1 resulting in inflammasome assembly which yields active caspase-1. Proinflammatory cytokines pro-IL-1 $\beta$  and pro-IL-18 are

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Part 2 of 2

processed by caspase-1 to release mature IL-1 $\beta$  and IL-18. Caspase-1 can initiate pyroptosis by cleaving GSDMD to generate active GSDMD-NT which forms pores in the plasma membrane by targeting phospholipids resulting in pyroptosis, although other caspase-1 substrates capable of inducing pyroptosis may also exist. IL-1 $\beta$  and IL-18 are presumably released by cell lysis during pyroptosis. (B) The non-canonical inflammasome pathway is triggered by cytosolic Gram-negative bacteria or bacterial LPS in the infected cells resulting in activation of caspase 11 in mice (caspase-4/5 in humans) which cleaves GSDMD initiating pyroptosis. In the first step, the GSDMD pores allow potassium release, resulting in the activation of the NLRP3 inflammasome and IL-1 $\beta$ /IL-18 maturation. In a second step, GSDMD pores cause pyroptosis, thereby driving the release of mature cytokines. (C) Granzymes initiate GSDMB-NT- and GSDME-NT-induced pyroptosis. GzmA or GzmB, which is released from killer cytotoxic lymphocytes, induces GSDMB- or GSDME-dependent pyroptosis in tumor cells, respectively. (D) The intrinsic or extrinsic apoptotic pathway can activate caspase-3 which cleaves GSDME to produce the pyroptotic GSDME-NT, leading to membrane permeabilization and releases of proinflammatory DAMP molecules such as high mobility group box protein 1 (HMGB1), subsequently inducing secondary necrosis in macrophages. ASC, apoptosis-associated speck-like protein containing a CARD; AIM2, absent from melanoma 2; GSDMD, gasdermin D; GSDME, gasdermin E; GSDMB, gasdermin B; GzmA, granzyme A; GzmB, granzyme B; HMGB1, high mobility group box protein 1; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-18, interleukin-18, LPS, lipopolysaccharide; NLRP3, Nod-like receptor (NLR) pyrin domain-containing 3; NLR4, NLR family CARD domain-containing protein 4.

pyroptosis include swelling and lysis of cells resulting in the release of cytoplasmic content which attracts the effector immune cells to elicit an inflammatory immune response [12]. The term pyroptosis is derived from the Greek words ‘pyro’ which means fever or fire and ‘ptosis’ which means to fall underlying its association with the secretion of IL-1 $\beta$  and IL-18. Earlier, pyroptosis was recognized as ‘caspase-1-dependent necrosis’, because of morphological resemblance and its stringent requirement of caspase-1 [13], however, the discovery of the inflammasome in 2002 [14] and subsequent identification of non-canonical inflammasome in 2011 [15] led to redefining of pyroptosis as an effector mechanism of the inflammasome activation.

A recent breakthrough in inflammasome biology and pyroptosis research was the identification of the gasdermin D (GSDMD) protein which has now emerged as a key executioner of pyroptosis. Several landmark studies in 2015 have independently reported that the central mediator of pyroptotic cell death is GSDMD. Activated inflammatory caspases such as human caspase-1 and -4, and murine caspase-1 and -11 cleave within a central linker region joining two domains of GSDMD producing a 31 kDa N-terminal fragment which can initiate pyroptosis by forming membrane pores [16–19].

## The gasdermin (GSDM) family

Nearly two decades ago, the GSDM family was mentioned in the literature for the first time as a gene family which can cause hearing loss in humans and alopecia in mice [20]. The name ‘gasdermin (gastro + dermato)’ was assigned based on the predominant expression of mouse GSDMA in the upper gastrointestinal (GI) tract and the skin [20]. At present, the family comprises six paralogous genes in humans: GSDMA, GSDMB, GSDMC, GSDMD, GSDME (DFNA5), and PJVK (DFNB59) (Table 1). Mice lack GSDMB and encode multiple isoforms of GSDMA (GSDMA1–3) and GSDMC (GSDMC1–4). In humans, GSDMA and GSDMB are located at chromosome 17q21.1, while GSDMC and GSDMD are at chromosome 8q24. In mice, paralogous genes GSDMA1, GSDMA2, and GSDMA3 are located on chromosome 11, while GSDMC1, GSDMC2, GSDMC3, and GSDMC4 are located at chromosome 15. Phylogenetic analysis reveals that the GSDME-like gene originated in Cnidaria which gave rise to the GSDM genes in the animal kingdom. From a duplication event of the ancestral GSDME gene, PJVK was originated which is related to the development of the complex inner ear in vertebrates. The presence of GSDMA in birds, reptiles, and platypus, and GSDMB, GSDMC, and GSDMD in mammals and placenta depicts high evolutionary pressure. However, these genes can be expanded or deleted depending on the species [21]. The predominant expression of GSDMs is observed in the skin, GI tract, and various immune cells. They are associated with various diseases such as deafness, alopecia, inflammatory disorders and cancer (Table 1) [22].

The GSDMs share nearly 45% sequence homology and are leucine-rich proteins. Their structure depicts well-conserved N-terminal and C-terminal gasdermin domains which are linked through an intervening flexible hinge region which is a potential substrate site for proteases including caspases and granzymes [23]. Based on

**Table 1. Gasdermin family members, expression pattern, and associated diseases**

Human members	Mouse members	Expression	Activating enzymes	Associated diseases	References
GSDMA	<i>Gsdma1</i> <i>Gsdma2</i> <i>Gsdma3</i>	Skin, esophagus, tongue, stomach, mammary glands, and umbilical cord	Unknown	Asthma, systemic sclerosis, alopecia, inflammatory bowel disease	[22,82–86]
GSDMB	<i>Absent</i>	Esophagus, stomach, liver, colon and Lymphocytes,	Granzyme A (GzmA)	Rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes	[82–84,86–88]
GSDMC	<i>Gsdmc1</i> <i>Gsdmc2</i> <i>Gsdmc3</i> <i>Gsdmc4</i>	Esophagus, trachea, stomach, intestines spleen, bladder and skin	Unknown	Unknown	[82–84]
GSDMD	<i>Gsdmd</i>	Esophagus, stomach, intestines, Immune cells,	Caspase-1/4/5/8/11, neutrophil elastase, cathepsin G	Sepsis, familial Mediterranean fever, rheumatoid arthritis, inflammatory bowel disease	[82–84,89–91]
GSDME (DFNA5)	<i>Dfna5</i>	Intestines, brain, kidney, heart, cochlea, placenta, and IgE-primed mast cells	Caspase-3, granzyme B (GzmB)	Autosomal dominant congenital deafness	[22,82–84]
GSDMF (DFNB59, PJVK)	<i>Dfnb59</i>	Inner ear hair cells, auditory system, broadly expressed in other tissues	Unknown	Autosomal recessive congenital deafness	[22,82–84]

the presence of two potential leucine zippers in GSDMs, it was presumed that these proteins may act as transcriptional factors, however, the absence of nuclear localization signal sequence and DNA binding motifs, and their presence in the cytosol have weakened this hypothesis [24]. Hence, the presence of leucine zippers remains to be experimentally proved. Except for PJVK, the N-terminal domain of all GSDMs can oligomerize to form plasma membrane pores which can lead to cell death [25–27]. In PJVK, the N-terminal domain is directly linked to a short C-terminal domain carrying a zinc-finger domain of the anonymous function [28].

## Gasdermins can induce various modalities of programmed cell death (PCD)

At present, the most studied modes of PCD include pyroptosis, apoptosis, secondary necrosis, and NETosis. Initially, these mechanisms were viewed as independent processes, however, emerging data suggest that these four PCD pathways execute substantial cross-talk and can be induced by the same effectors, i.e. gasdermin proteins [29]. Although, pyroptosis seems a generic term for GSDMs' induced cell death, in fact, many recent studies have reported that GSDMs can execute other modes of PCD in addition to pyroptosis. Inflammatory caspases were thought to act as bona fide activators of GSDMs, however, latest data indicate that GSDMs can induce pyroptosis not only via inflammatory caspases but also through other inflammatory caspase-independent pathways. These findings elucidate that the repertoire of GSDMs activation outspreads inflammasome caspases. Furthermore, current researches have also reported that some GSDMs can target other cellular membranes in addition to the plasma membrane, for instance, N-terminal of GSDMA, -D and -E can target mitochondria, N-terminal of GSDMD can also target nuclear envelope membrane and azurophilic granules and PJVK can target peroxisomes. These revelations suggest that GSDMs could be involved in other intracellular processes in addition to PCD [21].

## Gasdermins induce pyroptosis using inflammatory caspases, pro-caspases, and granzymes

Caspase-1 from canonical inflammasome and caspase-11 (human caspase-4/5) from non-canonical inflammasome use GSDMD as their substrate and cleave linker region after residue Asp275 and Asp276 in humans and

mice, respectively, to release GSDMD-NT. This cleavage-liberated 31 kDa GSDMD-NT relocates from the cytoplasm to the plasma, nuclear or mitochondrial membrane to assemble membrane pores [18,30,31]. The GSDMD pores can allow the flow of a variety of species from the cells including ions, therefore, potassium efflux from these pores can further induce NLRP3 inflammasome activation (Figure 1B), which detects membrane disruption and contributes to the formation of a canonical inflammasome [32]. This phenomenon of secondary inflammasome activation establishes a positive feedback loop and strengthens the prospect of GSDMD acting upstream or downstream of inflammasomes [22].

Pro-apoptotic caspases including caspase-3 and caspase-8 are implicated in the regulation of GSDMD in macrophages by current findings. Caspase-8 is long known for its role in the control of apoptotic cell death, however, emerging studies are unveiling its role in pyroptotic cell death as well during specific inflammatory responses and pathogenic infections [32–35]. It catalyzes GSDMD to produce active p30 fragments that drive cell death in macrophages morphologically similar to pyroptosis, however, cleavage of GSDMD into p20 and p43 fragments were also observed which was suggested to be carried out by caspase-3. Activation of caspase-3 takes place downstream of caspase-8 and by cleaving GSDMD into inactive p20 and p43 fragments, it counteracts the function of caspase-8 [33,34]. Another study further reported that caspase-3 and -7 can cleave GSDMD at D87 to produce inactive fragments [36]. Taken together, these studies entail that caspase-8 and caspase-3 function to balance GSDMD activation and inactivation which can be important during inflammation and infection. In contrast with other GSDMs, that can induce pyroptosis after cleavage, full-length GSDMB is found to induce pyroptosis by activating caspase-4 via interaction to its CARD domain [37].

Granzymes are serine proteases produced by cytotoxic T cells to induce PCD in cancerous or infected cells. Recent studies have shown that granzymes can be involved in the activation of some GSDMs and ultimately drive pyroptosis. The study by Zhang et al. has shown that granzyme B (GzmB) can cleave GSDME which triggers pyroptosis [38]. Furthermore, GzmB produced by killer cytotoxic lymphocytes also induces caspase-3 activated GSDME-dependent pyroptosis in cancer cells (Figure 1C). The danger signals released by pyroptotic cancer cells exert remarkable promoting effects on cytotoxic lymphocytes [39]. These results highlight the novel role of GSDMs in antitumor immunity which can help devise future anti-cancer strategies. These studies also reinforce that GSDMs can induce pyroptosis independent of canonical or non-canonical inflammasome activation.

Furthermore, new activating enzymes are also being reported for GSDMs, for example, cathepsin G produced by cytoplasmic granules of monocytes and neutrophils, and neutrophil elastase (ELANE) produced by aging neutrophils, are recently reported to cleave GSDMD [40,41]. The cleavage site of these enzymes is slightly different from that of inflammatory caspases, nevertheless, they produce ~30 kDa GSDMD-NT which can form membrane pores [22]. Given the fact that multiple activating enzymes work for many GSDMs such as for GSDME and GSDMD, a redundant backup mechanism is discernible for inducing pyroptosis to stimulate a robust immune response [39].

### Gasdermins induce autophagy, apoptosis, and secondary necrosis

Numerous studies have suggested that expression levels of GSDMs can control the type of cell death they will induce. Upon inhibited or insufficient scavenging, the apoptotic cells can enter into another phase at the end of the apoptotic pathway called secondary necrosis [42]. It has been reported that GSDME can switch apoptosis to so called secondary necrosis/pyroptosis upon apoptotic stimulations downstream of caspase-3 [43–45] and acts upstream of caspase-3 to augment caspase-3 activation [45,46]. In bone-marrow-derived macrophages, upon induction of the mitochondrial apoptotic pathway, caspase-3-mediated cleavage of GSDME at D270 produces a necrosis-inducing fragment of GSDME-NT (Figure 1D), which induces secondary necrosis following apoptotic plasma membrane blebbing. Upon ablating GSDME, this necrotic morphology is disappeared, suggesting that GSDME carries out necrotic membrane permeabilization [39,43,47]. Multiple studies have reported that following treatment of cancer cells with chemotherapeutic drugs, the mitochondrial intrinsic apoptotic pathway is activated leading to caspase-3 mediated cleavage of GSDME resulting in robust pyroptotic cell death, in addition to simultaneously inducing cellular apoptosis [47,48]. After chemotherapy, the simultaneous detection of biochemical markers for apoptosis and pyroptosis suggests the co-occurrence and interplay of apoptosis and pyroptosis. In the absence of GSDME, the dominance of apoptotic markers with prolonged apoptotic morphology were observed [44,47–49]. GSDME also acts as mitochondrial pore-forming protein (PFP) that augments activation of the apoptotic pathway by releasing high-temperature requirement protein A2 (HtrA2/Omi) and cytochrome c (Cyt c) from the mitochondria and activating the intrinsic apoptotic pathway

[46]. Furthermore, other GSDMs can also augment the mitochondrial apoptotic pathway as shown by the interaction of GSDMA3 with Hsp90/Hsp70 machinery complex which targets GSDMA3-NT into mitochondria for interaction with a chaperone Trap1 to trigger mitochondrial permeability, reactive oxygen species (ROS) production, and secretion of Cyt *c*, all of which result in cell death [50]. Association of GSDMA3 with TNF- $\alpha$ -induced apoptosis has also been reported by multiple studies [51–53].

A possible role of GSDMs in autophagy is proposed based on evidence that GSDMD-NT can bring about the release of elastase and formation of LC3<sup>+</sup> autophagosomes in NLRP3-activated neutrophils without plasma membrane perforation or lytic death [54]. Additionally, GSDMDA3-NT and GSDMD-NT elevated the mitochondrial expression of the autophagic marker LC3-II in HEK293T cells [55], hinting at a possible role of GSDMs in mitophagy.

### NETosis induced by the GSDMs

'NETosis refers to a ROS-dependent modality of regulated cell death restricted to cells of hematopoietic derivation and associated with neutrophil extracellular traps (NETs) extrusion' [56]. It is a programmed neutrophil cell death which serves as a crucial anti-pathogenic strategy. During NETosis, NETs which are extracellular web-like matrices are formed by neutrophils via secretion of granular and cytoplasmic proteins, associated proteases, and chromatin [57,58]. The antimicrobial proteins including neutrophil elastase (NE), cathepsin G, and histones in NETs can eradicate the trapped pathogens [59], thus contributing to antimicrobial defense. Although exact mechanisms employed by NETosis are not fully disclosed, yet incoming studies are reporting that non-canonical inflammasome-activated GSDMD triggers NETosis [60], however, neutrophils are known to resist canonical inflammasome-induced pyroptosis [61,62]. Furthermore, it has also been reported that during the formation of NETs, GSDMD can be activated via a caspase-independent mechanism [40,63]. Overall, these studies have disclosed the remarkable contribution of GSDMD in NETosis upon diverse stimuli including LPS, ROS, and Gram-negative bacteria.

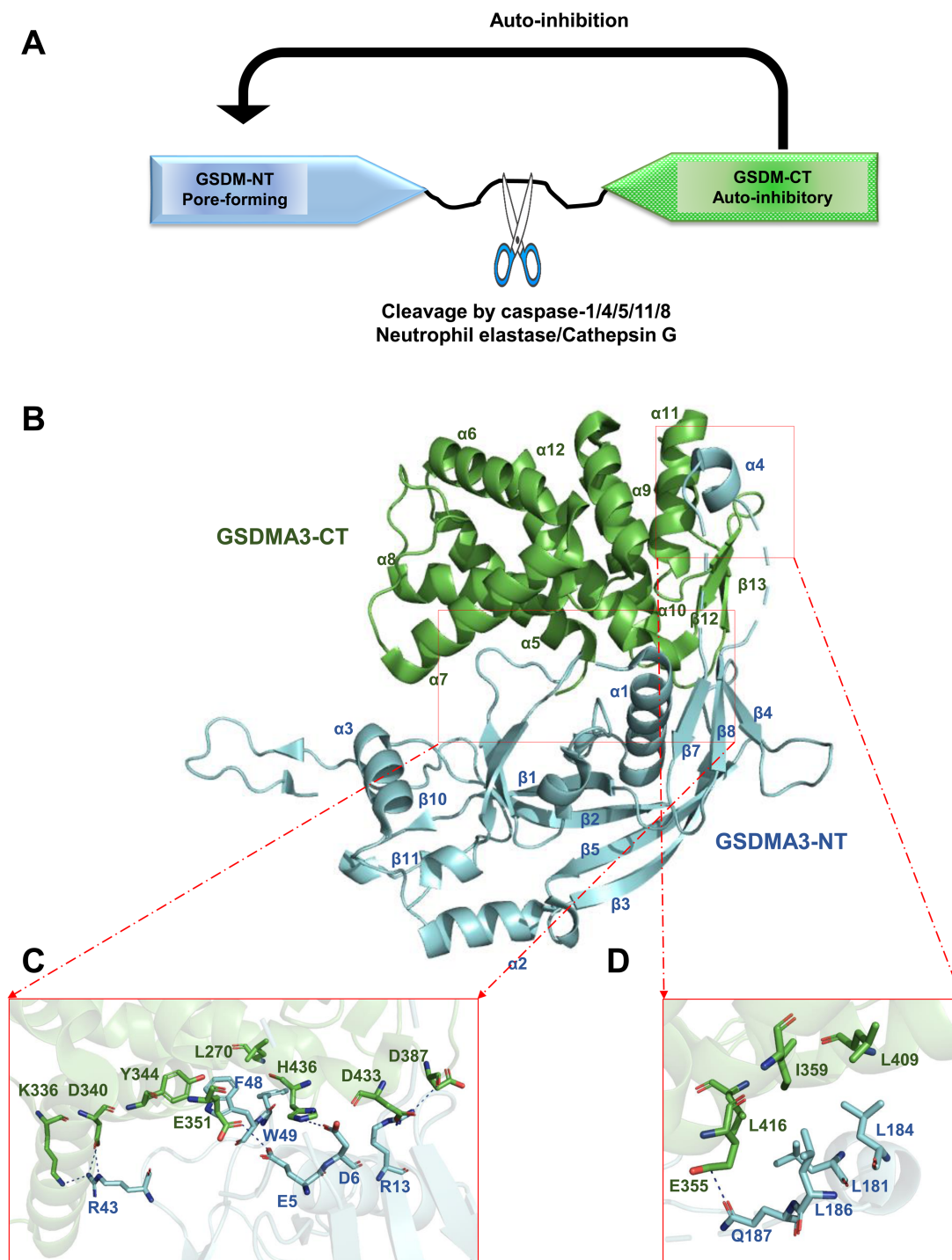
## Structural overview of GSDM architecture

The X-ray crystal structures of full-length mouse GSDMA3 and GSDMD depict that the overall structure of GSDMs is divided into N-terminal domain (GSDM-NT) and C-terminal domain (GSDM-CT) (Figure 2B) [18,27]. GSDM-NT predominantly consists of loops and  $\beta$ -strands ( $\beta$ 1– $\beta$ 11 in GSDMA3), additionally, it contains multiple  $\alpha$  helices ( $\alpha$ 1– $\alpha$ 4 in GSDMA3) (Figure 2B). During the pore formation process, this domain exhibits a dramatic change in its conformation [18]. Helix  $\alpha$ 1 and adjacent  $\beta$ 1– $\beta$ 2 hairpin loop are present deep in the  $\beta$ -sheet, which is flanked by helices  $\alpha$ 2 and  $\alpha$ 3 at one end. In GSDMA3, aromatic residues F48 and W49 of the hairpin loop are surrounded by L270, Y344, A348, and A443 in a deep groove forming a hydrophobic core (Figure 2B,C). From the other end of the  $\beta$ -sheet, helix  $\alpha$ 4 protrudes away via two loops to interact with the GSDM-CT [18]. In the crystal structure of GSDMD, the hydrophobic pocket consists of L292, E295, Y376, A380, S470, and A474 of GSDMD-CT in which two aromatic residues from GSDMD-NT are inserted (F50 and W51 in mouse GSDMD or F49 and W50 in human GSDMD). This hydrophobic core is a conserved feature of GSDM family members and is critical for autoinhibition [27].

GSDM-CT depicts a globular conformation made up of mostly  $\alpha$ -helices ( $\alpha$ 5– $\alpha$ 12 in GSDMA3) making its crystallization relatively easy as compared with full-length protein [18,27] and is covered by a small three-stranded  $\beta$ -sheet ( $\beta$ 12– $\beta$ 14 in GSDMA3) (Figure 2B). GSDM-NT and GSDM-CT are joined through a long flexible linker (residues 234–263) which extends away from the main body making it accessible to activating enzymes. Caspase-1 and -11 cleave the linker after amino acid 275 at a conserved (F/L)LTLD motif [16,17]. The structural homology search for GSDMA3-NT and GSDMA3-CT revealed no significant similarity to other known proteins, implying that it represents a new type of PFP [18].

### Autoinhibition of GSDMs

To induce autoinhibition, GSDMA3-CT masks GSDMA3-NT at three main sites using hydrophobic, hydrogen bonding, and electrostatic interactions. These three contact sites include  $\alpha$ 1 helix (predominantly via charge–charge interactions),  $\alpha$ 4 helix (predominantly via hydrophobic interactions), and the  $\beta$ 1– $\beta$ 2 loop of GSDM-NT (predominantly via charge–charge interactions). Helices  $\alpha$ 1 and  $\alpha$ 4 have been shown to play a vital role during lipid binding and membrane insertion, therefore, these helices are masked by GSDM-CT to make the full-length protein inactive [64] (Figure 2B). Although the  $\beta$ 1– $\beta$ 2 loop is also masked by the GSDM-CT, its role in pore formation is yet unclear. Surprisingly, GSDM-NT and GSDM-CT are found to



**Figure 2. Two domain architecture and crystal structure of GSDMA3.**

(A) The two domain architecture of the GSDM family. Before proteolytic activation, the pore-forming GSDMA3-NT is kept inactive by the auto-inhibitory GSDMA3-CT. (B) X-ray crystal structure of murine GSDMA3 (PDB: 5B5R) is shown as a cartoon model with labeled secondary structure elements. The structure shows that GSDMA3-CT, colored in green, folds back on the functional GSDMA3-NT, colored in light-blue, for autoinhibition. (C,D) The two domain interaction sites I and II are shown. Residues involved in the auto-inhibitory interactions are labeled and shown as sticks.

remain bound to each other even after cleavage at inter-domain linker region under *in vitro* conditions deprived of lipids [18], suggesting that lipid environment may be a pre-requisite for the separation of two domains. Moreover, mutagenesis of GSDM-CT to remove its contact with GSDM-NT restored the pore-forming ability of full-length protein which makes it clear that it is the dissociation of GSDM-CT from GSDM-NT but not the proteolytic cleavage which is important for the pore formation process [18]. Taken together, these results point towards many important questions such as whether proteolysis is essential for activating the GSDMs or not? What is the molecular basis of GSDM-NT/CT non-covalent complex's binding to membrane lipids? What is the role of post-translational modifications in autoinhibition and pore formation?

## Biological and structural basis of GSDM pore formation

The process of pore formation can be divided into three major steps for conceptual understanding, although these three steps may take place concomitantly. These steps include (i) lipid binding upon inter-domain proteolytic cleavage (ii) oligomerization, and (iii) membrane insertion [27,64]. Nevertheless, it is yet to be discovered if all the GSDMs follow this three-step model for pore formation. GSDMD-NT shows an affinity for cardiolipin (CL) or phosphatidylinositol phosphate PIP1 and PIP2 species, while it may exhibit a relatively reduced affinity for triple-phosphorylated PIP3 and the zwitterion head group phosphatidylserine (PS) [18,31]. *In vitro* lipid binding assays also revealed that non-charged head group lipid phosphatidylinositol (PI) or the positively charged head group lipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) do not have a binding affinity with GSDMD [18,31]. CL is exclusively found in bacterial membranes and mitochondria, while PS and PIPs are present in the inner leaflet of the plasma membrane. The selectivity of GSDMD to bind with acidic lipids highlight its ability to rupture plasma and mitochondrial membranes [46,50] and kill bacteria [31]. Furthermore, the presence of PS and PIPs in the cytosolic leaflet of the plasma membrane ensures that pore formation takes place on the cytosolic face of the plasma membrane while not damaging bystander cells [31]. Until now, it has been unclear if oligomerization of GSDMs can occur before membrane lipid binding or these two processes occur simultaneously [18,31], however, a recent study on GSDMD induced pyroptosis by Evavold et al. [65] has suggested that monomers of GSDMD-NT can insert into the plasma membrane and then assemble into pore-forming oligomers.

## AFM studies to explore the pore formation process

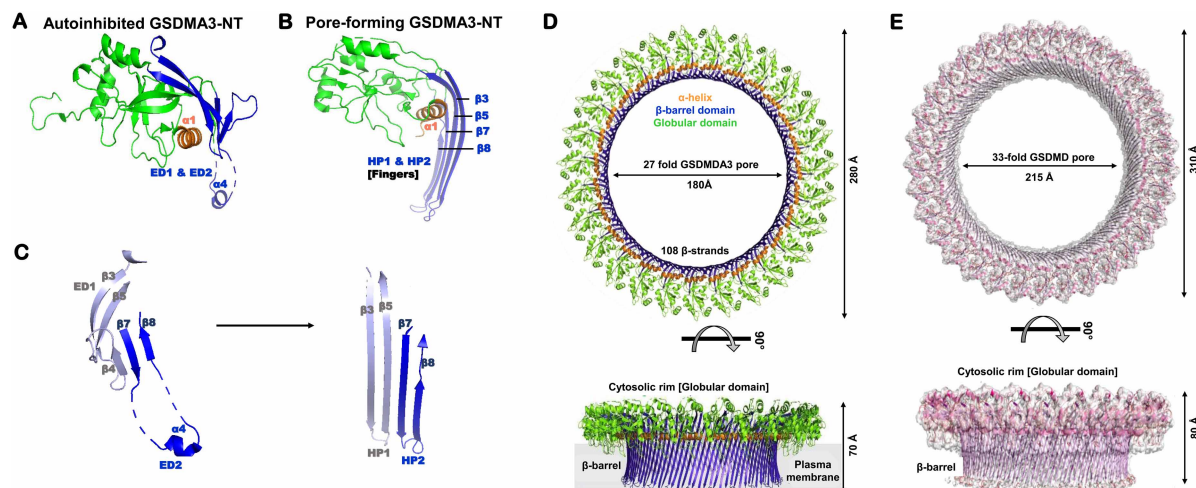
To better elucidate the process of pore formation, Mulvihill et al. used atomic force microscopy (AFM) which revealed that after cleavage of GSDMD by caspase-1, GSDMD-NT assembles arc-, slit-, and ring-shaped oligomers capable of forming transmembrane pores by binding to the PI-, PIP-, or PS-containing membranes. These oligomers incorporate additional oligomers into the assembly over time to establish stable and large membrane pores with an average diameter of 22.6 nm [66]. The arc-, slit-, and ring-shaped oligomers were assembled in the presence of phosphoinositide (PI(4,5)P2) but not phosphatidylinositol. Furthermore, PIP enhanced the formation of ring-like oligomers in the membrane without altering their diameters. Importantly, GSDMD-NT could not bind to the PIP-containing membrane in the presence of cholesterol and showed no oligomer formation. These findings further validate the statement that GSDMD forms pores in the inner side of the mammalian membranes, not the outer leaflet because it contains cholesterol [18].

## Structural basis of pore formation

Recently, cryoelectron microscopy was used to resolve the structures of GSDMA3 and GSDMD pores which provided excellent details of the pore formation process [64,67].

The cryo-EM structure of the GSDMA3 membrane pore was reconstituted on CL-containing liposomes which revealed that GSDMA3 shows heterogeneity in oligomerization by assembling 26–28 fold pores with dominant 27-fold pore. The 27-subunit pore has an inner diameter of 180 Å or 18 nm with a prominent  $\beta$ -barrel transmembrane (TM) region having 108  $\beta$ -strands and a globular cytosolic rim next to the  $\beta$ -barrel (Figure 3D). The major binding element of GSDMA3-NT was the positively charged  $\alpha$ 1 helix containing three Arg residues. This  $\alpha$ 1 helix is covered up by the GSDMA3-CT in full-length protein, hence, full-length GSDMA3 cannot induce membrane pore. A cryo-EM density blob observed next to the  $\alpha$ 1 helix was thought to represent the head of the acidic lipid CL [64]. X-ray structural data also revealed that mutating the basic residues of  $\alpha$ 1 helix diminishes the pore-forming activity of both GSDMD and GSDMA3. A previous study has shown that mutating the residues of  $\alpha$ 3 helix compromises the lipid interaction [31], however, the cryo-EM





**Figure 3. Mechanism of GSDM pore formation.**

(A) Crystal structure of autoinhibited GSDMA3 (PDB: 5B5R). (B) Ribbon diagram of GSDMA3-NT domain in the pore conformation. (C) Conformational changes from autoinhibited to membrane-inserted GSDMA3-NT. Extension domains ED1 shown in gray color and ED2 shown in blue color, which transition into the finger-like hairpins HP1 and HP2, respectively. (D) The cryo-EM structure of the 27-subunit murine GSDMA3 pore (PDB: 6CB8). In addition to the 108-strand  $\beta$ -barrel that inserts into the membrane, each subunit contributes a basic  $\alpha$ 1 helix (orange) that interacts with acidic lipids and a globular domain (green) above the transmembrane (TM) region (adapted from [64]). (E) Ribbon diagram and dimensions of the 33-subunit human GSDMD pore structure fitted into its cryo-EM density map. The 33-fold pore comprises a large transmembrane  $\beta$ -barrel and a globular domain on the cytosolic side of the pore (adapted from [67]).

map showed no density near  $\alpha$ 3 helix. The importance of the  $\alpha$ 3 helix is linked with its location which is near to a subunit oligomerization interface, therefore,  $\alpha$ 3 mutation may interfere with the oligomerization process. Furthermore, oligomeric GSDMD binds to lipids more strongly as compared with monomeric GSDMD mutant, hence, during lipid binding, oligomerization-mediated cooperativity may exist. The mechanism of oligomerization involves extensive intermolecular interactions between neighboring subunits mainly charge–charge interactions and hydrogen bonding [64].

GSDMA3-NT undergoes significant conformational changes during pore formation leading to the formation of four transmembrane  $\beta$ -strands which are two hairpin structures HP1 and HP2 formulated by two extension domains ED1 and ED2, respectively, of autoinhibited GSDMD-NT (Figure 3A–C). ED1 spans the  $\beta$ 3– $\beta$ 4– $\beta$ 5 region and during membrane insertion, it transforms into HP1. One TM  $\beta$ -strand of HP1 is formed by  $\beta$ 3 elongation and the second TM  $\beta$ -strand is formed by  $\beta$ 4,  $\beta$ 5, and the loop between them. ED2 spans the  $\beta$ 7– $\alpha$ 4– $\beta$ 8 region. The  $\alpha$ 4 helix and the flanking loops become straightened to form  $\beta$ -strand along with  $\beta$ 7 and  $\beta$ 8, leading to the formation of HP2. HP1 and HP2 of the same subunit and the neighboring subunits develop an extended hydrogen-bonding network between amides and carbonyl groups of the backbone which stabilizes the  $\beta$ -barrel. Along each  $\beta$ -strand, hydrophobic and hydrophilic amino acids assemble in a manner that the side of the  $\beta$ -barrel facing the pore channel has abundant polar amino acids while the side facing the membrane has mainly non-polar amino acids. The oligomerization interfaces generated by these conformational changes are crucial to assemble the membrane- inserting  $\beta$ -barrel and mutations at the oligomerization interfaces or in the  $\beta$ -barrel drastically affect the pore formation process [64]. The known function of the cytosolic globular region is to enable the intermolecular contacts, however, given its size and structure further research may disclose more functions.

Very recently, the cryo-EM structure of the GSDMD pore was resolved by Xia et al. which revealed that the GSDMD pore has 31–34-fold symmetry with 33-fold being dominant, in contrast with the 26–28 fold symmetry of GSDMA3 pore (Figure 3E). This variation suggests that different GSDMs can have different degrees of oligomerization and also modest plasticity in pore size. The inner and outer diameters of the GSDMD pore

are 215 Å and 310 Å, respectively, larger than that of GSDMA3. Each pore-forming subunit of GSDMD also contains two inserted  $\beta$ -hairpins and a globular domain, though, with a  $\sim 16^\circ$  angle difference from the globular domain of GSDMA3 pore hinting for a flexible junction between globular domain and  $\beta$ -barrel. GSDMD-NT also undergoes conformational changes to convert ED1 and ED2 into HP1 and HP1 during membrane insertion [67]. While in GSDMA3 positively charged  $\alpha 1$  helix is essential for membrane lipid binding, in the GSDMD pore, the  $\beta 1$ – $\beta 2$  loop having a hydrophobic tip flanked by basic residues was proposed to be important for interaction with membrane lipids. The hydrophobic tip inserts into the membrane acting as an anchor, while its flanking basic residues interact with membrane lipids. The sequence and structure of this hydrophobic tip are conserved among GSDM family members. The GSDMD pore conduit has a predominant negative charge and based on electrostatic filtering favors the release of IL-1 $\beta$  and IL-18 as compared with their precursors emphasizing the importance of charge as well as the size of the pore in the passage of cargo [67].

## Outcomes of GSDM pore formation and implication for lysis-independent functions

Following the formation of GSDM pores, the plasma membrane loses its normal permeability barrier leading to the catastrophic disturbance in the normal separation of potassium and sodium which aids in cell rupturing [68]. The end result of GSDM pore formation was originally assumed to be always terminal in the form of pyroptotic cell death [16,17,31,69], however, subsequent studies have shown that the main function of GSDM pore formation is not necessarily cell death [70,71], because, cell death following GSDM pore formation is also regulated and can be postponed or even circumvented. Cells can regulate the pore formation process by controlling the expression level of a particular GSDM to a low level, so that cell death can be avoided. Furthermore, variations in caspase activity may also contribute to sublytic pore formation [72]. Besides, the ESCRT machinery have been shown to remove GSDMD pores from the plasma membrane [73]. Multiple other studies have reported several mechanisms that can repair the plasma membrane damage aroused from pore-forming toxins, mechanical and laser-stress to restore the integrity of the plasma membrane [13]. Although, lysis-independent functions are known only for GSDMD as of now, however, given the similarity between GSDMs, it is conceivable that other members also perform such functions. GSDMD activation downstream of activated NLRP3 inflammasome has been shown to release IL-1 $\beta$  without detectable cell lysis in mouse macrophages [8,70,71]. These studies imply that sublytic GSDMD pore formation could be an unconventional method employed by cells to secrete cytokines, small cytosolic proteins or cysteine protease inhibitors cystatins [71,74]. However, consideration should be given to the fact that assays using LDH release as an indicator of cell death are not very sensitive as they cannot detect the death of small fraction of cells. Moreover, LDH is also released during other types of cell death, therefore, more sensitive methods which offer simultaneous real-time optical detection of cell death and IL-1 $\beta$  release at single-cell level should be employed [75]. Recently, a cell-surface protein NINJ1 was reported to be crucial for plasma membrane rupture, however, GSDMD pores could still be formed in the absence of NINJ1 suggesting that GSDMD pores can release IL-18 and IL-1 $\beta$  without inducing plasma membrane rupture [76]. Taken together, GSDM pores appear to act as master regulators for the secretion of leaderless proteins either via membrane lysis or through lysis-independent pore formation.

The size and numbers of particular GSDM pores are likely to be varied depending upon the type of cells [77]. Furthermore, the function of these pores is based on size restriction and charge properties of the conduit [67]. Noticeably, GSDM pore having a diameter of 10–15 nm can secrete IL-1 $\beta$  (4.5 nm) [18,31] and IL-18 (5.0 nm), while it is small enough to prevent the passage of larger molecules, i.e. 25–30 nm diameter ribosomes [78]. In the presence of few GSDM pores, a cell can use compensatory mechanisms to lower the cell volume for example using swelling-activated Cl $^-$ , K $^+$  and organic osmolyte channels that can export volume-increasing solutes and their accompanying water [79]. Normal emergency exocytic membrane fusion events also repair the pores in the membrane if their number is small, e.g. MLKL pore removal by ESCRT machinery during necroptosis [79–81]. On the other hand, if the number of GSDM pores exceeds the cell's compensatory capability, then a membrane rupture event is inevitable [78].

## Conclusion

Here, we have summarized the current knowledge about the GSDM protein family, their role in programmed cell death, and lastly structural and molecular aspects of the GSDM pore formation process. Elucidation of further aspects of cytotoxic activity of GSDMs has the potential to disclose novel molecular participants in the

process of cell death. Clearly, current studies have only touched the tip of the iceberg and much work still remains to be done until we get a complete picture of the biological relevance of GSDMs.

## Perspective

- Given the importance of gasdermin-induced cell death in tissue damage and inflammation, they have taken prominent place in cell death pathways. Elucidation of biological and structural aspects of gasdermins can help to design novel therapeutic strategies. We still need to know if all the GSDMs have a role in inflammation and antimicrobial immunity. How do their functions differ from each other? What structural factors contribute to the differences in the functions of various GSDMs?
- Recent cryo-EM structural data has provided valuable information regarding the pore formation process of GSDMA3 and GSDMD. Our knowledge regarding the role of GSDMA- and GSDMC-mediated pore formation is still inadequate. Future studies can focus on deciphering the pathological or physiological signals that drive the cleavage of GSDMA and GSDMC in humans.
- Since distinct GSDMs exhibit a cell-type and tissue-specific expression, hence, it will be of great importance to describe the cell-type and tissue-specific functions of the different GSDMs which may elucidate their role in various diseases. Furthermore, besides inflammasomes, are there other sensor systems with similar complexity upstream of GSDMs which can activate them?

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

## Author Contributions

A.Z. wrote the manuscript. H.I. proofread and helped with figure illustrations. T.J. provided guidance and revised the manuscript. All authors contributed to the article and approved the submitted version.

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## Abbreviations

AFM, atomic force microscopy; ALR, AIM2-like receptor; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; CARD, caspase recruitment domain; CL, cardiolipin; Cryo-EM, cryoelectron microscopy; Cyt c, cytochrome; DAMP, damage-associated molecular pattern; ED, extension domain; ELANE, neutrophil elastase; ESCRT, endosomal sorting complex required for transport; GI, gastrointestinal; GSDM-CT, gasdermin C-terminal domain; GSDM-NT, gasdermin N-terminal domain; GSDMs, gasdermins; GzmA, granzyme A; GzmB, granzyme; HP, hairpin; HtrA2, high-temperature requirement protein A2; IL-1 $\beta$ /18, interleukin-1 $\beta$ /18; LC3, microtubule-associated protein 1A/1B-light chain 3; LPS, lipopolysaccharide; NETs, neutrophil extracellular traps; NINJ1, ninjurin 1; NLR, nucleotide-binding domain and leucine-rich repeat-containing protein, NLRP3, NLR family PYD domain-containing protein 3; NLRC4, NLR family CARD domain-containing protein 4; PAMP, pathogen-associated molecular pattern; PC, phosphatidylcholine; PCD, programmed cell death; PE, phosphatidylethanolamine; PIP, phosphatidylinositol; PJVK, pejvakin; PS, phosphatidylserine; ROS, reactive oxygen species; TM, transmembrane; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

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