INVITED REVIEW

Mechanisms of Gasdermin family members in inflammasome signaling and cell death

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Abstract

The Gasdermin (GSDM) family consists of Gasdermin A (GSDMA), Gasdermin B (GSDMB), Gasdermin C (GSDMC), Gasdermin D (GSDMD), Gasdermin E (GSDME) and Pejvakin (PJVK). GSDMD is activated by inflammasome-associated inflammatory caspases. Cleavage of GSDMD by human or mouse caspase-1, human caspase-4, human caspase-5, and mouse caspase-11, liberates the N-terminal effector domain from the C-terminal inhibitory domain. The N-terminal domain oligomerizes in the cell membrane and forms a pore of 10-16 nm in diameter, through which substrates of a smaller diameter, such as interleukin (IL)-1β and IL-18, are secreted. The increasing abundance of membrane pores ultimately leads to membrane rupture and pyroptosis, releasing the entire cellular content. Other than GSDMD, the N-terminal domain of all GSDMs, with the exception of PJVK, have the ability to form pores. There is evidence to suggest that GSDMB and GSDME are cleaved by apoptotic caspases. Here, we review the mechanistic functions of GSDM proteins with respect to their expression and signaling profile in the cell, with more focused discussions on inflammasome activation and cell death.

Introduction

Gasdermins (GSDMs) are a family of functionally-diverse proteins which are expressed in a variety of cell types and tissues [1-3]. Earlier identification of GSDMs in the gastrointestinal tract and dermis led to its nomenclature, “gas-dermin” [4, 5]. Six GSDMs are found in humans and ten GSDMs are found in mice. Humans carry genes encoding Gasdermin A (GSDMA, previously known as GSDM1) [4-6], Gasdermin B (GSDMB, gasdermin-like, GSDML, or PRO2521) [5, 7], Gasdermin C (GSDMC, melanoma-derived leucine zipper extranuclear factor, or MLZE) [5, 8], Gasdermin D (GSDMD, gasdermin domain-containing 1, GSDMDC1, deafness, autosomal dominant 5-like, or DFNA5L) [5, 9], Gasdermin E (GSDME or DFNA5) [10] and Pejvakin (PJVK, autosomal recessive deafness type 59, DFNB59, putative Gasdermin F, or GSDMF) [11] (**Figure 1**).

Mice carry genes encoding three homologs of GSDMA (GSDMA1-3), four homologs of GSDMC (GSDMC1-4) and one homolog each of GSDMD, GSDME and PJVK [5]. Mice, however, do not carry a gene encoding GSDMB [5]. The presence of multiple copies of genes encoding the same GSDM member in mice likely arose during gene duplication events over the course of vertebrate evolution [5, 12] (**Figure 1**). Some studies exclude GSDME and PJVK from the GSDM family based on their divergent expression pattern and mutant-associated phenotypes compared with other GSDM family members, despite similarities in gene sequences and structural arrangement of these two family members to other GSDMs [5].

An emerging function of GSDMs is their ability to induce cell death and inflammation, and, in particular, the role of GSDMD in inflammasome signaling and pyroptosis. An inflammasome is a cytosolic multimeric complex which activates the cysteine protease caspase-1 to drive proteolytic processing of the proinflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18). The inflammasome also induces pyroptosis, a form of programmed cell death that is inflammatory due to the consequential release of IL-1β, IL-18 and danger signals (also known as danger-associated molecular patterns, DAMPs), such as DNA, ATP and high

mobility group box 1 (HMGB1), upon cell lysis [13]. Pyroptosis is characterized by cell swelling, nuclear condensation, disruption of the cell membrane, and release of inflammatory cytokines and DAMPs [14, 15]. The requirement of inflammatory caspases, namely human and mouse caspase-1, human caspase-4 and caspase-5, and mouse caspase-11, to induce pyroptosis distinguishes pyroptosis from other forms of programmed cell death, such as apoptosis and necroptosis which do not rely on inflammatory caspases, [13, 16, 17].

GSDMD is an executioner of pyroptosis owing to its ability to be cleaved by inflammatory caspases and its ability to form membrane pores [18-21]. Although there is evidence to support the idea that the pore-forming ability of GSDMD may be conserved throughout the GSDM family [1, 18], the mechanisms of how other GSDMs form pores is largely unknown.

All GSDMs, with the exception of PJVK, are comprised of a conserved two-domain arrangement: a C-terminal domain and an N-terminal domain [18, 22, 23]. Full-length GSDM proteins do not normally induce cell death due to the presence of the C-terminal autoinhibitory domain binding to the N-terminal effector domain [1, 18, 23]. Once the C-terminal domain is removed via proteolytic cleavage, the N-terminal domain of certain GSDMs can bind to lipid components and form pores in the cell membrane [1, 18, 19, 24]. These GSDMs include GSDMA, GSDMA3, GSDMB, GSDMC, GSDMD and GSDME [18, 23, 25-27]. GSDMD induces pyroptosis following cleavage by inflammatory caspases [25, 28, 29], whereas the roles of GSDMs other than GSDMD in pyroptosis are less certain despite their N-terminal domains possessing pore-forming ability. There is some evidence to suggest that GSDME can induce cell death [26, 30-33]. PJVK is homologous to the other GSDMs [11], but no information is available regarding its pore-forming function. In this review, we provide an overview of the latest advances in GSDM biology, with a focus on inflammasome signaling and cell death. We also discuss the expression profile and molecular regulation of GSDMs and highlight the importance of this protein family in human diseases.

Overview of pattern-recognition and inflammasome activation

The innate immune system functions as the first line of defense against microbial threats, requiring the concerted activity of germline-encoded pattern-recognition receptors (PRRs). PRRs detect pathogen-associated molecular patterns (PAMPs) found on pathogens or DAMPs in the form of endogenous self-molecules or foreign environmental irritants [34-37]. PAMPs are structural motifs that are conserved among and characteristic of pathogens, enabling the host to differentiate between self and non-self. Examples of PAMPs include lipopolysaccharide (LPS), flagellin, and components of the Type III secretion system [14]. Examples of DAMPs include host nuclear or mitochondrial DNA, fibrillary amyloid-β, ATP and HMGB1, which are released during cell death and tissue damage [14]. DAMPs can also include environmental irritants such as asbestos, silica and bee venom [35, 38]. Together, the suite of PRRs provide targeted host defense against a wide range of signaling cues.

The PRR family Absent in melanoma 2 (AIM2)-like receptors (ALRs), the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and the tripartite motif containing (TRIM) family member protein Pyrin, can form inflammasomes [36, 39]. Inflammasome-forming members of the NLR family include NLRP1, NLRP3, NAIPs and NLRC4 [14]. NLRP6, NLRP9 and NLRP12 have also been implicated in inflammasome signaling, but further evidence is required to establish the existence of these putative inflammasome complexes [14, 38].

Of the NLR family members, NLRP1 detects the anthrax lethal toxin of *Bacillus anthracis* [40]; NLRP3 can be activated via a canonical or noncanonical pathway [38]. The canonical NLRP3 inflammasome is activated by a wide range of both PAMPs and DAMPs, such as bacterial peptidoglycans, ATP and viral dsRNA as well as disturbance of intracellular homeostasis and production of reactive oxygen species (ROS) [41-48]. In the noncanonical NLRP3 inflammasome pathway, murine caspase-11, or human caspase-4 or caspase-5 directly bind to lipid A on the LPS of Gram-negative bacteria [49]. This interaction leads to pyroptosis

independently of caspase-1 [49-53]. The NAIP-NLRC4 inflammasome can be activated by flagellin and Type III secretion system needle and rod proteins [54-59].

Of the non-NLR family members that can assemble the inflammasome, AIM2 recognizes dsDNA [60-63] and the TRIM family member, Pyrin, can detect bacterial modification of host Rho GTPases [64]. There is also evidence to suggest that the ALR family member IFI16 can form an inflammasome [65], however, further validation is required to establish the existence of this inflammasome complex [14].

Inflammasome complexes trigger inflammation via caspase-1-dependent proteolytic processing of pro-IL-1β and pro-IL-18 into bioactive IL-1β and IL-18, respectively [66-68]. The inflammasome substrate IL-1β is a strong mediator of inflammation, often referred to as an endogenous pyrogen [69, 70], acting to induce fever and immune cell activity. IL-1β can also increase vasodilation and hyperalgesia [13, 70]. Uncontrolled IL-1β release can lead to auto-inflammatory diseases such as Cryopyrin associated periodic syndrome (CAPS) and Mediterranean fever [71-73]. The other inflammasome substrate IL-18 functions to promote inflammation primarily through stimulating the production of interferon-γ (IFN-γ), which is a classical anti-microbial inflammatory cytokine. Overproduction of IL-18 can also cause auto-inflammatory diseases such as rheumatoid arthritis, Crohn's disease and systemic lupus erythematosus, all of which are associated with a pathological IFN-γ signature [74].

Gasdermin D (GSDMD)

GSDMD is expressed in the oesophagus, stomach and skin [4, 6] (**Figure 2, 3**). The expression pattern of GSDMD at sites that are likely points of pathogen entry in the host suggested a putative role of GSDMD in host defense. In 2015, two independent groups used genomic screening techniques to identify GSDMD as a substrate of inflammasome-associated caspases, which upon cleavage generates an effector domain that induces pores in the cell

membrane leading to pyroptosis [25, 28] (**Figure 4**). One group used ethyl-*N*-nitrosourea (ENU) mutagenesis to generate genetically mutated mouse strains and found that bone marrow-derived macrophages (BMDMs) from a mouse strain carrying the mutation I105N within GSDMD were unable to undergo LPS-induced caspase-11-dependent pyroptosis [28]. Another group utilized a clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system to perform a genome-wide screen for genes involved in canonical caspase-1-dependent and noncanonical caspase-11-dependent pyroptosis [25]. In this study, they found that mouse BMDMs lacking the *Gsdmd* gene exhibited a reduction in the release of IL-1β in response to cytosolic LPS and other classical activators of the inflammasome [25]. A third group subsequently confirmed the role of GSDMD in pyroptosis, but further demonstrated that cleavage of GSDMD promotes pyroptosis and suppresses apoptosis [29]. Importantly, prolonged stimulation of BMDMs lacking GSDMD with canonical inflammasome activators eventually leads to pyroptosis [28], suggesting that GSDMD operates in concert with other pyroptotic factors to induce pyroptosis.

Further mechanistic studies revealed that GSDMD is activated by caspase-1, caspase-4, caspase-5, and caspase-11, all of which cleave GSDMD into a ~30 kDa N-terminal effector domain and a ~23 kDa C-terminal inhibitory domain [25, 28, 29]. The cleavage is the result of a nucleophilic attack on an aspartic acid motif present on GSDMD [25, 28, 29]. Once liberated, the GSDMD N-terminal domain can bind to phosphatidylinositol phosphates (PIPs) in the cell membrane [18-21, 24]. PIPs bound by the GSDMD N-terminal domain include PtdIns(4)P, PtdIns(4,5)P2 [19], PtdIns(3)P, PtdIns(5)P, PtdIns(3,4)P2, PtdIns(3,5)P2, and PtdIns(3,4,5)P3 [18]. The GSDMD N-terminal domain also binds phosphatidic acid (PA) and phosphatidylserine (PS), albeit to a lesser extent and strength to that of the aforementioned PIPs [19]. Further, binding to PtdIns(4,5)P2 enhances membrane insertion of the GSDMD N-terminal domain, whereas binding to cholesterol has the opposite effect [75]. The N-terminal domain of GSDMD does not bind phosphatidylethanolamine (PE) and phosphatidylcholine (PC), both of which constitute much of the outer and inner leaflets of the membrane bilayer

[19], suggesting that the N-terminal domain of GSDMD might not be able to interact with the outer plasma membrane of host cells, preventing unwarranted cell death and tissue damage. The specifics behind the mechanism of GSDMD-pore formation has been further characterized using atomic force microscopy, which revealed that in liposomes the GSDMD N-terminal domain forms arc-shaped oligomers initially, which then transition into slit-shaped oligomers and grows into ring-shaped oligomers, resulting in membrane pores [75].

Of particular interest to host defense is that the N-terminal domain of GSDMD can bind to cardiolipin, a lipid moiety found on bacterial plasma membranes as well as on the mitochondrial inner membrane [18, 19]. Indeed, recombinant protein of the GSDMD N-terminal domain induces robust killing of *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*, whereas the full-length protein and the C-terminal domain do not [19]. The relative contribution of the membranolytic ability of GSDMD in host defense is unclear, given that pyroptosis can also induce other forms of anti-microbial responses (discussed further below).

In addition to driving membrane rupture and pyroptosis, the pore formed by GSDMD can promote cytokine secretion prior to the cell undergoing complete lysis. Studies have shown that secretion of IL-1β can occur in the absence of cell lysis, in cell types such as human monocytes and mouse BMDMs, bone marrow-derived dendritic cells (BMDCs), neutrophils, and embryonic fibroblasts (MEFs) [76-80]. Indeed, the size of the GSDMD pore diameter has been estimated to be between 10-16 nm [18-21]. This proposed diameter of the GSDMD pore is wide enough to allow passage of mature IL-1β and IL-18, both of which have a molecular diameter of 4 nm [18, 19]. Studies using liposomes carrying IL-1β and IL-18 revealed that these cytokines can be released through GSDMD-induced pores; however, tetrameric LDH which has a diameter of 10 nm cannot [81]. The selectivity of the GSDMD pore for passage of molecules is unknown. Studies have reported that a broad set of proteins and DAMPs, including mitochondrial cytochrome *c*, lysosomal cathepsin B and pro-caspase-3, are released

during pyroptosis [82, 83], implying that the selectivity of the GSDMD pore is based entirely on size. Whether cells that are actively secreting cytokines via the GSDMD pore must proceed to pyroptosis when the amount of pores reach a certain threshold is still under investigation. In this context, cells that remain viable to mediate cytokine secretion rather than undergoing instantaneous pyroptosis would increase the magnitude of localized inflammation.

Further studies have shown that the cryoprotectin glycine can block cell lysis and release of lactate dehydrogenase (LDH), but not the release of IL-1β in BMDMs treated with inflammasome activators [81, 83]. Stimulation of BMDMs with agents such as bacterial peptidoglycan (PGN), the PGN fragment *N*-acetyl glucosamine (NAG), oxidized phospholipids, or a mutant strain of *Staphylococcus aureus* lacking O-acetyltransferase A (OatA), leads to the release of IL-1β requiring GSDMD, but without triggering pyroptosis [81]. Similarly, in BMDCs, exposure to the oxidized phospholipid oxPAPC activates caspase-11 and induces secretion of IL-1β without pyroptosis [80]. BMDCs lacking GSDMD exposed to oxPAPC secrete reduced levels of IL-1β compared to wild type (WT) BMDCs [83], suggesting that the GSDMD pore is required for secretion of IL-1β in BMDCs. These results suggest that secretion of IL-1β can occur prior to complete membrane rupture of BMDMs and BMDCs. It is important to note that oxPAPC has also been reported to inhibit IL-1β release in mouse BMDMs and human THP1 macrophages, but not in mouse BMDCs [84]. In these cases, oxPAPC is thought to bind directly to human caspase-4 and mouse caspase-11 and outcompete LPS for binding [84].

Mouse neutrophils do not undergo pyroptosis but maintain the ability to secrete IL-1β [77]; the mechanism potentially preventing the pyroptosis-inducing activity of GSDMD is unknown. Neutrophils express a lower level of *Gsdmd* mRNA compared to BMDMs and BMDCs [83]. However, neutrophils lacking GSDMD release less IL-1β after activation of either the NLRC4 or NLRP3 inflammasome compared with WT neutrophils, but more than that in neutrophils lacking both caspase-1 and caspase-11 [83]. These findings would indicate that GSDMD

pores necessitate the release of IL-1β, but these pores are not an absolute requirement for the liberation of this cytokine in neutrophils. A further study observed mouse neutrophils undergoing GSDMD-dependent cell death [85]. This study found that neutrophil elastase cleaves GSDMD at a site upstream of the caspase cleavage site, producing a functionally-active N-terminal domain of GSDMD that mediates neutrophil cell death [85]. How inflammatory caspases and neutrophils elastase coordinate their activities remains to be defined.

An anti-microbial function of GSDMD-mediated pyroptosis is the release of intracellular pathogens from dying cells, facilitating pathogen expulsion from their intracellular replicative niche. This expulsion mechanism exposes the pathogen to other phagocytes and immune cells for killing [86]. A further study revealed that pyroptosis leads to the formation of pore-induced intracellular traps (PITs) that hold pathogens captive within the lysed cell remnant [87]. Phagocytes such as neutrophils subsequently remove PITs [87].

Removal of an infected cell via pyroptosis from the host tissue is another mechanism of host defense. During infection with *Salmonella enterica* serovar Typhimurium, pyroptosis expels infected cells from the intestinal epithelium in a manner dependent on caspase-1, caspase-8 or caspase-11, but not GSDMD [88-90]. The lack of requirement for GSDMD in the physical extrusion of an infected cell is perhaps not surprising; removal of the cell requires actin rearrangement, which is initiated by caspase-dependent cleavage of cytoskeletal substrates [90, 91]. A role for GSDMD in the host defense against Rotavirus in mice has been observed [92], broadening the physiological relevance of GSDMD to anti-viral response. Pathogen expulsion, PIT formation as well as the removal of entire infected cells via pyroptosis are combinatory innate immune defenses that work synergistically to promote pathogen clearance.

Gasdermin A (GSDMA)

The human genome encodes a single GSDMA, whereas the mouse genome encodes three paralogs known as GSDMA1, GSDMA2 and GSDMA3 [4, 6, 7]. Human GSDMA is expressed in the esophagus, lower gastrointestinal tract, mammary gland, skin, and the stomach [4, 93] (**Figure 2**). Mouse GSDMA1 is expressed in the skin and the squamous epithelium of the stomach cardia; GSDMA2 is expressed in the skin; and GSDMA3 is expressed in the epithelium of glandular stomach [5, 6, 94, 95] (**Figure 3**).

Human GSDMA and mouse GSDMA3 adopt an auto-inhibited two-domain structure, in which the N-terminal domain is inhibited by the C-terminal domain [18, 96, 97]. Nine mutant alleles of mouse GSDMA3 have been shown to disrupt the interdomain interaction between the N-terminal domain and the C-terminal domain, leading to the activation of mouse GSDMA3 [18, 96]. Of these nine mutations, two encode a truncated GSDMA3 protein with an interrupted interdomain region; these two truncated mutations are 259RDW carrying three mistranslated residues inserted after residue 259, and 366stop, a premature stop at residue 366 [18]. Three other mutations, Y344C, Y344H and A348T, target residues that are required to mediate contact with the N-terminal domain directly; two other mutations, T278P and L343P, are in close proximity to the aforementioned three mutations [18]. The biological importance of mutations that impair inhibition of the N-terminal domain of GSDMA3 is implicated by their association with alopecia, skin inflammation, hyperkeratosis, hyperplasia, and progressive bulge stem cell depletion in mice [18, 98, 99]. Mice that harbor a mutation Y344H in GSDMA3 that impairs the auto-inhibitory function of the C-terminal domain have an increased spontaneous immune response, enlarged lymph nodes, and carry cells with residual bodies of indigestible materials [100, 101].

Similar to the N-terminal domain of GSDMD, the N-terminal domain of GSDMA and GSDMA3, when overexpressed in 293T cells, form pores in the plasma membrane and induce pyroptosis-like features [18]. The protease cleaving human or mouse GSDMA has not been identified (**Figure 4**). Cryo-Electron Microscopy analysis revealed that the N-terminal domain

of mouse GSDMA3 undergoes a conformational change upon membrane insertion, forming long β-strands spanning the plasma membrane [97]. In addition, the N-terminal domain of mouse GSDMA3 has a putative role in inducing pore-formation in the mitochondrial membrane [23]. In an overexpression study, the N-terminal domain of mouse GSDMA3 can associate with the chaperone protein, heat shock protein 90 (HSP90) [23] (**Figure 4**). Following this initial association, the N-terminal domain of mouse GSDMA3 is delivered to the mitochondria, mediated by the mitochondrial importer receptor TOM70 [23]. The N-terminal domain further interacts with and might inhibit the mitochondrial chaperone protein TRAP1, which can lead to reduced mitochondrial oxidative stress, suppressing formation of the mitochondrial permeability transition pore and mitochondrial-associated apoptosis [23, 102]. Transfection of mouse GSDMA3 carrying the mutation Y344H which impairs the auto-inhibitory function of the C-terminal domain in HEK293T cells leads to decreased mitochondrial membrane potential and increased levels of reactive oxygen species (ROS) [23]. Whether the N-terminal domain of mouse GSDMA3 can directly form pores in the mitochondrial membrane and induce mitochondrial membrane rupture remains to be investigated.

Mouse GSDMA3 is required for TNF-induced apoptosis in keratinocytes [103]. TNF upregulates the expression of GSDMA3 in keratinocytes in mice, which might allow GSDMA3 to enhance the expression of caspase-3 and apoptosis [103]. How mouse GSDMA3 upregulates caspase-3 and whether GSDMA3 requires proteolytic cleavage to exert all of its functions is still unknown. Overexpression of human GSDMA in gastric epithelial cell lines induces apoptosis, characterized by increased caspase-3 or caspase-7 activity [93]. It is possible that insertion of the N-terminal domain of human GSDMA into the mitochondrial membrane might lead to mitochondrial-associated damage, cytochrome c release and initiation of intrinsic apoptosis. In this context, expression of the mouse GSDMA3 protein carrying the Y344H mutation leads to reduced apoptosis and caspase-3 expression in keratinocytes [103]. In HEK293T cells, however, mouse GSDMA3 carrying the same mutation can induce cell death via autophagy [23]. Further, this mutant form of mouse GSDMA3 can

induce caspase-independent, mitochondrial permeability transition pore-mediated cell death in HEK293T cells [23]. The contribution of GSDMA3 in apoptosis is emerging, but how this pore-forming protein is regulated is unknown.

Gasdermin B (GSDMB)

Humans carry a gene encoding GSDMB whereas mice do not [4, 6]. GSDMB is specifically expressed in the bronchial epithelium of asthmatic lungs, and in the epithelium of esophagus and gastrointestinal tract [104, 105] (**Figure 2**). GSDMB has at least four splice variants, each encoding a protein with a molecular weight ranging from 35 to 50 kDa [5]. These isoforms have distinct expression profiles and subcellular localization patterns in different cell types [106-108]. For example, GSDMB-1 is the primary and longest isoform and is expressed in human cancer cell lines [106]. GSDMB-1 localizes to the nucleus of the human breast cancer cell line MCF7 and the human cervical cancer cell line HeLa, whereas it is exclusively found in the cytoplasm of the hepatocellular carcinoma cell line HepG2 [106]. Genome analyses revealed an association between single nucleotide polymorphisms (SNPs) in the gene encoding GSDMB and asthma, type 1 diabetes, inflammatory bowel disease, and ankylosing spondylitis [109-115]. Moreover, patients with gastric cancer [4], uterine cervix cancer [106] and breast cancer [108, 116] have increased expression of GSDMB.

The membrane-binding preference of GSDMB differs to that of other GSDMs. Both the full-length and the N-terminal domain of GSDMB can bind to phosphoinositides and glycolipid sulfatides [27]. The capacity to bind to sulfatides indicates that GSDMB may be able to interact with the extracellular leaflet of the plasma membrane, which differs to other GSDM family members. Further, the membrane-binding ability of both full-length and the N-terminal domain of GSDMB suggests that the C-terminal domain of GSDMB is unable to inhibit the membrane-binding ability of the N-terminal domain [27]. However, the N-terminal domain of GSDMB, but

not the full-length GSDMB, can induce pyroptosis-like features in human HEK293T cells [18] (**Figure 4**). Therefore, it might be possible that binding of the full-length GSDMB protein to the cell membrane is not sufficient to induce pores, and that activation or processing of the protein following membrane binding might yield the N-terminal domain that then catalyzes the pore formation process.

With respect to cleavage of GSDMB, a study suggests that GSDMB is cleaved by apoptotic caspase-3, caspase-6, and caspase-7, but not by inflammatory caspases [27] (**Figure 4**). This study found that GSDMB is cleaved in its N-terminal domain at the site $_{88}$DNVD$_{91}$, whereas GSDMD is cleaved in the interdomain linker region [27]. In contrast, another study found that GSDMB is cleaved by caspase-1 at site D236; transfection of plasmids expressing GSDMB and caspase-1 into 293T cells results in pyroptosis-like features [117] (**Figure 4**). However, transfection of a plasmid encoding a splice variant of GSDMB, rs11078928, encoding a truncated N-terminal domain into 293T cells fails to induce pyroptosis-like features [117]. This splice variant is associated with a lower risk of developing asthma in humans [117]. Whether apoptosis or pyroptosis inducers can trigger the cleavage of GSDMB endogenously in a cell, and whether pores formed by the N-terminal domain of GSDMB can release pro-inflammatory cytokines are important questions for future research.

Gasdermin C (GSDMC)

Humans have a single copy of the gene encoding GSDMC, whereas mice have four copies of the gene [105]. The gene encoding human GSDMC is expressed in the trachea, spleen, and epithelial cells of the stomach and esophagus [8, 105] (**Figure 2**). Human GSDMC is also preferentially expressed in metastatic melanoma cells [8]. In mice, the gene encoding GSDMC is expressed in the stomach, small intestine, cecum and colon [5] (**Figure 3**).

Similar to other GSDM family members, overexpression of the N-terminal domain of GSDMC induces pyroptosis-like features in 293T cells [18] (**Figure 4**). However, no inflammatory or

apoptotic caspases have been shown to activate GSDMC. Instead, GSDMC has been suggested to serve as a transcription factor [8]. Human GSDMC was originally named 'melanoma-derived leucine zipper, extranuclear factor' or MLZE owing to the presence of a putative leucine zipper in the C terminal domain [8]. The presence of this putative leucine zipper suggests that GSDMC might recognize a specific DNA sequence in nucleus and could potentially transmit signals between the nucleus and cytoplasm. Indeed, the C-terminal domain is found in the cytoplasm and nucleus, however, the full-length and N-terminal domain of GSDMC are localized to the cytoplasm [8].

There is no consensus on the biological function of GSDMC. A study reported that the full-length GSDMC protein inhibits cellular proliferation in gastric cancer cell lines, suggesting that it has a putative role in tumor suppression [105]. In contrast, small-interfering RNA-mediated knockdown of *GSDMC* in colorectal cancer cell lines, including DLD-1, LoVo, SW 480 and WiDr cells, inhibits cell proliferation; similarly, overexpression of *GSDMC* in SW 480 and WiDr cells promotes cell proliferation [118]. There is some evidence to suggest that the tissues of 11 patients with metastatic melanoma are more likely to stain positive for GSDMC [8]. However, further studies examining different types of cancer in a larger cohorts would be required to fully elucidate the role of GSDMC in cancer.

Gasdermin E (GSDME)

Human GSDME is expressed in the brain, heart, kidney and placenta [10] (**Figure 2**). Mutations leading to the expression of a truncated GSDME protein carrying an incomplete C-terminal domain are associated with hearing impairment in humans [10, 119-123]. Mouse GSDME is expressed in the cochlea, thymus, colon, lung, brain, spleen, and small intestine [10, 124] (**Figure 3**). Earlier studies suggest that the expression of the gene encoding human or mouse GSDME is promoted by the tumor suppressor p53, owing to the presence of one or

more putative p53 binding sequences at the intron 1 of *GSDME* [124]. In the human breast cancer cell line MCF7, genomic screening and microarray analysis revealed that *GSDME* is silenced by DNA methylation of its promoter region, and treatment with the demethylation agent 5-aza-2'-deoxycytidine fully restores p53-induced expression of *GSDME* [125]. The methylation of *GSDME* is thought to increase the risk of lymph node metastasis in patients with breast cancer [126]. Human *GSDME* is also methylated in colorectal cancer cell lines and primary colorectal cancer tissues [127]. Moreover, downregulation of *GSDME* is associated with acquired etoposide resistance in melanoma cells [128]. Therefore, GSDME might be a tumor suppressor and act by inducing programmed cell death in cancer cells.

GSDME is cleaved by caspase-3 [32, 33] (**Figure 4**). Caspase-3 is an apoptotic caspase, which can be activated by intrinsic and extrinsic apoptotic pathways, the former involves permeabilization of the mitochondrial membrane and the assembly of apoptosome leading to activation of caspase-9, and the latter requires activation of death receptors and caspase-8 [14]. Both apoptotic pathways lead to activation of caspase-3 and both induce cleavage of GSDME [32] (**Figure 4**). Caspase-3 cleaves human or mouse GSDME after residues 267–270 which encode a putative caspase-3 motif $_{267}$DMPD$_{270}$ or $_{267}$DMLD$_{270}$ [32, 33]. Following cleavage by caspase-3, the N-terminal domain of GSDME can perforate cell membranes and induce pyroptosis [32]. Overexpression of the N-terminal domain of GSDME results in pyroptosis-like features in human 293T cells [18]. Indeed, residues 1 to 56 of the N-terminal domain of GSDME carries a putative membrane-targeting domain encoding an amphipathic α-helix and a β-hairpin [32]. Further studies have shown that transfection of a plasmid encoding a truncated GSDME leads to increased levels of ROS and apoptotic and necrotic cell death in HEK293T cells [26, 30, 31]. However, the caspase-3 activators FasL and cytochrome c can trigger plasma membrane damage in GSDME-deficient macrophages, suggesting that GSDME may be redundant for late necrosis in this context [129].

In response to chemotherapy drugs, such as topotecan, etoposide, cisplatin and CPT-11 which can induce caspase-3-mediated apoptosis, GSDME-positive cell lines undergo pyroptosis, whereas GSDME-negative cell lines undergo apoptosis [33]. Transfection of a plasmid encoding GSDME in HeLa cells, a cell line which do not express GSDME, causes the cell line to undergo pyroptosis, instead of apoptosis, in response to TNF [33]. In macrophages and 293T cells expressing GSDME, apoptotic inducers vesicular stomatitis virus (VSV) and etoposide can activate GSDME in a caspase-3-dependent manner [32]. Moreover, mice lacking GSDME are resistant to toxicity induced by the GSDME-activating chemotherapy drug cisplatin compared to WT mice [33], suggesting that GSDME can be targeted to reduce potential side effects of chemotherapy. Collectively, GSDME has the ability to induce a pyroptosis-like cell death pathway downstream of caspase-3 activation. It is possible that when a cell fails to undergo apoptosis, GSDME is expressed and cleaved to serve as the executor of pytoptosis-like cell death in response to certain physiological stimulations. This strategy might provide an effective host response against pathogens capable of blocking apoptotic pathways.

Pejvakin (PJVK)

PJVK (also known as DFNB59) is a protein associated with hearing impairment in humans and mice [11, 130, 131]. Human PJVK is expressed in hair cells, supporting cells of the central nervous system, spiral ganglion neurons, spiral ganglion cells of the inner ear, and the cell bodies of neurons [11, 130, 132] (**Figure 2**). Two missense mutations within the N-terminal region of PJVK cause an impaired transmission of auditory signal by auditory neurons, leading to nonsyndromic recessive deafness in humans [11]. Patients with mutations in PJVK have impaired cochlear responses when exposed to low-energy sound, a phenotype which is recapitulated in PJVK-deficient mice [133]. Further, the mutation A290T in PJVK causes a

truncation of the C-terminal region, which results in dysfunctional outer hair cells leading to hearing impairment in mice [134, 135].

PJVK is localized to the peroxisomal membrane and is required for oxidative stress-induced peroxisome proliferation in HepG2 cells [133]. Cells in cochlea lacking PJVK have peroxisomal dysfunction and impaired antioxidant defenses, which contribute to susceptibilities to noise-induced oxidative stress in mice [133]. Two proteins have been found to bind the C-terminal region of PJVK; the actin cytoskeleton regulator, Rho-associated coiled-coil containing protein kinase 2 (ROCK2), and the scaffold protein, IQGAP1 [136]. ROCK2 is required to stabilize the actin cytoskeleton by modulating a wide range of substrates, such as myosin light chain (MLC) and LIM kinases [137]. PJVK binds to the coiled-coil domain of ROCK2 and further recruits substrates of ROCK2 [136, 138]. IQGAP1 is known to recruit signaling components to moldulate the actin cytoskeleton [139]. Whether PJVK promote or inhibit actin dynamics via its ability to bind to ROCK2 and IQGAP1 remains to be elucidated [136].

Conclusions and Future Perspectives

GSDM is a family of pore-forming proteins which have an emerging role in driving lytic cell death. Five members of the GSDM family, with the exception of PJVK, possess a pore-forming N-terminal domain which can induce pyroptosis-like features in overexpression systems [18]. Although these findings provide insights into the putative pore-forming roles of GSDMs, the cell types that express functionally-active GSDMs remain to be identified. Understanding the expression profile and regulation of GSDMs is critical, given that mutations in GSDMs are associated with a spectrum of clinical conditions, including alopecia, asthma, breast cancer, gastric cancer, colorectal cancer, and hearing loss [18, 25, 99, 108, 126, 140, 141].

GSDMD is cleaved by caspase-1, caspase-4, caspase-5 and caspase-11, yielding an N-terminal domain of the GSDMD protein that induces cytokine release and cell death

associated with activation of the inflammasome. Indeed, studies in mouse models have revealed a central role for GSDMD in the host defense against certain bacterial and viral infections. GSDME is cleaved by caspase-3 [32, 33] and some evidence suggests that GSMDB is cleaved by caspase-1, caspase-3, caspase-6, caspase-7 [27, 117]. Although the physiological role for GSDME in chemotherapy-induced toxicity has been implicated [33], the function of GSDMB is largely not known. Further, it remains unclear if proteolytic cleavage is a universal requirement to license activation of all GSDMs. Therefore, identifying the apical signals inducing liberation of the N-terminal effector domain of GSDM family members is an exciting area for future research. A better understanding of the mechanisms regulating GSDM activation could reveal new molecular targets for translation into therapies and treatment of human diseases.

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Competing interests

The author declares no competing interests.

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Figure Legends

Figure 1. Phylogenetic divergence of human and mouse Gasdermin family members.

BLAST sequence alignment of amino acid sequences of human and mouse Gasdermin family members extracted from the NCBI database. Scale indicates the number of substitutions for each amino acid in the sequence. Phylogenetic tree drawn by MEGA software version 7.

Abbreviations: hGSDMA, human Gasdermin A; mGSDMA1-3, murine Gasdermin A1, A2, A3; hGSDMB, human Gasdermin B; hGSDMC, human Gasdermin C; mGSDMC1-4, murine Gasdermin C1, C2, C3, C4; hGSDMD, human Gasdermin D; mGSDMD, murine Gasdermin D; hGSDME, human Gasdermin E; mGSDME, murine Gasdermin E; hPJVK, human Pejvakin; mPJVK, murine Pejvakin.

Figure 2. Expression of Gasdermin family members in humans.

Reported expression patterns of the Gasdermin family members in different organ sites in humans.

Abbreviations: GSDMA, human Gasdermin A; GSDMB, human Gasdermin B; GSDMC, human Gasdermin C; GSDMD, human Gasdermin D; GSDME, human Gasdermin E; PJVK, human Pejvakin.

Figure 3. Expression of Gasdermin family members in mice.

Reported expression patterns of the Gasdermin family members in different organ sites in mice.

Abbreviations: GSDMA1-3, murine Gasdermin A1, A2, A3; GSDMC1-4, murine Gasdermin C1, C2, C3, C4; GSDMD, murine Gasdermin D; GSDME, murine Gasdermin E; PJVK, murine Pejvakin.

Figure 4. The role of Gasdermin family members in inflammasome signaling, pore formation and cell death.

GSDMD mediates pyroptosis following cleavage by inflammatory caspases. The N-terminal domain of GSDMD binds membrane lipids of the plasma membrane and form pores, allowing release of the inflammatory cytokines IL-1β and IL-18 and induction of pyroptosis. Overexpression of GSDMA-N, GSDMA3-N and GSDMC-N can result in pore formation. Further, GSDMA3-N can associate with HSP90 for transportation to the mitochondria. The mitochondrial transport receptor TOM70 mediates the trasnoportation process and further stimulate the production of ROS. Mitochondrial dysfunction can activate caspase-9, which cleaves caspase-3. Alternatively, signaling through death receptors can activate the apoptotic caspase caspase-8, which can cleave caspase-3. Active caspase-3 cleaves GSMDE, generating GSDME-N to form pores in the plasma membrane. Both full-length GSDMB and GSDMB-N can bind membrane lipids, however, the pore-forming activity has only been observed when GSDMB-N is cleaved by caspase-1, but not by caspase-3, caspase-6 and caspase-7. The ability of PJVK to bind membrane lipids or form pores in the plasma membrane is uncertain.

Abbreviations: GSDM-N, Gasdermin N-terminal; GSDMA, Gasdermin A; GSDMB, Gasdermin B; GSDMC, Gasdermin C; GSDMD, Gasdermin D; GSDME, Gasdermin E; HSP90, heat shock protein 90; IL-1β, interleukin-1β; IL-18, interleukin-18; PJVK, Pejvakin; ROS, reactive oxygen species; TOM70, translocase of outer membrane 70; TRAP1, TNF receptor-associated protein 1.

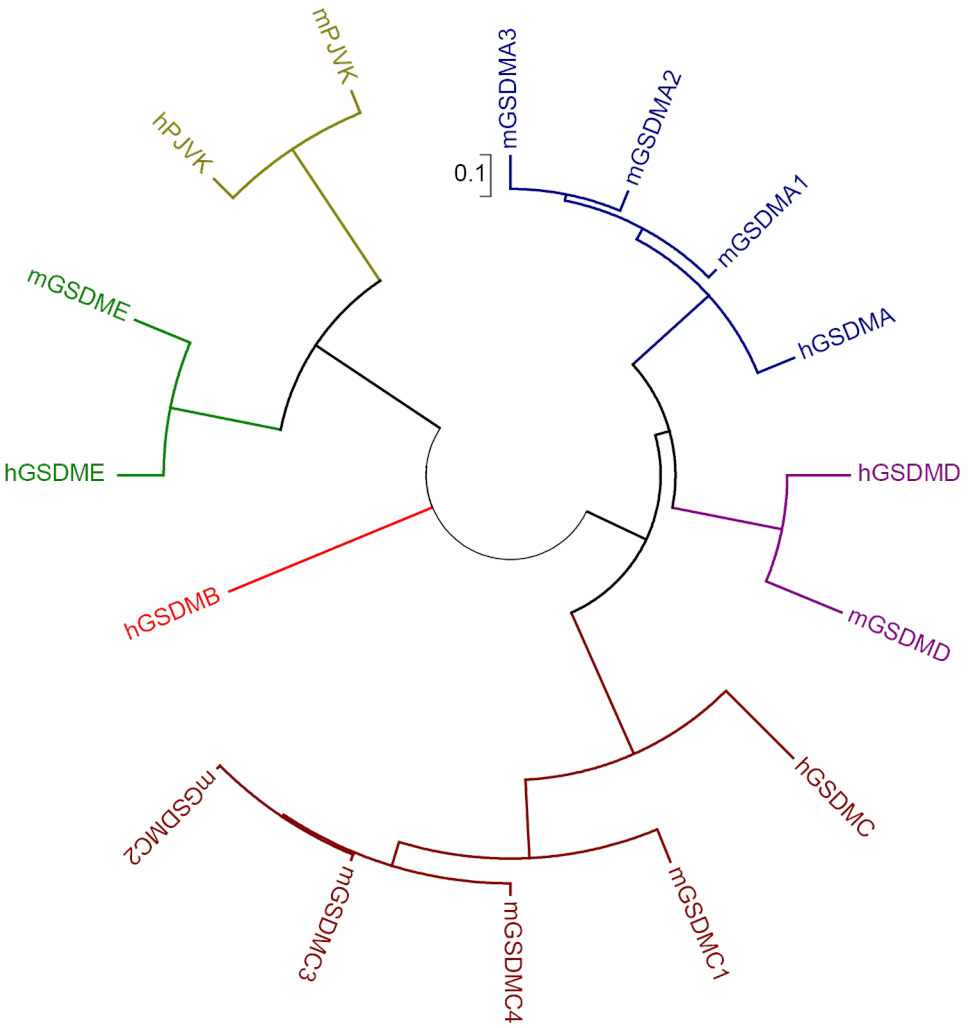
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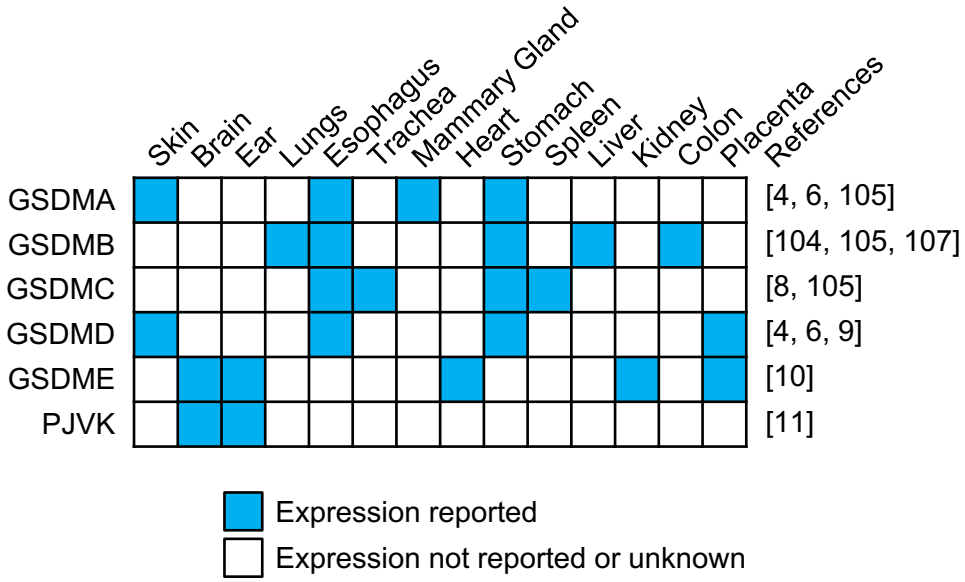
Figure 1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Heart
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Liver
Kidney
Colon
Placenta
References
GSDMA
GSDMB
GSDMC
GSDMD
GSDME
PJVK
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[8, 105]
[4, 6, 9]
[10]
[11]
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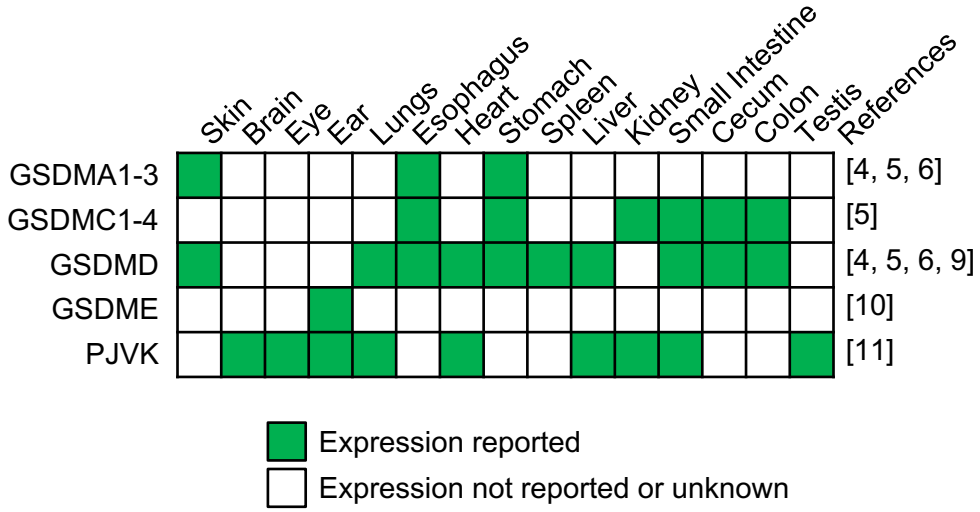
Figure 2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GSDMA1-3
[4, 5, 6]
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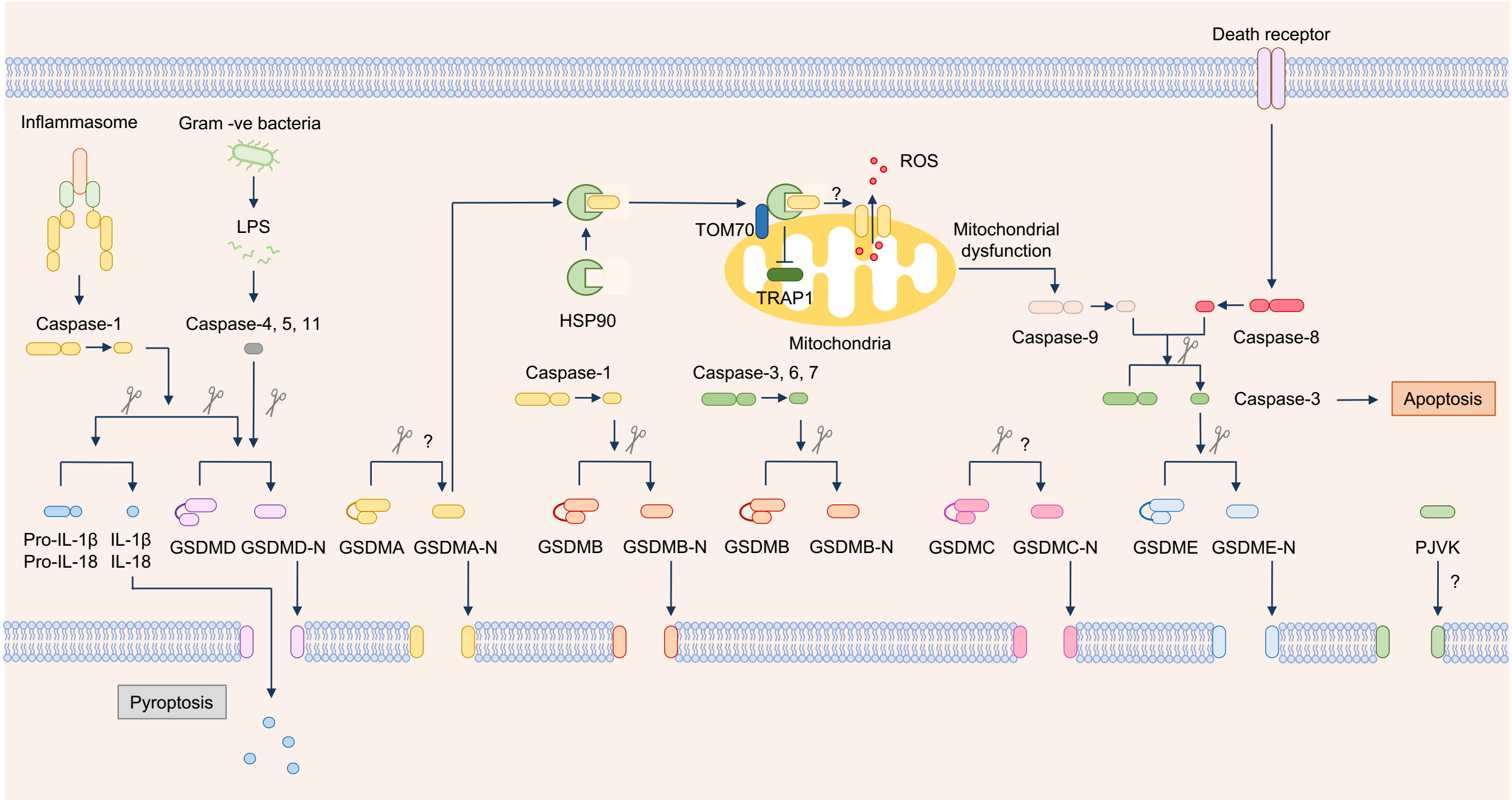
Figure 3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Gram -ve bacteria
LPS
ROS
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TOM70
HSP90
TRAP1
Mitochondrial dysfunction
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Caspase-1
Caspase-4, 5, 11
Caspase-9
Caspase-8
Caspase-3
Apoptosis
Caspase-1
Caspase-3, 6, 7
?
?
Pro-IL-1β
IL-1β
Pro-IL-18
IL-18
GSDMD
GSDMD-N
GSDMA
GSDMA-N
GSDMB
GSDMB-N
GSDMB
GSDMB-N
GSDMC
GSDMC-N
GSDME
GSDME-N
PJVK
?
Pyroptosis

Figure 4