REVIEW

Gasdermin D in pyroptosis

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Abstract Pyroptosis is the process of inflammatory cell death. The primary function of pyroptosis is to induce strong inflammatory responses that defend the host against microbe infection. Excessive pyroptosis, however, leads to several inflammatory diseases, including sepsis and autoimmune disorders. Pyroptosis can be canonical or noncanonical. Upon microbe infection, the canonical pathway responds to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), while the noncanonical pathway responds to intracellular lipopolysaccharides (LPS) of Gram-negative bacteria. The last step of pyroptosis requires the cleavage of gasdermin D (GsdmD) at D275 (numbering after human GSDMD) into N- and C-termini by caspase 1 in the canonical pathway and caspase 4/5/11 (caspase 4/5 in humans, caspase 11 in mice) in the noncanonical pathway. Upon cleavage, the N-terminus of GsdmD (GsdmD-N) forms a transmembrane pore that releases cytokines such as IL-1β and IL-18 and

disturbs the regulation of ions and water, eventually resulting in strong inflammation and cell death. Since GsdmD is the effector of pyroptosis, promising inhibitors of GsdmD have been developed for inflammatory diseases. This review will focus on the roles of GsdmD during pyroptosis and in diseases.

1. Introduction to GsdmD and pyroptosis

Gasdermin D, encoded by *GSDMD* on chromosome 8 (8q24.3), is a protein made of a 31 kDa N-terminus (GsdmD-N) and a 22 kDa C-terminus (GsdmD-C) connected by a peptide linker. Upon activation, the linker is cleaved to separate GsdmD-N from its autoinhibitory domain, GsdmD-C[1–3]. GsdmD-N forms a transmembrane pore that releases cytokines such as interleukin IL-1β[4,5] and IL-18[6] and also disturbs the regulation of ions and water[7], eventually resulting in pyroptosis. Pyroptosis is a form of inflammatory cell death *via* GsdmD-mediated cell lysis, which involves the participation of inflammatory caspases[1]. The inflammatory caspases are caspases 1, 4, and 5 in *Homo sapiens*, and 1 and 11 in *Murinae*[2]. Mouse caspase 11 is the counterpart to caspases 4 and 5 in humans. The most critical role of pyroptosis is to induce strong inflammatory responses that aid in host defenses against pathogen infection.

The pyroptotic pathways are important drug targets because they play critical roles in several diseases, including, but not limited to, sepsis[3], Alzheimer's disease[8], human immunodeficiency virus (HIV)[9], and gout[10]. Several compounds, such as VX-765, are currently being tested for pyroptosis-associated diseases. Chemical danger signals like cytokines are released upon pyroptotic cell death, while some may even be released before cell death[11]. These danger signals result in the expansion of blood vessels in the localized area, leading to an increase in blood flow. This leads to other symptoms of inflammation, such as swelling and excess heat in the inflamed area. Collectively, the induction of inflammation is a double-edged sword in that it could defend against intracellular bacteria leading to resolution; however, it could also promote pathological inflammations, leading to disease development such as aberrant blood clotting and sepsis[3,12,13].

The gasdermin family of proteins consists of 6 proteins: gasdermins A–E and deafness autosomal recessive type 59 (DFNB59, also called pejvakin). Except for DFNB59, the gasdermin family plays various roles in pyroptosis[14]. The mechanism of how pyroptosis plays a role in the innate immune system are discussed briefly in this review, and more details can be found in many excellent review papers[15–18].

2. Other members of gasdermin family and their roles in pyroptosis

The gasdermin family found in humans is composed of six members: gasdermins A–E, and DFNB59. Mice have no GsdmB but do have three GsdmA homologues (GsdmA1–3) and four GsdmC homologues (GsdmC1–4). Except for DFNB59, the family members have two domains, an N-terminal domain and a C-terminal domain connected by a peptide linker. These two domains of the gasdermins are similar in structure and have a sequence homology of ∼45%. The N-terminal domains are generally considered the effector domain

capable of forming transmembrane pores, while the C-terminal domains of GsdmA/D/E have been shown to play auto-inhibitory roles. Among the gasdermins, GsdmD and GsdmE have been better characterized for their activation and function.

2.1. GsdmA

GSDMA maps on chromosome 17 at 17q21 and has been associated with autoimmune diseases and cancers[19]. As mentioned above, mice have three homologues of GsdmA, and most of the mutation phenotypes in mice are mapped to GsdmA3. These mutations of GsdmA3 have been shown to cause strong skin inflammations[20,21] suggesting a role in pyroptosis. When the N-terminus is expressed by itself in cells, pyroptosis is triggered[22]; however, the cleavage enzyme of GsdmA is still unknown. GsdmA3 has been well characterized in structure, including full length (FL) GsdmA3[23,24] and the pore form of the N-terminal domain[24]. Similar to GsdmD, its C-terminus inhibits the N-terminus, the activation of which triggers the formation of the transmembrane pore to execute pyroptosis[23].

2.2. GsdmB

Like *GSDMA*, *GSDMB* maps at 17q21, and its polymorphisms are linked to autoimmune diseases such as asthma and inflammatory bowel disease (IBD)[25]. GsdmB enhances the activity of caspase 4 during noncanonical pyroptosis, suggesting its role in inflammation[26]. Paradoxically, GsdmB can be cleaved by apoptotic caspases 3/6/7, but not by inflammatory caspases, with previous evidence suggesting the cleaved N-terminal product may not contain the full N-terminal domain to form pores or directly participate in inflammation[27]. In a recent report, however, it was found that granzyme A from cytotoxic T cells and natural killer cells cleave and activate gasdermin B (GsdmB), triggering pyroptosis in tumor cells[28]. This suggests a more direct involvement in pyroptosis than what was previously understood. Intriguingly, *GSDMB* is overexpressed and considered an oncogene in several cancer types such as breast cancer[29], gastric cancer[27], and cervical cancer[30]. As such, further studies of GsdmB regarding its roles in pyroptosis and cancers are needed.

2.3. GsdmE

GSDME, mapping on chromosome 7 at 7p15, is also named deafness autosomal dominant 5 (*DFNA5*). Initially, it was associated with hereditary hearing loss, but without assigned physiological function[31]. Recently, GsdmE has been discovered to contribute to the regulation of both apoptosis and pyroptosis[32,33]. Its C-terminal domain also plays an inhibitory role in the N-terminal domain. Upon activation by caspase 3, the N-terminal

domain forms lytic pores to execute apoptosis and/or pyroptosis[32,33]. GsdmE is also activated by killer-cell granzyme B, which is released into tumor cells by cytotoxic lymphocytes. Subsequently, granzyme B cleaves GsdmE to induce pyroptosis in tumors[34]. Consistently, GsdmE mediated pyroptosis contributes to the cytotoxicity of many chemotherapeutic reagents[33], including a potential target for hepatocellular carcinoma patients, which uses the secondary metabolite miltirone[35].

2.4. DFNB59

DFNB59 is located at 2q31 and has not been associated with either pore formation or pyroptosis. Compared with other gasdermin members, DFNB59 has a shorter C-terminal domain that does not share sequence homology with other gasdermin proteins. Mutations of *DFNB59* cause a neuronal defect, leading to non-syndromic deafness[36,37]. It has been suggested that DFNB59 functions as a receptor/adaptor in pexophagy, which maintains redox homeostasis of auditory hair cells to prevent noise-induced damage[38].

2.5. GsdmC

GSDMC maps on chromosome 8 at 8q24.21. Limited is known about GsdmC, although artificial mutations cause cytotoxicity in cell lines[23]. It is highly expressed in the gastrointestinal (GI) tract and may play a role in GI cancers[19,39].

3. Introduction to pyroptotic pathways

There are two major pathways for pyroptosis: canonical and noncanonical pyroptosis (Fig. 1). Both pathways use GsdmD as the downstream effector[40–42]. The canonical pathway detects both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) as well as cytoplasmic disturbances, recently coined as homeostasis altering molecular properties (HAMPs)[43]. PAMPs and DAMPs are recognized intracellularly by cytoplasmic pattern recognition receptors (PRRs, Fig. 2), the activation of which assembles the inflammasome complex to execute pyroptosis. In contrast, the recognition of HAMPs is through the detection of molecular processes that perturbs cytoplasmic homeostasis, *e.g.*, the phosphorylation of pyrin inflammasome[44,45]. Generally, after the binding of a PAMP or DAMP, PRRs interact and activate the apoptosis-associated speck-like protein (ASC), which then oligomerizes and uses its caspase activation and recruitment domain (CARD) to bind to the CARD of pro-caspase 1. The binding complex of PRRs, ASC, and pro-caspase 1 is termed the inflammasome. A CARD−CARD interaction triggers the activation of pro-caspase 1 in order to cleave GsdmD and pro-IL-1β/IL-18 into their active forms, initiating pyroptosis. During pyroptosis, GsdmD-N selectively interacts with membrane lipids to form transmembrane pores, through which cellular contents, especially danger signals, are released[23,46]. The canonical pathway also utilizes toll-like receptors (TLRs) for priming certain PRRs to enhance immune responses. The noncanonical pathway detects intracellular lipopolysaccharides (LPS) of Gram-negative bacteria. LPS directly activates pro-caspase 11 (pro-caspase 4/5 in humans) by binding to CARD, resulting in the oligomerization and autoproteolysis of caspase 11[1,13]. Similar as caspase 1, active caspase 11 cleaves the linker region of GsdmD to separate GsdmD-N and GsdmD-C, initiating pyroptosis. The noncanonical pathway crosstalks with the canonical pathway *via* NOD-like receptor family pyrin domain containing 3 (NLRP3), which activates caspase 1 to cleave pro-IL-1β/IL-18, promoting inflammation. Collectively, these events trigger downstream effects, such as cell death by membrane disruption and the release of cytokines through GsdmD-N pores, as shown in Fig. 1[41,47]

3.1. Canonical cascade

NOD-like receptors (NLRs), which are the cytoplasmic pattern-recognition receptors (PRRs), can recognize PAMPs and DAMPs to initiate the canonical inflammasome pathway. After binding
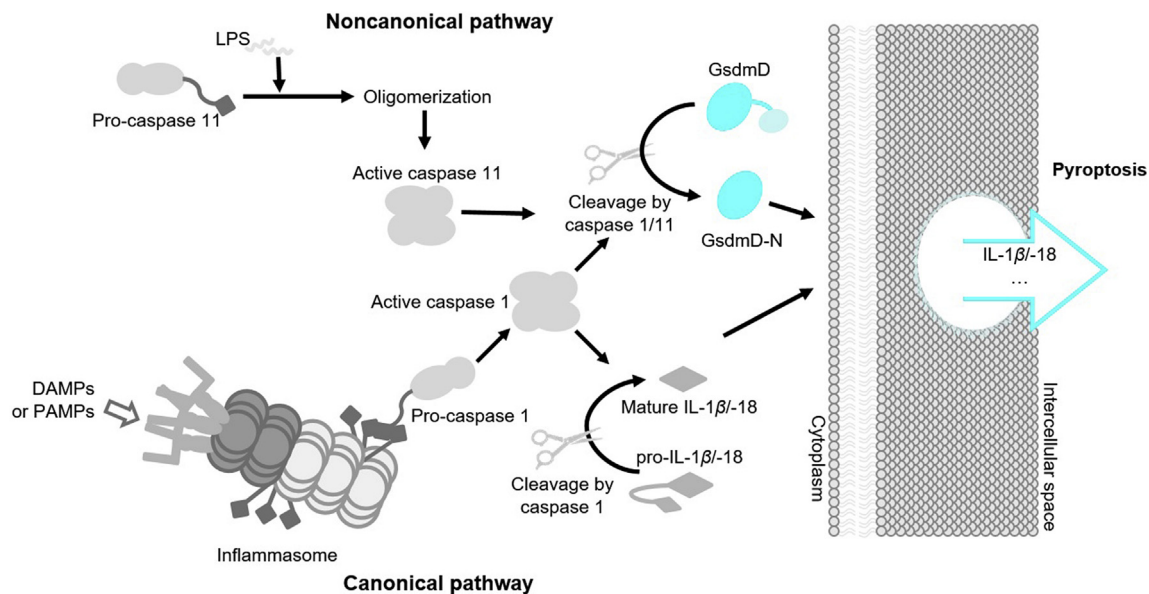

Figure 1   Activation of either caspase 1 or caspase 11 can lead to pyroptosis. Pro-caspase 11 is directly activated by LPS, which leads to activated caspase 11 that cleaves GsdmD. Pro-caspase 1 is activated by the CARD recruiting domain after receptor activation and inflammasome formation. Caspase 1 can activate GsdmD and IL-1β/IL-18.

with a DAMP or PAMP, NLRs are activated (Fig. 2), followed by the recruitment of the adaptor ASC complex. This results in the formation of inflammasomes, which recruit and activate pro-caspase 1. Activated caspase 1 then cleaves GsdmD to free its N-terminus, which forms the lytic pore as the pyroptotic effector[48]. Some well-characterized pathways are shown in Fig. 2 [10,49,50]. Except for NLRP1, NLRs typically contains three domains: an N-terminal adaptor domain (such as CARD or pyrin domain (PYD)), a central nucleotide-binding domain (NBD), and a C-terminal leucine-rich repeat (LRR) domain. NLRP1 contains two additional domains at its C-terminus, a CARD followed by a function to find domain (FIIND) domain. The LRR domains are used to sense bacterial components; the NBD domain is critical for oligomerization and activation; the N-terminal domain is responsible for CARD recruitment and CARD−CARD interactions as well as for activating caspase 1[51,52]. In addition to NLRs, absent in melanoma 2 (AIM2) and pyrin, two other cytoplasmic PRRs, detect bacterial components and form inflammasomes to activate caspase 1. Individual pathways are explained in more detail in the following paragraphs.

NLRP1, which was first identified in humans, was later found in mice and was the first discovered NLR family member discovered to be involved in the formation of an inflammasome complex. NLRP1 does not require ASC and thus is able to activate pro-caspase 1, leading to IL-β secretion without ASC oligomerization[53]. It is coded by a single gene in humans with three homologues NLRP1a, b, and c in mice[12,50,54]. NLRP1b is activated by anthrax lethal toxin after it cleaves an N-terminal peptide from NLRP1b[55], while human NLRP1 is not activated by the same toxin[56]. However, the molecular mechanism of cleavage-mediated NLRP1b inflammasome activation remains elusive. Using an in vitro system consisting of recombinant proteins, NLRP1 in humans was found to be activated by muramyl dipeptide and ATP[57]. In addition, inhibitors of dipeptidyl peptidases 8 and 9 (DPP8/9) can selectively activate NLRP1 and its related protein CARD8 in both mouse and human lymphocytes[58]. Interestingly, NLRP1b responds to metabolic stress to produce IL-18[59] (known to prevent obesity and diet-induced metabolic syndrome), which paradoxically can induce inflammation. The activation of the NLRP1 inflammasome is also essential to the secretion of high mobility group protein B1 (HMGB1), which stimulates inflammatory responses by modulating both the innate and the adaptive immune responses[50].

NLRP3 uses a similar overall mechanism as other NLRs such as NLRP1. However, the major difference is that NLRP3 may not directly interact with its activators; rather, it must first be primed by a cytokine receptor or another PRR[60]. NLRP3 can be activated by a wide range of DAMPs and PAMPs such as bacterial LPS, fungal zymosan, viral RNA extracellular ATP, reactive oxygen species, nigericin, crystallines, and amyloid-β plaque[60]. Lysosomal damage has also been attributed to NLRP3 activation, with lysosomal membrane damage resulting in K^+ efflux and the release of lysosomal protease cathepsin B, both of which initiate NLRP3 inflammasome activation[61]. Despite the

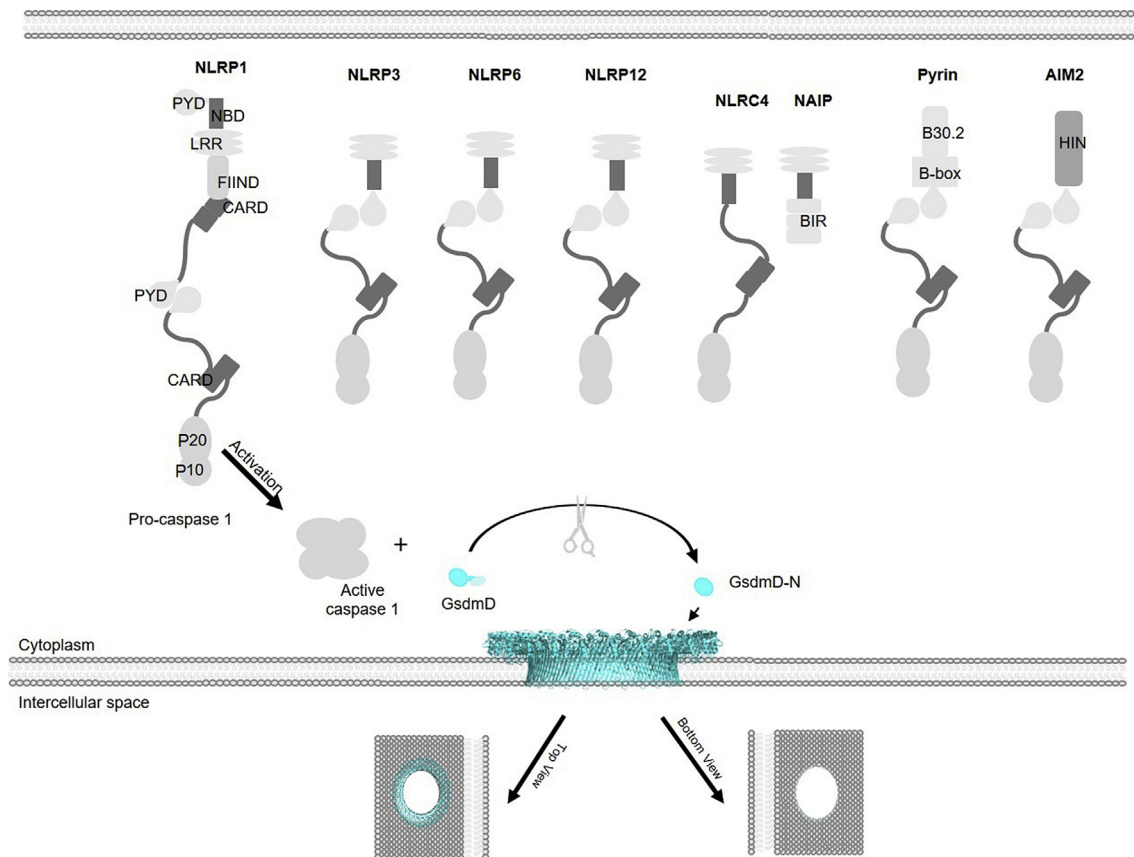**Figure 2** Inflammasomes in canonical pyroptosis pathway. Ligands are differentially recognized by PRRs, which then assembles corresponding inflammasome complexes to activate caspase 1. Activated caspase 1 subsequently cleaves GsdmD to free its N-terminus, which forms the transmembrane pore and functions as the executor of pyroptosis. Note: the oligomerization of inflammasome proteins is not shown.

diverse activators, a common downstream event is the disassembly of the *trans*-Golgi network (TGN), which recruits NLRP3 using phosphatidylinositol-4-phosphate (PtdIns4P) and activates NLRP3 to form the inflammasome complexes[62]. Another crucial player during NLRP3 activation is the protein NIMA-related kinase 7 (NEK7), which participates by regulating NLRP3 inflammasome formation following potassium release and preventing inflammasome formation during mitotic division stages of the cell cycle[63,64]. Co-crystal structures show that NEK7 directly binds to two neighboring NLRP3 subunits as a necessity for inflammasome formation[65]. After activation, the NLRP3 receptor recruits the ASC by PYD−PYD interaction, assembling NLRP3 inflammasome (Fig. 2). When NLRP3 is knocked out in mice or antagonized with inhibitors, there is a significant reduction of pyroptosis, suggesting NLRP3's key involvement in the pyroptotic pathway. NLRP3 crosstalks with other innate immune pathways[66,67], suggesting that the inhibition of the NLRP3 could have a broad inhibitory effect for inflammatory responses. As such, NLRP3 is an attractive drug target for patients with inflammatory diseases[68]. Research involving treatments for septic shock has recently shown cardamonin, a secondary herbal metabolite used in Chinese traditional medicine (CTM), showed inhibitory effects on NLRP3 activation within mouse bone marrow-derived macrophages (mBMDM) and human peripheral blood mononuclear cells (PBMC) by preventing ASC oligomerization[69]. It has also been shown that overactivation of the NLRP3 pathway causes gouty arthritis in mice models[10]. OLT1177, an inhibitor of NLRP3, is in the U.S. Food and Drug Administration (FDA) clinical trials phase II for gouty arthritis[68].

NLRP6 has an N-terminal PYD, an internal NBD, and a C-terminal LRR. Similar to other NLR inflammasomes, activated NLRP6 causes ASC speck formation[70] and subsequently caspase 1 activation. NLRP6 has been reported to play an inflammasome-dependent role in host defenses and inflammation and an inflammasome-independent role in intestinal homeostasis and cancer. However, the molecular mechanisms in these processes are not fully elucidated, and significant discrepancies exist[71,72].

Like NLRP6, NLRP12 plays both inflammasome-dependent and -independent roles. NLRP12 inflammasome participates in the canonical pyroptotic pathway by assembling the NLRP12 pyroptosomes using a similar mechanism to NLRP6 (Fig. 2). Conversely, NLRP12 is also capable of negatively regulating inflammation by promoting the degradation of NF-κB inducing kinase (NIK)[73]. The reduction of NIK leads to attenuated noncanonical NF-κB signaling, which is crucial to inflammatory responses to pathogens in the innate immune system[73]. NLRP12, similar to NLRP6, is highly-expressed within intestinal tissues, and plays a central role in maintaining intestinal inflammation, tumorigenesis and homeostasis[73,74].

Pyrin, not an NLR, has an N-terminal PYD, followed by a B-box domain, a coiled-coil domain, and a B30.2 domain at the C-terminus. Pyrin detects the inactivation of Rho GTPase, especially RhoA, which is targeted by various bacterial toxins[75]. Without bacterial infection, pyrin stays inactivated when phosphorylated by protein kinase1/2 (PKN1/2), and bound to protein 14-3-3. This inactive form is a monomer, which dimerizes to become active[76]. As a means of regulation, active RhoA promotes PKN1/2 to phosphorylate pyrin[76]; toxin-induced inactivation of RhoA leads to pyrin activation. Microtubules have also been shown to facilitate the dimerization of pyrin. Upon dimerization, pyrin triggers the ASC speck formation, which subsequently activates pro-caspase 1[75]. Mutations in pyrin have been correlated with inflammatory diseases, familial Mediterranean fever (FMF)[77], and hyper-immunoglobulinemia D syndrome (HIDS). AIM2, not an NLR, consists of an N-terminal PYD and a C-terminal positively charged HIN (hematopoietic expression, interferon-inducible nature, and nuclear localization) domain. AIM2 plays an important role in the innate immunity for the detection of cytoplasmic pathogenic dsDNA[43]. The HIN domain uses its positive charge to bind the negatively charged dsDNA. This binding is independent of DNA sequence but requires a minimum of 80 base pairs in length[49]. Once the HIN domain detects dsDNA in the cytoplasm, AIM2 is activated and uses its PYD to recruit ASC by PYD−PYD interaction[78]. The ASC speck forms and recruits pro-caspase 1 *via* CARD−CARD interactions, similar to NLRP3 inflammasome[49]. This activation pathway further facilitates immune response, ensuring escaped foreign DNA particles from phagosomes are targeted[79]. Like other inflammasomes, The AIM2 inflammasome is tightly controlled in cells and has been shown to be negatively regulated by interferon-inducible protein p202 within mBMDMs for the activation of pro-caspase 1[80].

NLRC4, composed of an N-terminal CARD, an NBD, and a C-terminal LRR, plays a major role in protection against certain Gram-negative bacteria with type III or type IV secretion systems[81]. NLRC4 forms complexes with NAIPs (NLR family apoptosis inhibitory proteins), and activates NLRC4 by binding to pathogenic proteins from flagellin or type III/IV secretion systems. Once the NLRC4 is activated, it oligomerizes and directly interacts with pro-caspase 1 *via* a CARD−CARD interaction (Fig. 2), bypassing the participation of ASC. This direct CARD−CARD interaction between the NLRC4 and pro-caspase 1 leads to its activation as well as the formation of GsdmD-N pores and interleukin release[81]. There are several gain-of-function mutations in NLRC4, which have been shown to result in the development of autoinflammatory diseases[82].

3.2. Noncanonical cascade

The noncanonical pathway plays a critical role in cell immunological responses to intracellular Gram-negative bacteria[13,41,83], where LPS is detected by caspase 4/5/11.

Intracellular LPS can be derived directly from intracellular bacteria or the extracellular space. Before encountering LPS, pro-caspase 11 has negligible catalytic activity. The CARD domain of pro-caspase 4/5/11 directly interacts with the lipid A moiety of LPS, causing a significant conformational rearrangement of pro-caspases 4/5/11. The conformational changes of these pro-caspases result in oligomerization using their CARD domains, further enabling limited activity for auto-proteolysis. It is important to note that the oligomerization of caspase 11 directly involves CARD, while the CARD of caspase 1 is used for ASC binding instead of oligomerization[84,85]. After oligomerization, caspase 11 auto-proteolyze after D285 with a sequence of MEAD|A to gain full activity in proteolyzing GsdmD[22,41]. In contrast to caspase 1, which cleaves pro-IL-1β/IL-18 and GsdmD, activated caspases 4/5/11 has been considered only recognize GsdmD and cleave at D275 of hGsdmD or D276 of mGsdmD[22,41] for generating pyroptotic pores (Fig. 1). Interestingly, caspase 4/11 were shown to be activated by guanylate-binding proteins 1−4, which activate innate immunity against intracellular pathogens, on the surface of intracellular bacterium to cleavage pro-IL-18[86].

Additionally, the active caspase 4/5/11 also crosstalk with NLRP3, giving rise to the formation of the NLRP3 inflammasome, which can then activate caspase 1 for the production of mature cytokines, IL-1β/IL-18[12]. This crosstalk is suggested to involve a drastic efflux of K$^+$ due to membrane rupturing caused by caspase 11 working as a signal to elicit NLRP3 inflammasome activation[87]. The noncanonical pathway has also been suggested to involve metabolic diseases due to mitochondrial dysfunction with the release of mitochondrial reactive oxygen species (ROS) and mitochondrial DNA (mtDNA). Activation of inflammasomes *via* both the canonical and noncanonical pathways display immune response variability enabling the host to elicit a variety of defense mechanisms dependent upon the type of pathogen infection[88].

4. Mechanism of GsdmD activation in pyroptosis

The activation mechanisms of hGsdmD and mGsdmD are considered identical, but the cutting sites on the linker-region are slightly different, with FLTD|GVP in hGsdmD and LLSD|GID in mGsdmD[41]. Neither hGsdmD nor mGsdmD has a high-resolution structure available in the pore form, although the structures of inactive FL proteins have been solved. The N-termini of mGsdmD and hGsdmD have a ~60% sequence similarity with the N-terminus of mGsdmA3 (Fig. 3A). As shown in Fig. 3B, there is a significant structure overlap among the inactive N-termini of hGsdmD (PDB:6N9O), mGsdmD (PDB:6N9N), and mGsdmA3 (PDB:5B5R), with an RMSD of 1.2 Å[23,24,89]. Therefore, the structure of mGsdmA3 in the pore form resembles that of a GsdmD pore (Fig. 3). Using cryo-electron microscopy (Cryo-EM), the structure of mGsdmA3 pore is solved with a resolution of 3.8 Å[24]. Cryo-EM resolved the GsdmD pore structure with a resolution of low nanometers because of insufficient homogeneity[48]. mGsdmA3 pore is formed by 26−28 subunits, while GsdmD uses a similar number of subunits for pore formation. The GsdmA3 pore, which is formed by a series of β-barrels, has a ~180 Å inner diameter and ~280 Å outer diameter (Fig. 3C). The inner diameter is large enough to pass proteins like IL-18, IL-1β, and metabolites. Additionally, galectin-3, an effector of the NLRP3 inflammasome, could also pass through the pore[90], suggesting the GsdmD pore may selectively release cellular proteins. The height of this cross-membrane β-barrel is ~70 Å (Fig. 3D), which is the typical thickness of mammalian membranes.

Without proper activation, the inflammatory caspases 1/4/5/11 are in catalytic inactive pro-forms, leaving GsdmD in the inactive form[40]. Previous studies have shown that by mutation of the aspartic acid within the linker, GsdmD stays in its inactive form without being cleaved, and a pyroptotic pore cannot form[91]. Additional studies have shown that overexpression of the C-terminus suppresses the creation of pores in the membrane.
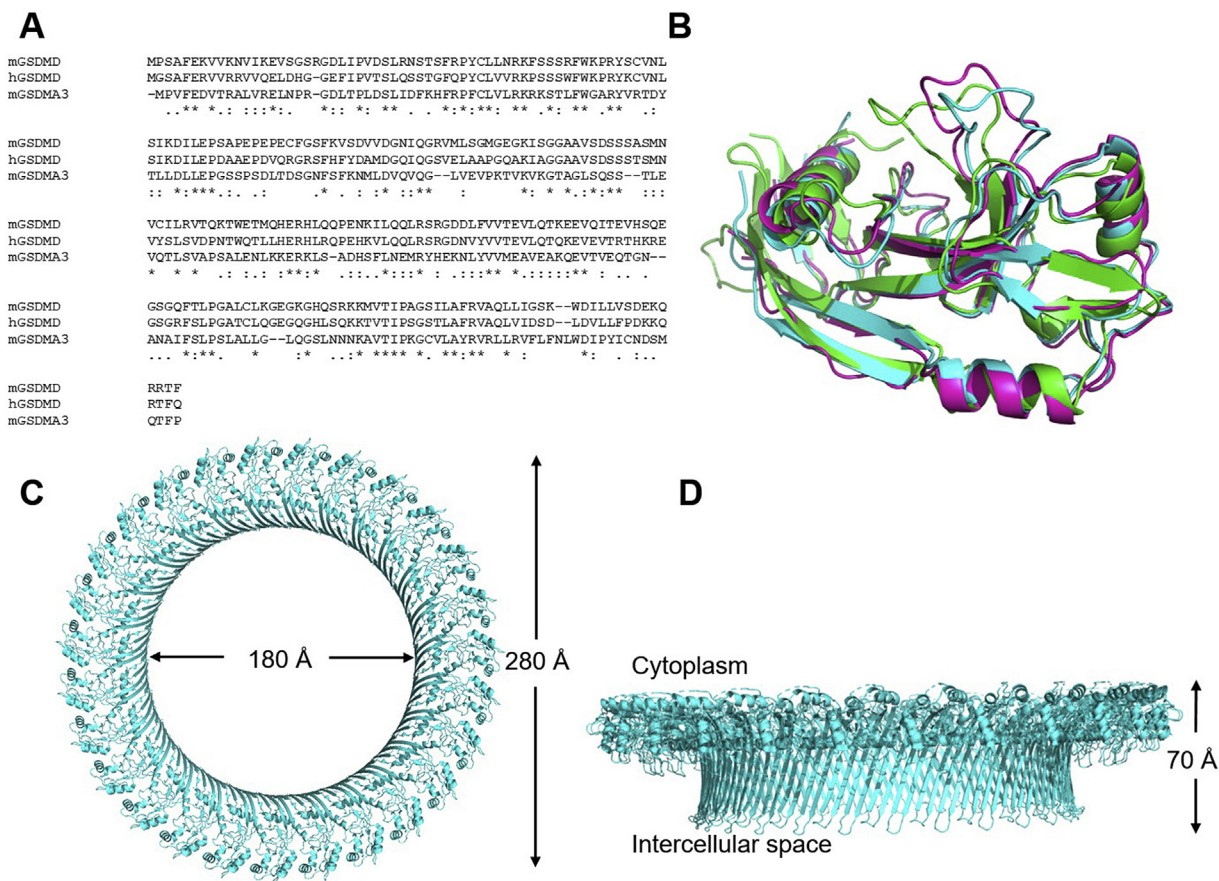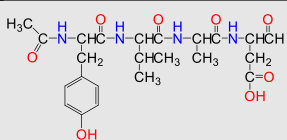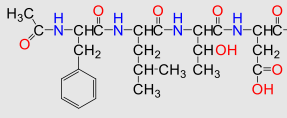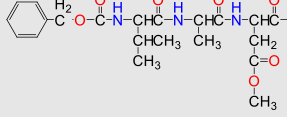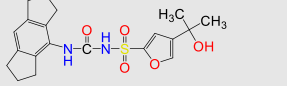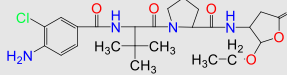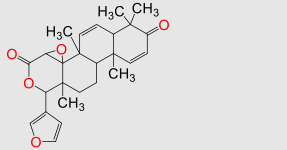
Figure 3 Similarities of the N-termini of hGsdmD, mGsdmD, and mGsdmA3, and the structure of the transmembrane pore of mGsdmA3. (A) The sequence and (B) structural alignments of the unactivated N-termini of hGsdmD, mGsdmD, and mGSDMA3 suggest the three proteins adopt similar structures after pore formation. Green: hGsdmD, PDB:6N9O; Pink: mGsdmD, PDB:6N9N; Cyan: mGsdmA3, PDB:5B5R. Note: There are ~30 amino acids at the end of N-terminal domain that are not all structurally available for all three proteins, and these amino acids are not shown in (A) and (B). The (C) top to bottom and (D) side views show the symmetric assembly of mGsdmA3 pore.

Table 1 Pyroptosis inhibitors, including their names, structures, targets, and type of inhibition.

Name of inhibitor	Structure of inhibitor	Target	Type of inhibitor
Ac-YVAD-CHO		Caspase 1/4	Suicide inhibitor
Ac-FLTD-CMK		Caspase 1/4/5	Suicide inhibitor
Z-VAD-FMK		Pan-caspase	Suicide inhibitor
MCC950		NLRP3	Allosteric or competitive inhibitor
VX-765		Caspase 1/4	Competitive inhibitor
7DG		Protein kinase-R	Activity-independent inhibitor

This suggests that GsdmD-C plays an inhibitory role for GsdmD-N and does not require the linker region for the inhibition of GsdmD-N[89,92].

Without the activation of GsdmD, pore-forming activity and pyroptosis are inhibited. Similarly, without the cleavage of pro-IL-1β or pro-IL-18 to their mature forms, inflammatory responses are limited. A full spectrum of pyroptosis includes the cleavage of pro-IL-1β, and pro-IL-18 as well as GsdmD, capable of eliciting significantly damaging effects to the local tissue and is not the best choice for immune cells to resolve minor insults. Under a mild challenge, DAMPs and PAMPs can be released independent of the activation of the inflammatory caspases and pyroptosis[13,47,93], suggesting that pyroptosis, the highly inflammatory form of cell death, is under tight regulation. This tight regulation of the inflammatory caspases prevents long-term damage such as organ damage[33,94,95], sepsis[3], Alzheimer's disease[8], atherosclerosis[96], and gout[10].

Compared to caspase 5, caspase 4's and 11's autocleavage is better characterized and shares a similar mechanism. Upon activation, the autocleavage of caspase 4/11 generates an enzymatically active caspase 4/11 dimers. However, it is still not fully elucidated if the dimer consists of p10+p32 or p10+p22. The p10 subunit binds to the GsdmD-C domain for substrate recognition before cleavage to separate GsdmD-N and GsdmD-C[97]. When GsdmD-C is replaced with mCherry with the cleavage site intact, free GsdmD-N cannot be generated as the mutant GsdmD is not recognized by the inflammatory caspases[97]. Consistently, in the caspase 4/GsdmD-C complex structure, the N-terminus of p10 is shown to stretch toward a hydrophobic patch of GsdmD-C, suggesting a direct interaction with GsdmD-C is required for

cleavage. After aligning the GsdmD structure along with the caspase 4/GsdmD-C complex, it shows that the cleavage site, P4−P1, of GsdmD is next to the autoprocessing (P4−P1 of caspase 4) site, suggesting the binding of caspase 4 and GsdmD-C in the crystal structure is physiologically relevant. Collectively, caspase 4 binds to GsdmD-C for recognition, followed by the cleavage and release of GsdmD-N from GsdmD-C[97]. In addition to covalent interaction using the peptide linker, GsdmD-C interacts with GsdmD-N using a large hydrophobic patch to prevent the conformational changes necessary for pore formation. Interestingly, some GsdmD-C is secreted out of the pore during pyroptosis. This could be a strategy to eliminate the noncovalent inhibition of GsdmD-C.

5. Examples of pyroptotic inhibitors

Since the uncontrolled activation of GsdmD can cause disease in both human and mouse models, caspase 1/4/5/11 are important drug targets for inflammation-related diseases[3,8,98,99]. Z-VAD-FMK is a cell-permeable inhibitor that was originally developed to bind irreversibly and inhibit inflammatory caspases[100]. The tripeptide, VAD, is for binding, while FMK is the warhead that can form a covalent bond with the catalytic cysteines, permanently preventing catalytic activities[101]. However, Z-VAD-FMK does not specifically inhibit only the inflammatory caspases 1/4/5/11 as originally designed; it also inhibits caspases 3/7/8 as a suicide inhibitor, inhibiting both pyroptosis and apoptosis. The promiscuity between caspases makes it unsuitable as a drug because of the off-target effects[100,101].

Acetyl-FLTD-chloromethylketone (Ac-FLTD-CMK) has also been developed to inhibit inflammatory caspases (Table 1) with K_d

values of 21.7 and 29.6 μmol/L to caspases 1 and 4, respectively. Unlike pan-caspase inhibitors, it does not bind to apoptotic caspase 3. Using ATP or nigericin-treated macrophage cells, Ac-FLTD-CMK significantly reduced the secretion of IL-1β[102], suggesting its inhibitory action for pyroptosis. In an *in vitro* caspase activity assay using a synthetic substrate, *N*-acetyl-Trp-Glu-His-Asp-7-amido-4-methylcoumarin (Ac-WEHD-AMC), IC$_{50}$ values of Ac-FLTD-CMK differ significantly for caspases 1, 4, and 5 with values of 0.0467, 1.49, and 0.329 μmol/L, respectively. The compound is water-soluble, similar to the previously mentioned pan-caspase inhibitors, although it slightly violates Lipinski's rule of five by having a molecular weight of 569 Da[103].

NEK7 directly interacts with NLRP3 for its activation[63,104] (Table 1). The expression level of NEK7 has been shown to be closely correlated with the degree of pyroptosis. As such, NEK7 has been suggested to be a marker for pyroptosis. Even though NEK7 plays roles in multiple pathways such as cell cycle regulation, NEK7 has been suggested to be a drug target for the prevention of inflammation in pyroptosis[105]. Studies have suggested a linkage between IBD and NEK7. During the development of IBD, which has been directly linked to NLRP3 and pyroptosis, NEK7 is upregulated through NF-κB and modulates NLRP3 activation by direct interaction[94,106].

Rv3364c, a protein secreted by *Mycobacterium tuberculosis*, was recently reported to inhibit host serine protease cathepsin G (CTSG), enabling *M. tuberculosis* survival in macrophages[107]. Rv3364c binds tightly to CTSG on the host cell membrane, suppressing its catalytic activity and downstream activation of caspase 1. This hinders the maturation of caspase 1 and inhibits pyroptosis within infected macrophages. Consistently, the exposure of *M. tuberculosis* resulted in the recruitment of a large number of infected macrophages but with a lower pyroptotic rate. However, when exposed to *M. tuberculosis* with Rv3364c knocked out, cellular immunity by pyroptosis was restored. This decreased the number of infected macrophages, with the rate of pyroptosis increasing back to normal levels. The restoration of pyroptosis levels was then able to prevent intracellular *M. tuberculosis* from proliferating[13,107]. Currently, inhibitors mimicking Rv3364c binding patterns are under development for inhibition of inflammation[107].

N-[[(1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)amino]carbonyl]-4-(1-hydroxy-1-methylethyl)-2-furansulfonamide (MCC950) is a promising inhibitor targeting the NLRP3 pathway[108]. MCC950 directly binds to the Walker B motif of NLRP3, which prevents ATP hydrolysis and associated conformational changes of NLRP3. This locks NLRP3 in the inactive form and prevents inflammasome activation[109]. Subsequently, the formation of an NLRP3-induced ASC oligomerization (spike) is abrogated, preventing the downstream maturation of caspase 1. As a result, the maturation and release of cytokines and the formation of pyroptotic pores are all inhibited[110,111]. The IC$_{50}$ for MCC950 is 7.5 nmol/L for the release of IL-1β from IMBM cells. MCC950 decreases the expression of caspase 1/11, NLRP3, and ASC in mice heart, lungs, and brain upon exposure to LPS[108]. Further experiments are looking at a wide range of NLRP-3 associated diseases, and it has promising inhibitory effects for NLRP3 in blood samples from patients with Muckle−Wells syndrome[108,110,111].

(*S*)-1-((*S*)-2-{[1-(4-Amino-3-chloro-phenyl)-methanoyl]-amino}-3,3-dimethyl-butanoyl)-pyrrolidine-2-carboxylic acid ((2*R*,3*S*)-2-ethoxy-5-oxo-tetrahydro-furan-3-yl)-amide (VX-765) is a prodrug of (*S*)-3-({1-[(*S*)-1-((*S*)-2-{[1-(4-amino-3-chlorophenyl)-methanoyl]-amino}-3,3-dimethyl-butanoyl)-

pyrrolidin-2yl]-methanoyl}-amino)-4-oxo-butyric acid (VRT-043198), a competitive inhibitor of caspase 1/4. VX-765 has been shown to be useful for inflammatory diseases, such as hepatic ischemia-reperforation[112], Alzheimer's disease[8,111], and bacterial infections[49] (Table 1). VRT-043198, the active form of VX-765, has IC$_{50}$ values of 0.2, 14.5, and 10.6 nmol/L when tested using purified caspase 1, 4 and 5 respectively in the presence of a peptide substrate[113]. In animal studies, VX-765 has been shown to protect LPS-challenged mice by inhibiting caspase 1, thus decreasing the production of IL-1β/IL-18. VRT-043198 selectively inhibits inflammatory caspases without inhibiting apoptotic caspases. This selectivity for inflammatory caspases makes it safer for the treatment of inflammatory diseases, in contrast to the pan-caspase inhibitors[113]. This drug is currently in phase II trials for psoriasis, while phase II trials for Alzheimer's disease have ceased due to the discovery of insignificant efficacy levels. In mice, VX-765 also showed a significant benefit in skin inflammation[8,114].

7-Desacetoxy-6,7-dehydrogedunin (7DG) is a selective inhibitor of protein kinase-R (PKR), which plays a kinase-independent role in pyroptosis[115] (Table 1). 7DG inhibits PKR by binding to its C-terminus, preventing ASC oligomerization in both the NLRP1 and NLRP3 inflammasome pathways. When macrophages are challenged with *Bacillus anthracis* (anthrax) lethal toxin (LT), 7DG treatment almost completely blocked pyroptotic cell death with an IC$_{50}$ of 5 μmol/L[112]. Although this compound has shown high efficacy in blocking pyroptosis, PKR also participates in apoptosis and plays a vital role in the actin dynamics within cells[116], making it an unideal drug target.

Disulfiram, an inhibitor of the enzyme acetaldehyde dehydrogenase, is an FDA-approved drug to help patients overcome alcoholism (Table 1)[117]. It has been suggested to be useful for cancer prevention in alcohol-dependent people, showing a significant decrease in the development of cancer[118]. Disulfiram was first discovered to inhibit pyroptosis when existing clinical drugs were screened in a cell-free assay, measuring Pd^{3+}-release from liposomes in the presence of active caspase 11 and GsdmD. Subsequent studies showed that disulfiram inhibited caspase 1, caspase 11, and the ring form of GsdmD-N by covalently attaching to the catalytic cysteines of caspases 1 and 11, and to the cysteine (human/mouse Cys191/Cys192) within the channel of the GsdmD-N pore[119,120]. When LPS-primed macrophages were treated with disulfiram following nigericin challenge, disulfiram significantly inhibited pyroptosis in these cells with an IC$_{50}$ of 7.67 μmol/L. *In vivo*, disulfiram increased viability in mice challenged with high doses of LPS[119]. Disulfiram has been shown to have inhibitory effects on a variety of proteins with differing functions. It is speculated that disulfiram's function is dependent on the expression level of the target proteins. For instance, when there is a strong pyroptotic response, it tends to target GsdmD, whose expression is upregulated. Meanwhile, it primarily targets acetaldehyde dehydrogenase during alcoholism treatment due to acetaldehyde dehydrogenase being the rate-limiting enzyme for ethanol metabolism and its high expression levels in alcohol-dependent people[117,119]. Because disulfiram is a safe drug in the clinic, its repurposing is promising to combat inflammation[117,119].

Dimethyl fumarate (DMF) is the methyl ester of fumarate, an intermediate of the citric acid cycle. It is currently used as an orally administered drug to treat relapsing multiple sclerosis. DMF succinates cysteine C192 of mGsdmD to *S*-(2-succinyl)-cysteine, which inhibits activation by inflammatory caspases. This ultimately results in the inhibition of pyroptosis. When DMF was used to treat mice models with multiple sclerosis and FMF,

clinical scores of the treated mice were critically decreased, suggesting that the diseases were alleviated with reduced neuropathology. Interestingly, DMF succinates GsdmE in GsdmD knockout BMDMs, where GsdmE-dependent cell death was blocked due to its succination[121].

While many of these studies have shown promise *in vivo* treating inflammatory diseases of mice, it is important to note that little data has been reported where clinical research has been done in human inflammatory diseases. The listed drug candidates above show promising *ex vivo* results. However, this can differ greatly *in vivo*.

6. GsdmD and membrane repair

Recent evidence has been brought to light that not all cells with an activated GsdmD protein undergo pyroptosis, suggesting an alternate repair mechanism cells have in place. Utilizing mBMDMs and HeLa cells, it has been shown that calcium influx through GsdmD pores served as a signal for membrane repair downstream of GsdmD cleavage[122]. The influx of calcium recruits the endosomal sorting complexes required for transport (ESCRT) to the GsdmD pores, where the GsdmD-damaged plasma membrane is repaired. ESCRT uses annexin V$^+$ vesicles to remove the damaged membrane that contains embedded GsdmD pores. A competitive dominant-negative ESCRT mutant revealed an increase in both pyroptosis and IL-1β secretion upon exposure to LPS as compared to the control, further supporting the repairing role of ESCRT[122,123]. Hence, the ESCRT machinery repairs the GsdmD-damaged membrane to counteract pyroptotic cell death and cytokine release.

7. GsdmD in other pathways

Proteins of the canonical and noncanonical pyroptotic pathways have been suggested to be the drug targets for inflammatory diseases such as sepsis[3,124]. While GsdmD is commonly known to be activated *via* the caspase 1 dependent canonical pathway or *via* caspase 4/5/11-dependent noncanonical pathway, recent studies have suggested alternate proteases to cleave GsdmD. The alternative proteases for GsdmD have shed light on alternative drug targets for pyroptosis and inflammatory responses.

As previously mentioned, GsdmD is cleaved at D275 in humans (D276 in mice) by caspase 1 in the canonical pathway and caspases 4/5/11 in the noncanonical pathway. Recent investigations showed that an apoptotic caspase, caspase 8, also could cleave GsdmD at the same site, providing an alternative method of activation. This further alludes to caspase 8's plasticity within immune functions, including the ability to both induce apoptosis and inhibit necroptosis, programmed necrosis[125]. Caspase 8-dependent cleavage of GsdmD was first discovered during *Yersinia pseudotuberculosis* infections, where mitogen-activated protein kinase kinase kinase 7 (MAP3K7/TAK1) and IκB kinase complex (IKK) are inhibited by the *Y. pseudotuberculosis* outer protein J (YopJ). This prevents downstream caspase 1 cleavage, accompanied by an increase of interleukin 1 (IL-1) secretion. In this process, GsdmD cleavage is driven by a RIP1-caspase 8 signaling cascade[126]. Consistently, *in vitro* experiments utilizing macrophages with TAK1 inhibition successfully induced the cleavage of GsdmD by caspase 8 and pyroptosis, independent of caspase 1/11[127]. This alternate pathway has further been tested utilizing a study of IBD in a mice model during which suppression

of FAS-associated death domain protein (FADD) elicited an enhanced inflammatory response within intestinal epithelial. The exaggerated inflammation results from caspase 8-mediated GsdmD cleavage and pore formation, not from ASC-caspase1-dependent cleavage[128]. It also suggests that FADD is a key player in suppressing pyroptosis in favor of non-inflammatory apoptosis within intestinal epithelial tissues. Consistently, recent evidence also suggests that caspase 8 is able to directly activate IL-1β in response to the activation by TLR, death receptor and dectin-1 pathways[129].

While GsdmD cleavage is well established in macrophages and monocytes, recent studies suggest that neutrophil specific serine proteases (NSPs) are also capable of cleaving and activating GsdmD within neutrophils independent of caspases. Neutrophil expressed elastase (ELANE), a serine protease in cytosolic granules, has recently been found to cleave GsdmD and produce a functional N-terminal fragment capable of pore formation on plasma membranes. The absence of ELANE or GsdmD increases neutrophil's lifespan, suggesting ELANE and GsdmD activation assist in neutrophil death[130]. The cleavage by ELANE occurs at C268, seven residues upstream of the caspase 1 cleavage site of D275 of hGsdmD. Another NSP that cleaves GsdmD is CTSG, which cleaves at L274 of mGsdmD, two amino acids upstream from D276 of mGsdmD. The cleaved GsdmD triggers pyroptotic death and releases inflammatory cytokines, including IL-1β, from neutrophils. GsdmD cleavage by CTSG is negatively regulated by Serpinb1 and Serpinb6, suggesting a strict regulation in neutrophil death pathways and cytokine release[131,132]. GsdmD-induced pyroptosis in neutrophils has also been correlated with the release of neutrophil extracellular traps (NETs) where caspase 11 and GsdmD rupture the neutrophil plasma membrane to extrude NETs as the last step to execute NET associated cell death (NETosis)[133].

While cleavage and activation of GsdmD *via* both caspase-dependent and caspase-independent mechanisms have been noted, cellular inhibitory pathways of GsdmD inflammasome activity have also been studied. Two key players within apoptotic pathways, caspases 3 and 7, have recently shown negative regulation of GsdmD in human macrophages[134]. These two caspases have been suggested to cleave GsdmD *via* an alternate pathway resulting in a truncated N-terminal domain dictating a loss of function. The cleaving by caspases 3 and 7 occurs at D87 of hGsdmD, as opposed to D275 of caspase 1/4/5 site. This loss of function within GsdmD has been proposed to execute anti-inflammatory processes by blocking pyroptosis and promoting apoptosis[134]. Consistent with this, a recent study shows that caspase 8-dependent GsdmD-induced cell death is limited by caspase 3 cleavage of GsdmD in mBMDM, while mutation of D88 in mGsdmD leads to an increase in pyroptosis[135].

In addition to pyroptosis and cytokine release, GsdmD also restricts Type 1 interferon (IFN-1) responses to DNA in the cytosol, which is to limit the production of damaging interferon. Like AIM2, cyclic GMP-AMP synthase (cGAS) is a dsDNA sensor in innate immune cells and promotes cGAS−STING (stimulator of interferon genes) for IFN-1 production[136]. Recent research suggests possible crosstalks between AIM2 and cGAS pathways in that the activation of GsdmD leads to a potassium efflux through GsdmD-N pore, which inhibits cGAS sensing and IFN-1 production[7]. In endothelial cells, GsdmD can activate cGAS by releasing mtDNA upon LPS treatment[137]. Intriguingly, STING, when triggered by bacterial infection-induced DNA damage, can also activate GsdmD. In this process, STING is first

phosphorylated and then interacts with inositol 1,4,5-trisphosphate receptor type 1 (ITPR1). The STING−ITPR1 complex enables calcium influx from the stressed endoplasmic reticulum (ER). This influx of calcium ions further activates caspase 1/11 for GsdmD cleavage, which releases coagulation factor 3 (F3) to trigger blood coagulation and sepsis[138]. These findings suggest the diversity of roles of GsdmD in both immune and non-immune cells.

8. GsdmD and diseases

Studies using non-small cell lung cancer (NSCLC) cells show that knockdown of GsdmD increases caspase 3-induced apoptosis of tumors without apoptotic stimuli, while mouse models show a decrease in tumor growth upon inhibition of GsdmD[139]. When the expression levels of GSDMD mRNA were analyzed from patients with lung cancers, a high level of GSDMD was shown to be associated with NSCLC. When NSCLC was subcategorized as lung adenocarcinoma and lung squamous cell carcinoma, GsdmD expression is correlated with the former for larger tumors and poor prognosis[139]. It has also been shown that acid reflux can cause pyroptotic cell death, which subsequently causes esophageal cancers. Similarly, overexpression of GsdmD has also been associated with cancer development in the bladder[140]. Paradoxically, pyroptosis is suppressed in many cancer types, with studies showing that pyroptosis has hindering effects on cancer proliferation and metastasis[141], such as in ovarian cancer[142], colorectal cancer[143], and gastric cancers[144]. GsdmD is either down-regulated or inhibited within these cancers, preventing the death of the cancerous cells.

Alzheimer's and Parkinson's diseases are neurodegenerative diseases in aging populations. Chronic neuronal inflammation plays a major role in the neural degeneration seen in Parkinson's and Alzheimer's diseases[8,145]. A linkage of GsdmD to both Alzheimer's and Parkinson's diseases have been suggested. After treatment with VX-765, a caspase 1 inhibitor, mice with Alzheimer's disease display more activity and a higher memory score than without treatment[8]. On a cellular level, neuronal death is protected by VX-765. This further suggests a connection to the inflammatory death of neurons *via* GsdmD mediated pyroptosis[8].

Vascular disease has also been shown to be related to inflammasome activation and pyroptosis pathways. Atherosclerosis is a chronic inflammatory disease that is related to endothelial dysfunction in vascular walls. This disease is characterized by the buildup of fat- and calcium-rich plaques, where cell death is triggered, and inflammatory cells are recruited. Although the full mechanism of plaque formation is not elucidated, it was found to directly activate the AIM2 pyroptotic pathway[146] resulting in GSDMD activation and pyroptosis of vascular smooth muscle cells[146]. When AIM2 was overexpressed, ApoE$^{-/-}$ mice fed a high-fat diet had larger plaque lesions where pyroptosis was prominent[146]. Another study showed that aspirin, known for decades to have anti-inflammatory effects, inhibits NLRP3 activation in mice vascular endothelial cells. The results warrant further research for the protective effects of aspirin for cardiovascular diseases associated with NLRP3 inflammasomes[147].

In 2010−2012, arthritis was found in an estimated 23% (more than 52 million) of adults in the U.S., with the most common treatment medications being non-steroidal anti-inflammatory drugs (NSAIDs)[148]. Gouty arthritis (gout) is a well-studied disease with respect to its relationship to GsdmD. Gout is caused by uric acid crystal formation in joints during purine degradation. This disease is exacerbated by activation of the NLRP3/GsdmD pathway in neutrophils, causing the release of activated ILs[149]. The prevention of IL activation in the NLRP3 pathway alleviates gout symptoms[149]. Calcium pyrophosphate deposition disease, commonly referred to as pseudogout, participates in pyroptosis the same way as gout, with the main difference being calcium pyrophosphate deposit instead of uric acid deposit[95].

FMF, also known as autoimmune encephalomyelitis, is the most common hereditary autoinflammatory disorder. FMF is caused by mutations on the MEFV gene, encoding pyrin. The symptoms of FMF are fever, serositis, and ventral pain. Cells that have mutations within MEFV have uncontrolled pyroptotic death, which is triggered by the activation of the pyrin inflammasome. Consistently, the viability of GsdmD$^{-/-}$ cells, which contain MEFV mutations, are significantly higher than cells expressing GsdmD, and the viability of GsdmD$^{-/-}$/caspase 1$^{-/-}$ cells is even greater[150]. Anti-inflammatory drugs are currently used to treat this chronic disease.

Diabetic kidney disease (DKD), also called diabetic nephropathy, is caused by prolonged uncontrolled diabetes. DKD is the major cause of end-stage renal disease. Pyroptotic cell death of renal tubular cells has recently been suggested to cause the progression of DKD. Upregulation of TLR4 and GsdmD is found in DKD patients and DKD animal models, which is subsequently associated with TLR4/NF-κB signaling pathway[151].

Steatohepatitis is a type of nonalcoholic fatty liver disease. A pathologic hallmark is chronic inflammation of the liver and buildup of fat within liver cells. GsdmD has recently been revealed to play a critical role in steatohepatitis in that GsdmD$^{-/-}$ mice with nonalcoholic fatty liver disease have less hepatic fibrosis and cytokine production. When GsdmD is over-expressed, the mice have an increase of GsdmD-N, pyroptosis, and a decrease in overall activity score[152]. In addition to its role in inflammation, GsdmD also regulates lipogenesis and the NF-κB signaling pathway in steatohepatitis. Currently, there is no drug approved to treat steatohepatitis, and GsdmD is a potential drug target.

While GsdmD has been found to be involved in provoking liver diseases such as steatohepatitis, it has also been shown to improve hepatocyte viability in noninfectious liver injuries such as acetaminophen-induced liver damage. A recent study utilizing GsdmD$^{-/-}$ mice shows that GsdmD$^{-/-}$ mice have a significantly higher level of liver damage when exposed to toxic levels of acetaminophen[153]. This is correlated with an increase in caspase 8-induced apoptosis or/and necroptosis in GsdmD$^{-/-}$ mice, favoring apoptosis over pyroptosis[153]. This research further suggests that GsdmD may play a role in diseases by regulating pyroptosis, apoptosis and necroptosis.

IBD, including ulcerative colitis (UC) and Crohn's disease (CD), are driven by chronic inflammation in the intestines[42,94]. IBD are common chronic diseases that share symptoms of nausea, diarrhea, constipation, and severe abdominal pain. GsdmD-mediated pyroptotic cell death occurs if these diseases are left untreated, causing prolonged damage to the bowel. It has been shown that GsdmD is over-expressed in patients with IBD, triggering the death of the epithelial lining in the bowel[94]. Treatment with an anti-pyroptotic compound, ABX464, has reversed bowel damage of the epithelial cells in mice with CD and has been shown to be safe for patients with UC[154].

HIV is the primary cause of acquired immunodeficiency syndrome (AIDS). The disease can be treated with a wide range of drug cocktails, limiting the progression of AIDS. Current medications allow for an increase in CD4$^+$ T-cells to the point of AIDS

remission. CD4$^+$ T-cell death in HIV is caused by the NLRP3 pathway by the formation of GsdmD-N pores on the membrane[9].

Both IL-1β and IL-18 play roles in antiviral infections using a caspase 1 dependent pathway; however, the roles of GsdmD in viral infections remain largely elusive. Recent work with enterovirus 71 (EV71), the virus known for causing hand-foot-and-mouth disease in children, has shown that EV71 disables GsdmD using its viral protease 3C in host cells. Viral protease 3C, known for inducing apoptosis, can cleave GsdmD at Q193, 82 residues upper to D275, the caspase 1 cleavage site. Cleavage by 3C resulted in a stunted N-terminal fragment inducing a loss of function, preventing the induction of pyroptosis within infected cells[155]. The result suggests that GsdmD may play a role in regulating viral infection, possibly by favoring pyroptosis over apoptosis within virally infected cells. The study shines a light on the antiviral roles of GsdmD as well as viral abilities to evade immune responses.

Sepsis occurs when a patient's body responds overwhelmingly to infection. Common clinical symptoms include high lactic acid levels in the blood, fever, hypotension, and, if left untreated, expiration. Uncontrolled pyroptosis leads to sepsis, where GsdmD-N pores release a large number of cytokines (IL-18/IL-1β), causing an overwhelming inflammatory response system-wide. Mice treated with LPS go into septic shock, which is an established sepsis model[156]. LPS-induced caspase 11 inflammasome has been well-established regarding involvement with sepsis. Mice studies showed that inhibition of either GsdmD or caspase 11 had increased viability when exposed to the Gram-negative endotoxins[157]. Upon co-treatment of LPS and a GsdmD inhibitor, disulfiram, the number of mice that expired due to sepsis is significantly lowered[120]. As such, proteins of the noncanonical pathway are critical targets for inhibitor screening treating sepsis.

One study has also found positive results utilizing adrenaline, a fight-or-flight hormone, to treat sepsis. Adrenaline reduces IL-1β release in human monocytic cell line THP1 and significantly decreases the septic death of LPS-treated mice[158]. Adrenaline plays its role by binding to its α-2B adrenergic receptor (ADRA2B), and inducing the production of cyclic adenosine monophosphate (cAMP). Subsequently, cAMP activates protein kinase A (PKA), which then phosphorylates caspase 11 to inhibit its activity for the cleavage of GsdmD[158]. A flavonoid, scutellarin, has also been suggested as a possible treatment for sepsis also believed to inhibit pyroptosis by affecting the cAMP/PKA signaling pathway[159].

Lipid peroxidation, which activates caspase 11 for GsdmD cleavage, can induce sepsis *via* GsdmD-mediated pyroptosis in infections. Glutathione peroxidase 4 (GPX4), an antioxidant enzyme, protects mice from septic death by degrading hydrogen peroxide to antagonize lipid peroxidation[160]. Among the eight glutathione peroxidases (GPX1–8), GPX4 is the only one upregulated in peritoneal macrophages and peripheral blood mononuclear cells from mice with polymicrobial infections. Consistently, vitamin E administration showed a similar protective effect from GsdmD activation by lipid peroxidation[160].

9. Conclusions

Pyroptosis induces strong inflammation to defend against intracellular pathogens, and its proper function leads to pathogen resolution. However, excessive pyroptotic activities will lead to numerous inflammatory diseases, including autoimmune disorders. Hence, pyroptotic proteins are targets for drug development for inflammatory diseases and sepsis. In addition, GsdmD is upregulated in many cancer types, suggesting that inhibitors for GsdmD may be used for cancer treatment. As vital players in pyroptotic pathways, NLRP3, GsdmD, and caspase 1/4/5/11 are actively pursued drug targets. Because the inflammatory caspases are cysteine dependent proteases, suicide inhibitors, containing a tri-/tetra-peptide and a covalent interaction warhead, are the frequently designed inhibitors for caspase 1/4/5/11. The tri-/tetrapeptides, usually selected by peptide library screening, are preferred sequences at P3−P1/P4−P1 of the cleavage site. Recently discovered GsdmD inhibitors covalently attach to C191 and inhibit its function of leaking cell contents; however, they show a lack of specificity by attaching caspase 1/11[120] or GsdmE[121]. Future drug development should aim for specific inhibitors of caspase 4/5/11 or GsdmD. Caspase 1 activates GsdmD and inflammatory cytokines IL-18 and IL-1β for pyroptosis. However, caspase 1 inhibitors do not significantly inhibit pyroptosis in mice. Hence, inhibitors upstream of caspase 1 are considered more potent than inhibitors directly interacting with caspase 1 or GsdmD. As such, inhibitors antagonizing NLRP3 assembly have been developed and cause the failure of ASC oligomerization and subsequent caspase 1 activation.

Caspases 1 and 11 seem to use the same chemical mechanism to cleave GsdmD, which requires the binding to GsdmD-C first. However, it is puzzling why caspase 1, not caspase 11, could cleave pro-IL-1β/IL-18. It is unclear how many overlapping roles gasdermins play in inflammation and cancer, although they all participate in pyroptosis in a different context. The expression of gasdermins is differential in tissues. However, it still requires further understanding of the tissue-specific function of each gasdermin, which plays a critical role in related inflammatory diseases and cancers. Enzymes, especially proteases, are critical players in pyroptosis. Inhibitor design should include further characterizations of the kinetic mechanisms of caspase 1/11, aiming to design differential inhibitors as future drugs.

In conclusion, pyroptosis, as a form of programmed cell death, has implications in many diseases. As a topic of a robust field, more specific roles of pyroptotic proteins in inflammation and related diseases are to be elucidated. This will provide further insight into the mechanisms and treatment of inflammatory conditions and cancers.

Author contributions

All authors participated in writing the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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