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The gasdermins, a protein family executing cell death and inflammation

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Preface: (102 out of max 100 words)

The gasdermins are a new family of pore-forming cell death effectors that cause membrane permeabilization and pyroptosis, a lytic pro-inflammatory type of cell death. Gasdermins consist of a cytotoxic N-terminal domain and a C-terminal repressor domain connected by a flexible linker. Proteolytic cleavage between these two domains releases the intramolecular inhibition on the cytotoxic domain, allowing it to insert into cell membranes and to form large oligomeric membrane pores, which disrupt ion homeostasis and induce cell death. In this review, we discuss the recent developments in gasdermin research with a focus on the mechanisms that control gasdermin activation, pore formation and the consequences of gasdermin-induced membrane permeabilization.

Key points (list max 6)

- The gasdermins are evolutionary conserved family of cell death effectors, which comprises 6 members (gasdermin A, B, C, D, E and Pejvakin) in humans and 10 members in mice (gasdermin A1-3, C1-4, D, E and PJVK). They derive their name from the distinct expression pattern of gasdermin A in the gastrointestinal tract and the dermis.

- The archetypical member of the family is gasdermin D, which was shown to cause a necrotic type of cell death known as 'pyroptosis' that is initiated after inflammasome complex assembly.

- All gasdermins are defined by a distinct two-domain architecture, consisting of a N-terminal cytotoxic domain, a flexible linker and a C-terminal inhibitory domain. Interaction between the N- and C-terminal domains keeps the gasdermins in an autoinhibited state.

- Several proteases promote gasdermin activation by cleaving within the internal linker and thereby relieving autoinhibition by the C-terminus. Most prominent among these are the inflammatory caspases, a family of cysteine proteases activated within inflammasome complexes.

- Following cleavage, the N-terminal gasdermin domain targets and inserts into cellular membranes by interacting with the negatively charged headgroups of certain phospholipids, phosphatidylinositol phosphates and cardiolipin. Upon membrane insertion the N-terminal domain oligomerizes to form large anti-parallel β-barrel pores with 27-fold symmetry.

- Gasdermin-induced pyroptosis play a prominent role in many hereditary diseases, (auto)inflammatory disorders and in cancer, highlighting the importance of gasdermins as a novel therapeutic target.

Outline of main text (currently 7000 words of 5000)

Introduction

The gasdermins are a family of genes first reported in the early 2000s as candidate genes for several alopecia-like skin mutations in mice[1,2]. The name gasdermin was coined based on the exclusive expression profile of the gasdermin A (GSDMA) proteins in the mouse gastrointestinal tract and the epithelium of the skin. Early studies also noted that the gasdermins share strong sequence similarity in their N-terminal region with Deafness autosomal dominant nonsyndromic sensorineural 5 (DFNA5), a protein that was linked in 1998 to autosomal dominant nonsyndromatic hearing loss in humans[3]. Based on this homology several other gasdermin family members and gasdermin-like proteins were identified and currently the family comprises 6 paralogous genes in humans: *GSDMA*, *GSDMB*, *GSDMC*, *GSDMD*, *GSDME* (a.k.a *DFNA5*) and *PJVK (a.k.a DFNB59*). By contrast, rodents lack *Gsdmb,* but mice have three *GSDMA* homologues (*Gsdma1–3*), four *GSDMC* homologues (*Gsdmc1–4*), *Gsdmd, Gsdme* and *Pjvk*.

While the gasdermin family members were easily identified based on sequence homology, the exact biological function of these proteins remained unknown for over 15 years. Still, links to cell death and inflammation started to emerge soon after their identification. For example, over the years a total of nine mutations in *Gsdma3* were reported to cause inflammation and alopecia in the mouse[4]. Gene deletion of *Gsdma3* on the other hand did not cause any discernible skin phenotype[4], indicating that these mutations conferred a gain-of-function that caused bulge stem cell depletion, hyperkeratosis and inflammation. The most direct evidence for a role of gasdermins in cell death came from studies showing that expression of the mutated, C-terminally truncated form of human GSDME, which causes autosomal dominant nonsyndromatic hearing loss and results from exon 8 skipping, causes cell cycle arrest in yeast cells and necrotic death in human cells[5,6]. Yet, how gasdermins induce cell death and what type of cell death is controlled by these proteins remained unclear.

The mechanism of gasdermin function was revealed by two studies in 2015 that identified GSDMD as the sole executor of pyroptosis[7,8]. Pyroptosis was initially defined as a caspase-1-dependent necrotic death first reported in the late 1990s and early 2000s in pathogen-infected cells[9–11]. Later studies showed that pyroptosis is the main effector mechanism of pro-inflammatory caspases, a group of proteases that is activated within so-called inflammasome complexes[12]. Two distinct pathways, named the canonical and non-canonical inflammasome, sense pathogen- or host-derived danger signals and initiate the activation of caspase-1 and -4 in humans, or caspase-1 and -11 in mice. These caspases cleave GSDMD, thereby releasing the N-terminal domain of GSDMD from an intramolecular inhibitory interaction with its C-terminal domain[7,8,13]. The N-terminal domain targets cellular membranes such as the plasma membrane, where it assembles large pores that permeabilize the membrane and eventually induce pyroptosis[14–17].

Since the discovery of the gasdermins as the executors of pyroptosis, a host of literature started to characterize their function in inflammasome biology, apoptosis and beyond. In this review, we discuss the latest insights into gasdermin activation and regulation, the assembly of the gasdermin pore and the biological functions of the gasdermin protein family.

The gasdermins, a family of membrane pore forming proteins

The human gasdermin protein family is encoded by six genes whose overall sequence similarity ranges from 23.9 to 49.4% (**Figure 1a**). The family can be further subdivided, since GSDME and PJVK also belong to the deafness associated genes (DFN) and their protein sequences cluster together, apart from the other human gasdermins (GSDMA-D) (**Figure 1a**). Evolutionarily, GSDME and PJVK are also the most ancient gasdermin members, since similar sequences are found in lower vertebrates and in some invertebrates[18,19]. *GSDMA* genes sequences are found in mammals as well as in birds and reptiles, while *GSDMB*, *GSDMC* and *GSDMD* genes are exclusively found in mammalian genomes and are closely related to *GSDMA* (**Figure 1a**), indicating that they arose through gene duplication[18]. Mice and rats lack *Gsdmb*, but mice feature several orthologues of *Gsdma* and *Gsdmc* (**Figure 1a**)[20].

Structurally, gasdermins consist of two distinct domains connected by a flexible linker (**Figure 1b**), except for PJVK that present a smaller C-terminal domain. The N-terminal gasdermin domain ($GSDM^{NT}$) displays the highest sequence similarity among all family members (similarity ranging from 28.8 to 50.5%). By contrast, the C-terminal gasdermin domains ($GSDM^{CT}$) present variable lengths and lower similarity (ranging from 1.3 to 46.3%) (**Figure 1c**)[7,8]. $GSDMD^{NT}$ harbors the intrinsic pore-forming/pyroptosis-inducing activity, while $GSDM^{CT}$ interacts with $GSDMD^{NT}$ and thereby inhibits its activity in the absence of an activating signal[7,8,14].

GSDMA (a.k.a. gasdermin-1, GSDM, GSDM1, or FKSG9, UniProt #Q96QA5) was first cloned from the mouse skin, and its expression is mainly restricted in humans to epithelial cells of the esophagus, bladder and skin[2,21] (**Table 1**). T and B lymphocytes also express detectable GSDMA protein[22]. Mice feature three *GSDMA* orthologous genes (*Gsdma1*, *Gsdma2*, and *Gsdma3*), but the expression is also restricted to epithelia and the skin, including epidermis, hair follicles, and stomach[4,23–25].

GSDMB (a.k.a. gasdermin-like, GSDML, PP4052, or PRO2521; UniProt #Q8TAX9) was found by database homology searches using the GSDMA sequence[26]. GSDMB is the most divergent member of the gasdermin family (**Figure 1a**) and is not present in the mouse and rat genome, although some species of rodents have a *Gsdmb* ortholog. GSDMB expression has been mainly detected in airway epithelium, esophagus, stomach, liver, small intestine, and colon, among other tissues[27,28] (**Table 1**). Different GSDMB splice variants have been detected in humans, with one transcript encoding a GSDMB with caspase-1 cleavage site in the interdomain linker (encoded on exon 6)[28]. Consequently, caspase-1 has been found to cleave this isoform in a recombinant cellular system and induce lytic cell death[28].

GSDMC (a.k.a. melanoma-derived leucine zipper-containing extranuclear factor, or MLZE; UniProt #Q9BYG8) was first identified as a gene with an upregulated expression in metastatic mouse melanoma cells[29] and latter it was identified as a member of the gasdermin family[25]. The mouse genome presents four *Gsdmc* orthologue genes[25] (**Figure 1a**). The expression of GSDMC is restricted to esophagus, skin, spleen and vagina (**Table 1**)[27]. Artificially truncated $GSDMC^{NT}$ is able to induce pyroptosis[14], but so far it is still unknown what protease could cleavage and activate GSDMC.

GSDMD (a.k.a. gasdermin domain-containing 1, GSDMDC1, deafness, autosomal dominant 5-like, DFNA5L, or FKSG10; UniProt #P57764) was first identified by searching the human genomic database

using the GSDMA sequence[26]. GSDMD is expressed in different human tissues, as well as different subsets of leukocytes (**Table 1**)[22,27]. *GSDMD* orthologous genes are only present in mammalian genomes and all contain a large central domain with a cleavage site for inflammatory caspases (caspase-1/-4/-5) (**Figure 1b**). It is however worth noting that in lower vertebrates, as in zebrafish caspase-a or caspase-b (caspy and caspy2, respectively), the homologs of mammalian caspase-1 and caspase-4/-5, are reported to induce cell death and participate in immunity [30–32], suggesting that an unidentified functional homolog of GSDMD might be present in these lower vertebrates. However, additional or alternative cell death pathways in fish (as necroptotic- or apoptotic-like) could be also responsible of this inflammatory phenotype. In mammals, caspase-1 cleaves the precursor pro-IL-1β to generate the mature and bioactive IL-1β cytokine. In parallel, the cleavage of GSDMD by caspase-1/-4/-5 results in the formation of the highly lytic GSDMD^{NT} protein fragment that allows the release of mature IL-1β (**Figure 2**)[7,8,33]. Lower vertebrate sequences for IL-1β lack a conserved caspase-1 processing site[32,34], albeit inhibition of fish caspase-a/-b, as well as, fish IL-1β is detrimental for the host during infection[32,35,36]. The appearance of GSDMD and pro-IL-1β, both with a caspase-1 processing site in mammals, could thus have conferred to the inflammasome the control over the two key steps for IL-1β signaling, its processing and release. By contrast, these two steps might be controlled by homologous proteases (as caspase-a/-b) and a GSDMD homolog in lower vertebrates.

GSDME (a.k.a. inversely correlated with estrogen receptor expression 1, ICERE-1, non-syndromic hearing impairment protein 5, or DFNA5; UniProt #O60443) was initially cloned as a candidate gene for autosomal dominant non-syndromic hearing loss[3] and lately was found to possess sequence and structural similarities to the gasdermins[6]. GSDME is variably expressed in different human cells and tissues, including brain, endometrium, placenta and intestine, among others (**Table 1**). In mice and humans, GSDME is processed by caspase-3 and was proposed to induce pyroptosis with apoptotic morphology [14,37]. GSDME is also expressed in different species of lower vertebrates, and for example two orthologous genes for GSDME (*GsdmEa* and *GsdmEb*) can be found in bony fish. Caspase-3 cleavage site is present in zebrafish *GsdmEa*, but not in *GsdmEb*, suggesting that *GsdmdEa* could be considered as the functional homolog of GSDME[14]. It is yet unknown if *GsdmEb* is processed in fish, but if it is processed by fish caspy or caspy2 it might act as a functional homologue of mammalian GSDMD (see above). Interestingly, deletion of *GsdmEb* in zebrafish results in a malformation of the semicircular canals of the ear[38], suggesting that *GsdmEb* could confer the hearing loss associated with human GSDME.

PJVK (a.k.a. autosomal recessive deafness type 59 protein, DFNB59, or GSDMF; UniProt #Q0ZLH3) is also a protein with mutations associated to deafness, but was initially cloned from human testis[39]. PJVK shares a high similarity with GSDME, and PJVK orthologous genes are present in early chordates and invertebrates, suggesting that gasdermin family of proteins could have evolved from these antecessors. PJVK expression is high in testis, but it is also broadly expressed in other tissues, including the hair cells of the inner ear and other cells of the auditory system (**Table 1**)[39–41]. So far, it is unknown if PJVK is able to be processed by a protease or if the N-terminus or full-length PJVK forms membrane pores.

Signaling pathways controlling gasdermin activation

In 2015, several studies independently identified GSDMD as the executor of pyroptosis, a type of necrotic cell death that is induced after the activation of the canonical or non-canonical inflammasome pathways (**Figure 2**)[7,8,13]. Inflammasomes are multi-protein signaling complexes that are assembled in the cytosol upon detection of host- or pathogen-derived danger signals and that promote cytokine release, pyroptotic cell death and inflammation[12]. Signaling downstream of inflammasomes is controlled by a family of cysteine protease that known as inflammatory caspases, and that comprise caspase-1/-4/-5 in humans and caspase-1/-11 in mice[12]. These proteases induce pyroptosis by cleaving GSDMD within its central linker domain (**Figure 1b**), resulting in the generation of a 31 kDa N-terminal and a 22 kDa C-terminal fragment[7,8,13]. Pyroptosis is induced by GSDMD^{NT}, since expression of the N-terminal fragment alone is sufficient to induce pyroptosis[7,8,13]. By contrast, the C-terminal fragment acts as a repressor as it is able to bind GSDMD^{NT} when overexpressed and thereby block cell death[8].

These results gave rise to a model, in which caspase-mediated cleavage releases the cytotoxic GSDMD^{NT} from an intramolecular auto-inhibition by GSDMD^{CT} and thus allows it to induce pyroptosis[7,8,20]. In support of this model, it was found that the defining two-domain architecture consisting of an N-terminal cytotoxic and a C-terminal inhibitory domain connected by a linker is shared by all gasdermin family members (with the exception of PJVK that features a truncated C-terminal domain), and that ectopic expression of the N-terminal domain of GSDMA, -B, -C or -E induces necrosis with similar morphology as GSDMD-induced cell death[14]. Thus, the gasdermin family emerged as new group of cell death effectors, that is defined by its N-terminal pyroptosis-inducing domain (**Box 1**).

Consistent with being a substrate of inflammatory caspases, GSDMD features a caspase cleavage motif (FLTD in humans, LLSD in the mouse) in its interdomain linker (**Figure 1b**)[7,8]. Such a caspase-1 cleavage site is not found in the other gasdermins with the exception of a minor splice variant of GSDMB[28], but other protease cleavage sites might be present. In fact, human and mouse GSDME feature a caspase-3 cleavage motif in their linker region (**Figure 1b**), and consequently GSDME was reported to be cleaved at this site upon induction of apoptosis (**Figure 3**)[37,42]. Based on this finding, Rogers *et al.* proposed that GSDME causes secondary necrosis[37], a process whereby late apoptotic cells progress to necrosis and lose their membrane integrity. Subsequent studies have now challenged this hypothesis and shown that GSDME can only induce pyroptosis with apoptotic features upon overexpression or in cells types that have naturally high GSDME levels[42–44], and that *Gsdme*-deficient macrophages still undergo secondary necrosis[43,44]. Interestingly, GSDME is not the only gasdermin that can get activated after apoptosis induction. Pharmacological or pathogen-induced inhibition of TAK-1 or the treatment with SMAC mimetics can elicit GSDMD cleavage independently of caspases-1 or -11[44–46]. It has been proposed that under these conditions GSDMD is directly processed by caspase-8 (**Figure 3**)[44,45], consistent with the fact that caspase-8 can process GSDMD *in vitro,* albeit with slower kinetics[44]. It has been also suggested that caspase-8-driven GSDMD pore formation is the cause for potassium efflux and NLRP3 inflammasome activation in apoptotic cells[45], but other findings suggest that this is mediated by caspase-driven activation of pannexin-1 channels[44]. Interestingly, GSDMD activity in apoptotic cells is negatively regulated by caspase-3 which inactivates the protein by cleaving within the N-terminal domain (**Figure 3**)[37,44,47], and thereby generating an inactive p20 fragment. These

findings reveal a potential role for GSDMD in causing lytic cell death and inflammation after apoptosis induction, but the physiological function of this new signaling pathway and the negative regulation by caspase-3 still remain to be determined.

The repertoire of pathways leading to gasdermin activation has lately been expanded beyond the caspase family. Two studies show that in activated neutrophils GSDMD can be cleaved by neutrophil elastase[48,49], a serine protease that is important for the maturation and the anti-microbial function of neutrophils. Although elastase cleaves GSDMD at several sites upstream of the FLTD motif cleaved by inflammatory caspases (LLSD in mouse), the cleavage still generates a functional pore-forming fragment[49]. The exact function of GSDMD in neutrophils is still debated: *Gsdmd*-deficient mice for example are more resistant to *E. coli* challenge, probably due to the extended lifetime of neutrophils[49]. On the other hand, GSDMD was proposed to be required for NETosis[48,50], a process involving the release chromatin structures, so-called neutrophil extracellular traps (NETs), to the extracellular space by activated neutrophils. Thus, GSDMD could have both detrimental and beneficial functions in neutrophils, depending on the type of infection and whether neutrophil survival or death is required for pathogen restriction.

Although so far proteolysis is the only known physiological mechanism by which gasdermins get activated, several lines of evidence indicate that the removal GSDM^{CT} is not an absolute requirement for gasdermin activation. Disruption of the auto-inhibitory interdomain interaction by certain mutations can also result in gasdermin activation[14], indicating that the presence of the C-terminal domain *per se* does not interfere with pore formation. The crystal structure of GSDMA3 reveals that key features of the interdomain interaction are the α1 helix and β1-β2 hairpin loop of GSDMA3^{NT} that insert deeply into a groove with a hydrophobic core within the GSDMA3^{CT}[14]. Consistently, mutations in the hydrophobic core of the C-terminal domains of GSDMA, GSDMA3, GSDMC, GSDMD and GSDME all cause pyroptosis[14], and several of the alopecia-causing *Gsdma3* mutations map to the C-terminal domain and the interdomain interaction interface. It is thus possible that physiological signaling pathways could similarly elicit gasdermin activation by relieving autoinhibition, for example by phosphorylation or other post-translational modifications.

Mechanism of gasdermin pore formation

Pyroptosis features plasma membrane rupture caused by gasdermin pore formation[51–53]. *In vitro* binding assays demonstrate that the Gasdermin N-terminus can directly interact with membrane lipids. [14–17]. The GSDMD^{NT} has been shown to preferably target acidic phospholipids, phosphoinositides and cardiolipin, albeit it can also weakly bind to phosphatidic acid and phosphatidylserine[14,16]. Other gasdermin-N domains, such as those of GSDME, GSDMA and murine GSDMA3 exhibit the similar lipid-binding property[14,42], suggesting a common membrane-targeting mechanism for the entire gasdermin family. Phosphoinositides are only present in the cytoplasmic leaflet of plasma membrane. In line with this, the GSDM^{NT} domain can only cause pyroptosis from inside of cells (extracellular addition of an activated gasdermin does not cause membrane lysis)[14] Cardiolipins, resembling the phosphoinositides in bearing the negatively charged head groups, are present in the inner membrane of the mitochondria in eukaryotes as well as that of the bacteria. Consistently, expression of a GSDM^{NT}

domain in *E. coli* exhibits severe toxicity, and recombinant GSDM^{NT} protein can lyse the protoplasts of *Bacillus megaterium*[14]. It has been shown that the unleashed GSDMA3^{NT} and GSDMD^{NT} can disrupt mitochondria[54,55] and that exposure of bacteria to recombinant GSDMD^{NT} inhibits cell growth[16], but it remains to be clarified how the gasdermin pore-forming domain can get access to the inner membrane location in both situations. Besides phospholipids being sufficient for the membrane targeting of the GSDM^{NT} domain, other membrane lipids, even showing no specific and robust binding to GSDM^{NT}, might also impact the action of the GSDM^{NT} domain through influencing the physical properties of a membrane[51]. For instance, the presence of sphingomyelin can greatly promote the liposome binding of GSDMD^{NT}, whereas inclusion of cholesterol into the lipid membrane notably reduces the association with GSDMD^{NT} [17,56]. While lipid binding can only be observed with free GSDM^{NT} domain for most gasdermins, full-length GSDMB exhibits the similar lipid-binding ability as its GSDMB^{NT} alone, suggesting that the GSDMB^{CT} domain does not impede the lipid binding of GSDMB. In addition to phosphoinositide binding, GSDMB shows an exclusive specificity to sulfatide[57], but the physiological relevance remains to be determined.

Given the high sequence similarity among all GSDM^{NT} domains, it is perceivable that most if not all gasdermins shall employ a similar mechanism to form pores on the membrane (**Figure 4.**). The high-resolution crystal structure of full-length GSDMA3[14] and the recently determined cryo-EM structure of GSDMA3^{NT} pore extracted from the reconstituted liposomes[14,58] provide an excellent template to derive a detailed understanding of gasdermin pore formation. In the GSDMA3 crystal structure, the GSDMA3^{NT} domain adopts an extended twisted β-sheet structure flanked by several helices, which represents a novel globular fold distinct from known pore-forming proteins. The helical GSDM^{CT} domain is juxtaposed closely at the side of the GSDM^{NT} domain. Within the GSDM^{NT} domain, helix α1 and a short β hairpin located at the concave of the β-sheet structure interact strongly with the GSDM^{CT} domain. A long loop stretches out from one end of the β-sheet to connect to the GSDM^{CT} domain. At the other end of the β-sheet is a short helix held by two flexible loops, which protrudes from the globular fold and interacts with another part of the GSDM^{CT} domain[14]. The two inter-domain interactions lock full-length GSDMA3 into an autoinhibited state. Upon disruption of the autoinhibition, the GSDM^{CT} domain is released from the concave surface to free the GSDM^{NT} domain for membrane pore formation. Compared with that in the autoinhibited state, the GSDMA3^{NT} pore structure shows drastic conformational changes, mainly in two structural elements[58]. The short helix that contacts the GSDM^{CT} domain in the autoinhibited structure, together with its flanking loops, refolds into two β strands, forming a β hairpin. In the neighboring region, another β strand and its flanking loops also refold into β strands and form an additional β hairpin. The four newly formed β strands each merge with an existing β strand in the core β-sheet structure. The two anti-parallel β-hairpins form a long four-stranded amphiphilic β-sheet that extends away from the core globular fold of the gasdermin-N domain. These conformational changes generate three oligomerization interfaces, which drives the GSDM^{NT} domains to form a ring-shaped pore with their amphiphilic β-sheets bundling together to assemble a membrane-inserting β barrel. Mutations in the β barrel or at the oligomerization interfaces, such as E15K and L192D in GSDMD[14], severely compromises the pore-forming activity of the GSDM^{NT} domain[58]. Each of the GSDMA3 pores contains about 26 to 28 GSDM^{NT} protomers with a predominant 27-fold symmetry[58] (**Figure 4**). The

inner and outer diameters of the GSDMA3 pore are about 18 and 28 nm, respectively, which is roughly consistent with that measured by other methods[14–17]. The GSDMD pores are more heterogeneous with an inner diameter ranging from 10 to 20 nm[14,56,58], suggesting a context-dependent stoichiometry in pores formed by different $GSDM^{NT}$ domains. The size of GSDMD pore is spacious enough to allow mature IL-1β to pass through upon canonical inflammasome activation[14,33,59].

The conformational changes leading to pore formation are triggered by binding of the $GSDM^{NT}$ domain to membrane phospholipids. A possible lipid-binding site has been observed in the cryo-EM structure of GSDMA3 pore[58]. A deep and positively charged pocket between helix α1 and the membrane-inserting β sheet is filled with extra electron densities that can be modeled with the head group of cardiolipin used to reconstitute the GSDMA3 pore. The surface of this pocket is fully masked by the $GSDM^{CT}$ domain in the autoinhibited GSDMA3 structure[57]. As interdomain cleavage does not unlock the autoinhibitory interaction (in the case of GSDMD and GSDME)[14,42], it remains to be determined how the charged phospholipid heads can access the completely buried pocket to trigger subsequent conformational changes. Alternatively, there might exist another lipid binding site in the $GSDM^{NT}$ domain that serves as a priming role for full binding to the phospholipids in the membrane. The structural changes observed in the GSDMA3 pore are reminiscent of those seen with the membrane attack complex perforin-like/cholesterol dependent cytolysin (MACPF/CDC) family although overall structures of the two types of pore-forming proteins are quite different[14,58,60]. For the MACPF/CDC family, the monomeric pore-forming domain oligomerizes into a soluble prepore prior to subsequent conformational changes-mediated membrane insertion and formation of mature pores[61,62]. Using high-resolution atomic force microscopy (AFM) method, a recent study analyzed the dynamic process of GSDMD pore formation, which reveals a mechanism of direct pore growing in the membrane[56]. $GSDMD^{NT}$ monomers are embedded into the lipid membranes and assemble arc- or slit-shaped intermediate oligomers in the membrane before growing into a ring-shaped transmembrane pore (**Figure 4c**). The process is continuous and does not involve a prepore transition stage[56], a feature distinct from the MACPF/CDC family.

Consequences of gasdermin pore formation: membrane permeabilization and pyroptosis

Sub-lytic pore formation. Although gasdermin pores eventually cause pyroptosis in experimental systems, it is possible that cell lysis is not always the main function of these pores. Such 'lysis-independent' functions have to date only been described for GSDMD pores, but it is conceivable that this mechanism applies to other family members. Research over the last couple of years has shown that the results of GSDMD pore formation can differ depending on cell type, the level of GSDMD expression, activation and timing, and the efficiency of counteracting mechanisms. In mouse macrophages, for example, activation of the NLRP3 inflammasome with the N-acetyl glucosamine (NAG) fragment of bacterial peptidoglycan (PGN) or OxPAPC can elicit a GSDMD-dependent release of mature IL-1β from live cells, i.e. in absence of detectable cell lysis[33,59,63,64]. Similarly, LPS induces IL-1 release from living human monocytes[65–68], and neutrophils also release IL-1 in a lysis-independent manner after canonical inflammasome activation[33,69]. These studies not only suggest that sublytic

GSDMD pore formation can occur, but also that sublytic GSDMD pores may be a pathway for direct unconventional secretion of the leaderless cytokines IL-1β and IL-18, allowing their release in absence of cell lysis.

This lysis-independent function of gasdermin pores might also be used to release other proteins or regulate signaling pathways[33]. The relatively large size of the GSDMD pores (estimated inner diameter up to 20 nm) did not only allow the direct release of IL-1β/-18 (~5 nm molecular diameter), but also of other small cytosolic proteins, such as small GTPases, galectins or the cysteine-type endopeptidase inhibitor cystatins[33,70]. Furthermore, as gasdermin pores are large unselective membrane channels, ion fluxes caused by gasdermin pores can have profound impact on cellular signaling pathways even before cell death become detectable. For example, potassium efflux caused by GSDMD pores triggers NLRP3 after LPS-induced activation of the non-canonical inflammasome pathway[71,72]. Since potassium-driven NLRP3 activation is a cell-intrinsic mechanism[7,72], potassium efflux and NLRP3 activation must occur before the cell undergoes GSDMD-driven pyroptosis. Similarly, it has been proposed that GSDMD-dependent potassium efflux activates NLRP3 after sublytic activation of AIM2 by *Legionella pneumophila*[73], and that potassium efflux impairs type I interferon responses independently of terminal cell death .

So how do cells regulate the level of gasdermin activation or pore formation to extend their lifetime, and could membrane pore formation even be reversible? GSDMD expression is strongly regulated by IRF2[75], which could allow cells to reduce overall GSDMD levels to avoid cell death. Furthermore, caspase activity varies considerably between cell types and activation triggers, which could give rise to conditions of sublytic GSDMD activation. In addition, studies on pore forming toxins, or mechanical or laser-induced membrane damage have demonstrated that plasma membrane damage is not a terminal event, and identified several types of membrane repair mechanism that restore membrane integrity within seconds or minutes[76]. Consistently, it was reported that GSDMD pores on the plasma membrane can be removed by the recruitment of the endosomal sorting complexes required for transport (ESCRT) machinery to the areas of damaged plasma membrane[77]. ESCRTs are recruited to the plasma membrane in response to the influx of Ca^{2+} through the GSDMD pores and promote the budding and release of vesicles that contain the damaged membrane[77]. If ESCRTs or other membrane repair systems are active in neutrophils or under conditions of sublytic inflammasome activation still needs to be demonstrated on a case-by-case basis.

Of note, vesicle release by ESCRTs[77] or other membrane budding mechanism[78], could also represent an alternative pathway for unconventional protein secretion. Early studies into inflammasome activation noted that cells exosome shedding increases quickly after inflammasome activation and that mature IL-1β can be found in exosomes shed from inflammasome-activated cells[79–81]. Newer studies also show that such exosome formation and exosome-mediated IL-1β release is dependent on GSDMD[77,82]. If such vesicles unspecifically release cytosolic proteins, or if IL-1β and other protein are preferentially packed into such vesicles remains to be determined. In summary, gasdermin pores have emerged as master regulators of unconventional protein secretion, which depending on the level of GSDMD activation promote the release of leaderless proteins by either 1) direct membrane

translocation (pore function), 2) vesicle release (induction of membrane repair) or 3) passive release by membrane lysis (pyroptosis).

Pyroptotic cell death. In most circumstances, the increasing levels of GSDMD processing and GSDMD pores will eventually overcome regulatory mechanism and induce cell death. This specific type of necrotic cell death controlled by the activation of inflammatory caspases was originally known as pyroptosis[11], however since the GSDM^{NT} domains all induce pyroptosis without caspase activation[14], the term 'pyroptosis' could be redefined as a gasdermin-dependent type of cell death (**Box 1**). In cell culture, pyroptotic cells are characterized by extensive membrane blebbing followed by ballooning of the membrane and eventual loss of membrane integrity, probably due to osmotic lysis[83][83]. The exact events that lead to cell lysis are not yet fully understood, and it is possible that gasdermin pore formation in organellar membranes, such as in mitochondrial or nuclear membranes[50,84], contributes to cell death execution and to the morphological changes associated with pyroptotic cells. The GSDMD^{NT}, for example, binds cardiolipin[14,16] and has been found to target the mitochondrial membrane to enhance the production of reactive oxygen species[85]. GSDMA3^{NT} is also able to damage the mitochondria and induce mitophagy[55]. In neutrophils, GSDMD is also able to bind an disrupt nuclear membrane thereby promoting DNA extrusion during NETosis[50]. Therefore, even under conditions of highly efficient plasma membrane repair[77], gasdermin pores affecting intracellular organelles might still elicit cell death.

Evidences for pyroptotic cell death *in vivo* comes from the study of autoinflammatory patients suffering Cryopyrin Associated Periodic Syndrome (CAPS), where inflammasome oligomers were detected systemically in the blood during inflammatory flares [86,87], denoting a potential pyroptosis of cells with activated inflammasomes. The activation of inflammasome *ex vivo* in monocytes from CAPS patients result in a small percentage of cells with active inflammasomes [88], suggesting that not all monocytes will undergo pyroptosis and will not result in a dramatic reduction of blood monocytes. In fact, GSDMD-driven pyroptosis influences the pathogenesis in a mouse model of CAPS[89]. More studies are needed to evaluate the contribution of pyroptosis *in vivo*.

Pyroptosis is often referred to as an inflammatory form of cell death[11], but there is little experimental evidence showing that pyroptosis per se causes more inflammation than apoptosis or other types of necrotic cell death. Similarly to apoptotic or necroptotic cells, pyroptotic cells release a number of molecules that can act as 'find me' signals, and present 'eat me' signals like phosphatidyserine on their surface[77,90]. Thus, it can be presumed that pyroptotic corpses are efficiently removed by efferocytosis. However, unlike other types of necrotic cell death, pyroptosis is in most cases caused by inflammatory caspases and thus associated with the release of mature IL-1β and other IL-1 family members[12]. It is likely, that it is the release of these factors that confers a highly pro-inflammatory phenotype to GSDMD-dependent pyroptosis and makes it distinct from other types of necrosis. However, it cannot be excluded that pyroptotic cells also release other unique danger signals that are generated for example as a result of organellar rupture or caspase activity. Several studies have characterized the secretome associated to pyroptosis, with the identification of >900 proteins released upon caspase-1 activation and plasma membrane permeabilization[70,91]. However, systematic studies comparing the secretome and immunological outcome of pyroptosis to other types of death are not available for the moment. In

conclusion, it can be assumed that pyroptosis results in different immunological outcomes, resulting in a low- or high-level inflammatory response (**Figure 5**), and that this inflammatory response will also depend the type of gasdermin that is activated, the mechanism of activation (protease driven or other), the cellular environment and cell type where pyroptosis is executed and how efficiently are gasdermin damaged-membrane repaired.

Role of gasdermins in health and disease

Gasdermin A. The expression of GSDMA is elevated in the gastrointestinal tract and skin, but silenced in primary gastric cancers and in gastric cancer cell lines[2]. Restoration of GSDMA in human gastric cancer cell lines induce apoptosis[21], suggesting that GSDMA could act as a tumor suppressor gene. However, it is unknown if GSDMA require a cleavage for its pro-apoptotic activity in cancer cells or what is the physiological function of GSDMA in the healthy gastric epithelia. The expression of GSDMA3^{NT} in cultured cells induce cell death by plasma membrane permeabilization, and interestingly there is also an upregulation of the class-II LC3, suggesting that GSDMA3 induced pyroptosis could be paralleled by an autophagy component [8,55]. Cell death induced by the GSDMA3^{NT} could be suppressed by the co-expression of the GSDMA3^{CT}, conferring to this domain an inhibitory function similarly to other gasdermins[55]. Different mutations in *Gsdma3* are associated with skin-related phenotypes, including keratosis and hair-loss[4,23,92,93]. However, *Gsdma3*$^{-/-}$ mice had no visible developmental skin abnormalities, suggesting that these mutations function as gain-of-function[55]. The physiological function of *Gsdma3* in the skin seems associated with the development of the hair follicle [94,95]. Gain-of-function mutations in *Gsdma3* disrupted the autoinhibition conformation of GSDMA3, allowing GSDMA3^{NT} domain to induce cell death[8], and therefore these mutations in *Gsdma3*, similar to GSDMA3^{NT}, result in pyroptosis with an autophagy component due to a decrease in mitochondrial activity[8,55]. It is yet unknown if GSDMA3^{NT} or *Gsdma3* gain-of-function mutations could directly target the mitochondrial membrane and form pores, inducing mitochondrial failure and promoting mitophagy in parallel to pyroptosis.

Gasdermin B. GSDMB has been involved in tumor progression and its expression is increased in gastric, cervix and breast cancers, as well as in hepatocarcinomas[96–98]. In HER2-positive breast cancer patients, increased *GSDMB* gene expression in tumor cells has been linked to a poor prognosis, with reduced survival and increased metastasis, and also poor therapeutic responses to HER2-targeted therapy, being *GSDMB* a co-expressed gene with *ERBB2*[98,99]. GSDMB present several splice variants in humans, and in particular the isoform 2 is strongly associated to pro-tumorigenic and pro-metastatic phenotypes of breast cancer cells[98]. So far it is unknown how GSDMB could promote cancer cell survival, since the GSDMB^{NT} is able to induce pyroptosis when over-expressed in cultured cells. Different genome-wide association studies have revealed a correlation between *GSDMB* SNPs and an increased susceptibility to diseases such as asthma, Crohn's disease, and ulcerative colitis[100–105]. The full length GSDMB as well as GSDMB^{NT} are both able to bind to the acidic phospholipids phosphoinositides as well as the sulfoglycolipid sulfatide[57], and in particular GSDMB has been proposed to function in the cellular transport of sulfatide. Different mechanisms explain the role of

GSDMB SNPs in the pathogenesis of asthma, while some studies suggest that SNPs within GSDMB^{CT} result in an alteration of the GSDMB structure affecting the levels of sulfatide within cells[57], others found that a splice variant in GSDMB associated with a lower risk of asthma, results in the deletion of an exon that lack a caspase-1 cleavage site and therefore the ability of GSDMB to induce pyroptosis of the airway epithelial cells[28]. Further studies are required to fully understand the role of GSDMB in cancer, infection and autoimmune diseases.

Gasdermin C. GSDMC was originally found to be strongly expressed in metastatic melanoma cells, and thus it has been initially named melanoma-derived leucine zipper-containing extranuclear factor (MLZE)[29,106]. Along the same lines, it was recently reported that knock-down of GSDMC reduces the proliferation of colorectal cancer cell lines[107]. However, another study suggested that GSDMC, which is also expressed in the suprabasal region of the esophagus and the isthmus/neck region of the stomach, is suppressed in many esophageal squamous cell carcinomas (ESCCs), suggesting that it could act as a tumor suppressor gene[106]. Thus, so far, no clear picture has emerged whether GSDMC promotes or suppresses cancer development, and whether this function require the activation of its N-terminal pore forming domain.

Gasdermin D. Cell culture studies have shown that while GSDMD acts as the sole executor of cell death downstream of the non-canonical inflammasome pathway, GSDMD is not essential for cell death after canonical inflammasome activation, indicating the existence of an alternative or back-up cell death program[7,13]. This difference is also highlighted by GSDMD functions *in vivo*. Kayagaki et al. for example demonstrated that *Gsdmd*-deficient mice are as protected against LPS-induced lethality as *Casp11*-deficient animals[7]. Furthermore, both *Casp11*- and *Gsdmd*-deficient mice showed increased susceptibility compared to WT animals to infections with either *Salmonella enterica* serovar Typhimurium ∆*SifA* or *Brucella abortus*[50,108], which both activate the non-canonical inflammasome *in vivo*. By contrast to these findings, it is not as clear to what extent GSDMD is essential for caspase-1-driven cell death and cytokine release. *Gsdmd*$^{-/-}$ mice are less susceptible to infection with *Francisella novicida* compared to *Casp1*- or *Aim2*-deficient animals[109,110]. Similarly, it was reported that peritoneal IL-1β levels are higher in *S.* Typhimurium-infected *Gsdmd*$^{-/-}$ mice than *Casp1Casp11*$^{-/-}$ controls[111]. By contrast, *Gsdmd*-deficiency fully protected mice harboring the FMF-associated *Mefv* V276A allele of Pyrin against autoinflammatory disease and completely abolished all NOMID-associated inflammatory symptoms in mice expressing the D301N gain-of-function mutation of NLRP3[89,112]. It thus appears that GSDMD might play a context-dependent role *in vivo*, and that under certain circumstances alternative cell death pathways are engaged in *Gsdmd*$^{-/-}$ animals that allow to protect animals against microbial challenge after canonical inflammasome activation.

Gasdermin E. *GSDME* was first reported as a gene associated with nonsyndromic hearing loss in humans[3]. All known mutations in *GSDME* results in the skipping of exon 8, thereby producing a truncated protein with cytotoxic activity. As GSDME^{NT} has pore forming activity and exon 8 skipping results in a loss of the GSDME^{CT} repressor domain, it can be assumed that *GSDME*-associated hearing

loss is caused by cell death[6,42]. However, since GSDME is expressed in all tissues, it is unclear why cells in the inner ear preferentially undergo cell death upon auto-activation of GSDME. It is possible that differential expression levels (see below) of GSDME might render some cell types more susceptible to active GSDME. The physiological activation mechanism of GSDME appears to be cleavage by apoptotic caspases-3 and -7[37,42]. Chemotherapy drugs that activate caspase-3, such as topotecan, etoposide, cisplatin and CPT-11, were subsequently shown to induce pyroptotic cell death in cell lines expressing high levels of GSDME, while inducing apoptosis in GSDME-negative cells[42]. Thus mouse BMDMs, which express low levels of GSDME[46], do not undergo GSDME-dependent pyroptosis upon engagement of intrinsic or extrinsic apoptosis, even though the GSDME is processed[43,44]. Nevertheless, GSDME might be responsible for some of the side effects of chemotherapy treatment, as mice deficient in *Gsdme* are resistant to tissue damage and weight loss induced by cisplatin injection compared to WT animals[42]. Thus, GSDME has the ability to convert apoptosis into a pyroptotic cell death in a highly cell-type specific manner.

Pejvakin. Mutations in PJVK are also associated to hearing impairment in both humans and mice. Unlike mutations in GSDME, which cause autosomal dominant hearing loss consistent with a gain-of-function mutation, all known mutations *PJVK* cause autosomal recessive hearing impairment due to dysfunctional outer hairs cells[39,40,113]. Interestingly, it was shown that *Pjvk*$^{-/-}$ mice display auditory phenotypes with early-onset progressive hearing loss, similarly to patents carrying mutations in *PJVK*[114]. This is distinct from all other gasdermin family members, where knock-out mice display no discernible phenotype, and suggests that the mutations in *PJVK* actually cause a loss-of-function phenotype. Although a pore-forming function for the PJVK^{NT} has not yet been demonstrated, it might be possible that its physiological function requires PJVK to be constantly active, i.e. forming channels, even in absence of a stimulus. In fact, the PJVK^{CT} is shorter and with no clear homology when compared for other gasdermin family members (**Figure 1c**), and thus might not act as an inhibitory domain. Interestingly, it has been suggested that PJVK localizes to the membrane of peroxisomes in inner hair cells[114], and that it directly recruits LC3B to drive autophagy-mediated removal of damaged peroxisomes (pexophagy) following oxidative stress caused by noise overexposure[115]. PJVK-driven pexophagy is followed by peroxisome proliferation, thereby protecting auditory hair cells from oxidative damage. *Pjvk*$^{-/-}$ mice show indeed signs of peroxisomal dysfunction and impaired antioxidant defenses, and furthermore peroxisomes in *Pjvk*$^{-/-}$ hair cells show structural abnormalities after the onset of hearing[114]. It is thought that this contributes to an exceptionally susceptibility of *Pjvk*$^{-/-}$ mice and human patients to sound, and thus to progressive hearing loss.

Inhibition of gasdermins and its application in inflammasome-associated diseases

Given the key function of GSDMD in inflammasome-induced cell death and cytokine release, the inhibition of the gasdermin pore is emerging as a novel target for anti-inflammatory therapy. The notion that pyroptosis could be inhibited arose from the first studies on this type of cell death, which revealed that cell lysis can be blocked by osmoprotectants or high concentrations of glycine (in the mM range)[116]. However, this strategy does not prevent the passage of molecules directly through the GSDMD pore

(like uptake of dyes or IL-1β release), and as such cannot be suitable when targeting GSDMD-associated inflammation[33,59]. The first compound described to block the execution of pyroptosis and IL-1β release was punicalagin, a complex antioxidant polyphenol found in pomegranate[117]. Punicalagin reversibly inhibits plasma membrane permeabilization and mature IL-1β/IL-18 release after caspase-1 activation with an IC_{50} in the low micromolar range, without affecting the generation of $GSDMD^{NT}$ [117,118]. Punicalagin does not affect NLRP3 or AIM2 inflammasome activation, but blocks plasma membrane fluidity and could interfere with the correct insertion of the $GSDMD^{NT}$ into the plasma membrane, its oligomerisation and/or pore formation [117]. Recently, a report suggested that punicalagin affects NLRP1 and NLRC4 inflammasome activation[119], however since punicalagin interfere with plasma membrane fluidity and the uptake of exogenous particles and proteins to the cell, when applied before stimulation, it might also interfere with the cellular delivery of flagellin or lethal factor as direct activators of the inflammasome. However, further studies are needed to understand the mechanism of action of punicalagin, and its potential specificity to GSDMD. After punicalagin wash-out, a rapid cell lysis is observed, and this lysis was blocked by the use of lanthanides (La^{3+} and Gd^{3+})[117]. These metallic chemical elements were also found to inhibit plasma membrane pores preceding pyroptosis in macrophages[120], however it is not known if lanthanides affect $GSDMD^{NT}$ oligomerization on the plasma membrane, since they do not block IL-1β release.

Recently, there are several compounds reported to directly target GSDMD. For example, necrosulfonamide (NSA), a cysteine-reactive drug previously shown to inhibit the necroptosis executor mixed lineage kinase domain like pseudokinase (MLKL) in human cells, also inhibits pyroptosis in human and mouse cells[121]. NSA binds GSDMD and inhibits $GSDMD^{NT}$ oligomerization on the plasma membrane without affecting Toll-like receptor signaling, inflammasome activation, GSDMD cleavage or cytokine maturation. NSA block plasma membrane permeabilization and IL-1β release in the low micromolar range upon inflammasome activation and protect mice in a model of sepsis. Mechanistically, NSA covalently modifies Cys^{191} of GSDMD[121], a residue essential for pore formation[58]. Interestingly, NSA did not affect cell death induced by $GSDME^{NT}$, consistent with the lack of a cysteine in a similar position in GSDME to form pores in the plasma membrane. Another compound, LDC7559, was reported to inhibit GSDMD in human neutrophils undergoing elastase-dependent NETosis or pyroptosis in mouse or human cells[48], yet its mechanism of action is so far unknown.

Overall, the characterization of specific gasdermin blockers has just started and validates pyroptosis as a viable pharmaceutical target. The clinical relevance of blocking the inflammasome-IL-1 pathway has been demonstrated in numerous *in vivo* models as well as in trials, being some IL-1 blockers already approved for the treatment of autoinflammatory and chronic inflammatory diseases. The development of novel gasdermin blockers not only will be important to understand the role of the pyroptotic pore in different disease scenarios, but will also pave the way to develop novel treatments for inflammatory diseases.

Conclusions and future perspectives

Since the identification of GSDMD as the effector of pyroptosis, our understanding of this emerging family of cell death effectors has rapidly progressed. Structural and mutagenesis studies on full-length

gasdermins, and the structure of the GSDMA3^{NT} pore, have revealed the mechanism of autoinhibition and membrane insertion. Furthermore, a host of studies have revealed the involvement of gasdermins in cell death beyond inflammasome-induced pyroptosis, such as in NETosis and during apoptotic cell death. On the other hand, the known function of gasdermins have been extended by the identification of lysis-independent function of gasdermin pores, pointing towards a critical role in unconventional protein secretion.

It is clear that these studies only reveal the tip of the iceberg, and that in the coming years the gasdermin family will assume a central player in immunity, cancer therapy and beyond. Nevertheless, many questions still remain unanswered. For example, it is crucial to identify the mechanism by which the different gasdermins are activated, what cell types produce these active gasdermins, and what biological effects they elicit. It is unknown if different gasdermin elicit different effects, particularly the function of GSDMB and GSDMC are largely unknown, and if all gasdermins function in inflammation and host defense against microbial infection.

With these advances in knowledge, pharmacological modulation of gasdermin activity will become essential to treat different diseases. GSDMD and GSDME play important roles in causing inflammation after infection or chemotherapy, and mutations in gasdermins and inappropriate activation are associated with a variety of clinical conditions, including alopecia, asthma, breast cancer, gastric cancer, autoinflammation, colorectal cancer and hearing loss. Thus, the development of specific gasdermin inhibitors could lay the foundation the development of new therapies for genetic and inflammatory diseases.

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Display items (max. 7):

BOXES:

Box. 1: Pyroptosis: a gasdermin-induced necrotic cell death

Pyroptosis was initially defined as 'caspase-1 dependent necrosis' by Cookson and Brennan, based on its morphological features and the strict requirement for caspase-1 but not for other caspases[11]. The term pyroptosis was chosen, from the Greek 'pyro' (meaning fire or fever) and 'ptosis' (meaning to fall), to underline the pro-inflammatory nature of this cell death, and its link to the release of mature IL-1β and IL-18. With the subsequent identification of the inflammasome complex in 2002[122] and the discovery of the non-canonical inflammasome pathway in 2011[71], pyroptosis was re-defined as an inflammasome dependent cell death and an effector mechanism of the inflammasome. However, it also became clear that pyroptosis is not necessarily linked to the release of mature IL-1β and IL-18, since caspase-11/-4 induce pyroptosis with all the characteristic morphological features even in absence of caspase-1 . This was further highlighted by the identification of GSDMD as the pyroptosis executor, and experiments showing that expression of the N-terminal domain of GSDMD or other gasdermins was sufficient to induce pyroptosis without the need for caspase activation[7,8,15].

Additional reports have started to even further uncouple pyroptosis and gasdermin activation from inflammatory caspases and inflammasomes. For example, it was shown that neutrophil elastase and caspase-8 can cleave and activate GSDMD to cause death[44,45,48,49] and that caspase-3 process GSDME to cause pyroptosis-like cell death[37,42]. Since in all cases cell death morphologically resembles pyroptosis and depends on the activation of a gasdermin-family member, it seems clear that the term pyroptosis again requires redefinition. We therefore propose to define 'pyroptosis' as a 'gasdermin-induced necrotic cell death', and propose to apply this term to all gasdermin family members that can cause cell death by membrane permeabilization. We also suggest to use this term independently of the actual mechanism of gasdermin activation and the cell type it occurs in. We acknowledge that the upstream signaling events or the type of cells affected can modify the ability of pyroptosis to provoke inflammation or an immune response – pyroptosis caused by caspase-1 will involve release of mature IL-1β while GSDME-dependent pyroptosis might not – however this is linked to the activation mechanism per se and not to the function of gasdermins as cell death executor.

FIGURES:

Fig. 1. The gasdermin protein family

a | Phylogenetic tree showing the divergence of the human, mouse and rat gasdermin proteins. Scale indicates the number of substitutions for each amino acid in the sequence. Phylogenetic tree was generated from UniProt sequences by the European Bioinformatics Institute (EMBL-EBI) Clustal Omega tool and drawn by FigTree software version 1.4.3. **b** | Domain structure of human GSDMD and GSDME featuring caspase cleavage sequences in the linker (top) and structure of the membrane inserted N-terminal domain (bottom). **c** | Alignment of human gasdermin proteins. Green and orange

(in b, c) mark the first α-helix that specifically interact with phospholipids and the four parallel β-strands that insert into the membrane, respectively.

Fig. 2. Role of GSDMD in canonical and non-canonical inflammasome activation

a | Canonical inflammasomes are assembled by cytosolic pattern recognition receptors Pyrin, AIM2, NAIP-NLRC4, NLRP3 and NLRP1. These sensors recognize pathogen-associated molecular patterns, endogenous danger signals or alteration to cellular homeostasis caused by cell death, injury or infection. The receptors recruit the adaptor protein ASC and pro-caspase-1 through homotypic interactions between PYDs and CARDs domains. Caspase-1 is activated within the inflammasome and active caspase-1 processes GSDMD as well as cytokines such as pro-IL-1β and pro-IL-18 (not shown). Upon permeabilization of the plasma membrane by GSDMD pores, cells undergo a lytic, proinflammatory cell death (pyroptosis) that promotes the release of mature IL-1β/-18. In absence of cell lysis, GSDMD pores can also allow direct release of cytokines. **b** | The non-canonical inflammasome pathway results in the activation of caspase-11 in mice or caspase-4 and caspase-5 in humans. Binding of lipopolysaccharide (LPS) from Gram-negative bacteria induces the oligomerization and activation of these caspases, allowing them to cleave GSDMD. The GSDMD pores allow in a first step potassium release, resulting in the activation of the NLRP3 inflammasome and IL-1β/-18 maturation. In a second step GSDMD pores cause pyroptosis, thereby driving the release of mature cytokines.

Fig. 3. Activation of gasdermins by apoptotic caspases

Certain extrinsic stimuli, such as genotoxic stress with concurrent IAP loss or pathogen-induced TAK-1 inhibition, can promote the RIP1-dependent assembly of cytosolic caspase-8-activating complexes (complex IIb/ripoptosome). Active caspase-8 drives apoptosis by activating effector caspases, caspase-3 and caspase-7, but also cleaves GSDMD to generate an active N-terminal fragment. Active GSDMD can induce pore formation, is however restricted by caspase-3-dependent cleavage at aspartate D87 (D88 in mice), which generates the inactive p20/p10 fragments of GSDMD. Caspase-3 can also cleave and activate GSDME, which can convert apoptosis into pyroptosis in cells with high GSDME expression levels. GSDME-induced death is distinct from secondary necrosis, which proceeds in a GSDMD/GSDME-independent manner.

Fig. 4. Mechanism of gasdermin membrane insertion and pore formation

Interdomain interaction between the $GSDM^{NT}$ and $GSDM^{CT}$ keep the protein in an autoinhibited state. Within the $GSDM^{NT}$ domain, helix α1 and a short β hairpin located at the concave of the β-sheet structure interact strongly with the $GSDM^{CT}$ domain. In addition, a long loop stretches out from one end of the b -sheet to connect to the $GSDM^{CT}$. Once autoinhibition is disrupted, such as by caspase cleavage of GSDMD and GSDME, the $GSDM^{CT}$ is released from the concave surface, thereby freeing the $GSDM^{NT}$ for membrane insertion and pore formation. Membrane-targeting requires phospholipids with negatively charged head groups, as found on the inner leaflet of the plasma membrane. Compared to the autoinhibited state, the pore conformation of $GSDM^{NT}$ shows drastic conformational changes,

involving the refolding of new b-strands that merge with the twisted b -sheet structure. These changes also generate new oligomerization interfaces that drive the assembly of a membrane-spanning b-barrel, the $GSDM^{NT}$ pore.

Fig. 5. Immunological outcomes of gasdermin pore formation and pyroptosis.
Pyroptotic cells release a number of intracellular molecules that can activate the immune system by acting as alarmins and 'find me' signals. If gasdermin pores are repaired and the gasdermin activating signal terminated, the release of intracellular content can be transient and restricted to small molecules able to pass across gasdermin pores (DMAPs). In the presence of pathogens or damage associated signals that activate NF-κB, pyroptosis will be the result of the activation of the NLRP3 inflammasome and associates with the activation of caspase-1 and release of pro-inflammatory cytokines (IL-1β, IL-18), as well as small intracellular proteins (DAMPs) that permeate across gasdermin pores. In this situation, if gasdermin pores at the plasma membrane are not repaired, pyroptosis will terminate with a burst in pro-inflammatory cytokine release, together with the release of large intracellular components (as inflammasome oligomers), resulting in a highly pro-inflammatory pyroptosis. Therefore, pyroptosis can most likely result in different immunological outcomes, resulting in a low- or high-level inflammatory response.

TABLES:

Table 1: Gasdermin expression profile

	GSDMA	*GSDMB*	*GSDMC*	*GSDMD*	*GSDME*	*PJVK*
Digestive system:						
Colon		•		•		
Esophagus	•	•	•	•		
Liver		•		•		
Pancreas				•		
Salivary gland		•		•		
Small intestine		•		•	•	
Stomach		•		•		
Reproductive system:						
Fallopian tube					•	
Ovary		•			•	•
Prostate	•					
Testis		•			•	•
Uterus		•		•	•	
Vagina		•	•	•	•	
Respiratory system:						
Airway epithelium		•				
Lung		•		•		
Urinary system:						
Bladder	•	•		•	•	

Kidney				•		
Circulatory system:						
Artery		•		•	•	
Heart				•		
Central Nervous System:						
Brain		•		•	•	
Pituitary						•
Other tissues:						
Adipose tissue		•		•	•	
Breast		•		•		
Skin	•	•	•	•		
Spleen		•	•	•		
Thyroid		•			•	
Immune cells:						
T cells CD4	•	•		•		•
T cells CD8		•		•		•
B cells		•		•		•
NK cells		•		•		
Monocytes				•		

711 Median expression of transcript per million (TPM) >10 for tissues, or >5 for cells; from the Genotype-
712 Tissue Expression (GTEx) Project for tissues[124], or the Database of Immune Cell Expression for
713 cells[125].
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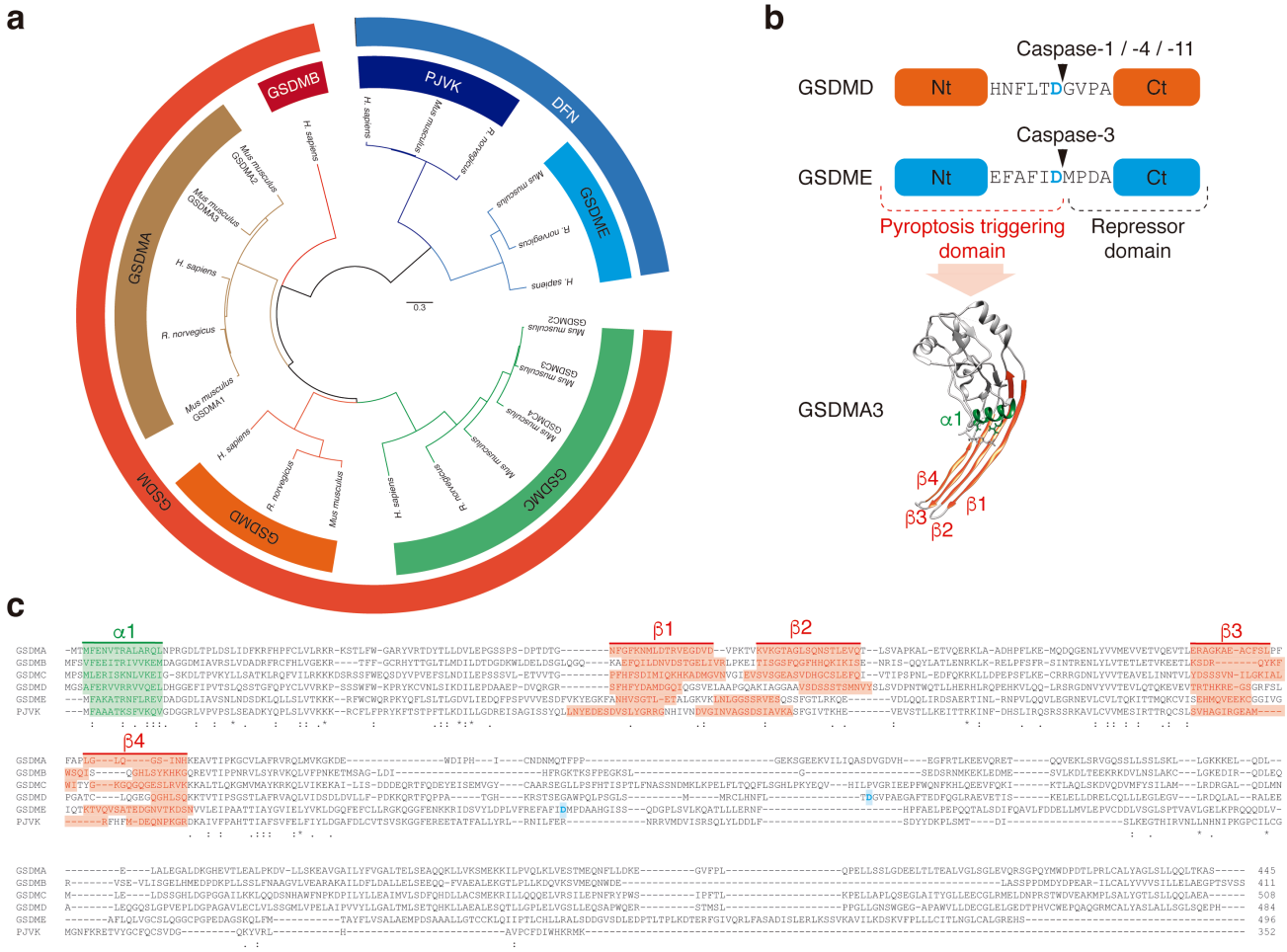
Broz P., Pelegrin P. and Shao F. Figure 1


a
GSDMB
PJVK
DFN
GSDME
GSDMC
GSDMD
GSDMA
H. sapiens
Mus musculus
R. norvegicus
Mus musculus GSDMA2
Mus musculus GSDMA3
Mus musculus GSDMA1
0.3
b
Caspase-1 / -4 / -11
GSDMD
Nt
HNFLTDGVPA
Ct
Caspase-3
GSDME
Nt
EFAFIDMPDA
Ct
Pyroptosis triggering domain
Repressor domain
GSDMA3
α1
β1
β2
β3
β4
c
α1
β1
β2
β3
β4
GSDMA -MTMPENVTRALARQLNPRGDLTPLDSLIDFKRFHPFCLVLRKR-KSTLFW-GARYVRTDYTLLDVLEPGSSPS-DPTDTG-------NPGFKNMLDTRVEGDVD--VPKTVKVKGTAGLSQNSTLEVQT--LSVAPKAL-ETVQERKLA-ADHPFLKE-MQDQGENLYVVMEVVETVQEVTLERAGKAE-ACFSLPF
GSDMB MPSVFEEITRIVVKEMDAGGDMIAVRSLVDADRFRCFHLVGEKR----TFF-GCRHYTTGLTLMDILDTDGDKWLDELDSGLQGQ----KAEFQILDNVDSTGELIVRLPKEITISGSFQGFHHQKIKISE--NRIS-QQYLATLENRKLK-RELPFSFR-SINTRENLYLVTETLETVKEETLKSDR-------QYKF
GSDMC MPSMLERISKNLVKEIG-SKDLTPVKYLLSATKLRQFVILRKKKDSRSSFWEQSDYVPVEFSLNDILEPSSSVL-ETVVTG--------FPHFSDIMIQKHKADMGVNVGIEVSVSGEASVDHGCSLEFQI--VTIPSPNL-EDFQKRKLLDPEPSFLKE-CRRRGDNLYVVTEAVELINNTVLYDSSSVN-ILGKIAL
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PJVK ---MFAAATKSFVKQVGDGGRLVPVPSLSEADKYQPLSLVVKKK--RCFLFPRYKFTSTPFTLKDILLGDREISAGISSYQLLNYEDESDVSLYGRRGNHIVNDVGINVAGSDSIAVKASFGIVTKHE-------VEVSTLLKEITTRKINF-DHSLIRQSRSSRKAVLCVVMESIRTTRQCSLSVHAGIRGEAM----
GSDMA FAPLG---LQ----GS-INHKEAVTIPKGCVLAFRVRQLMVKGKDE----------WDIPH---I----CNDNMQTFPP----------GEKSGEEKVILIQASDVGDVH----EGFRTLKEEVQRET------QQVEKLSRVGQSSLLSSLSKL---LGKKKEL--QDL--
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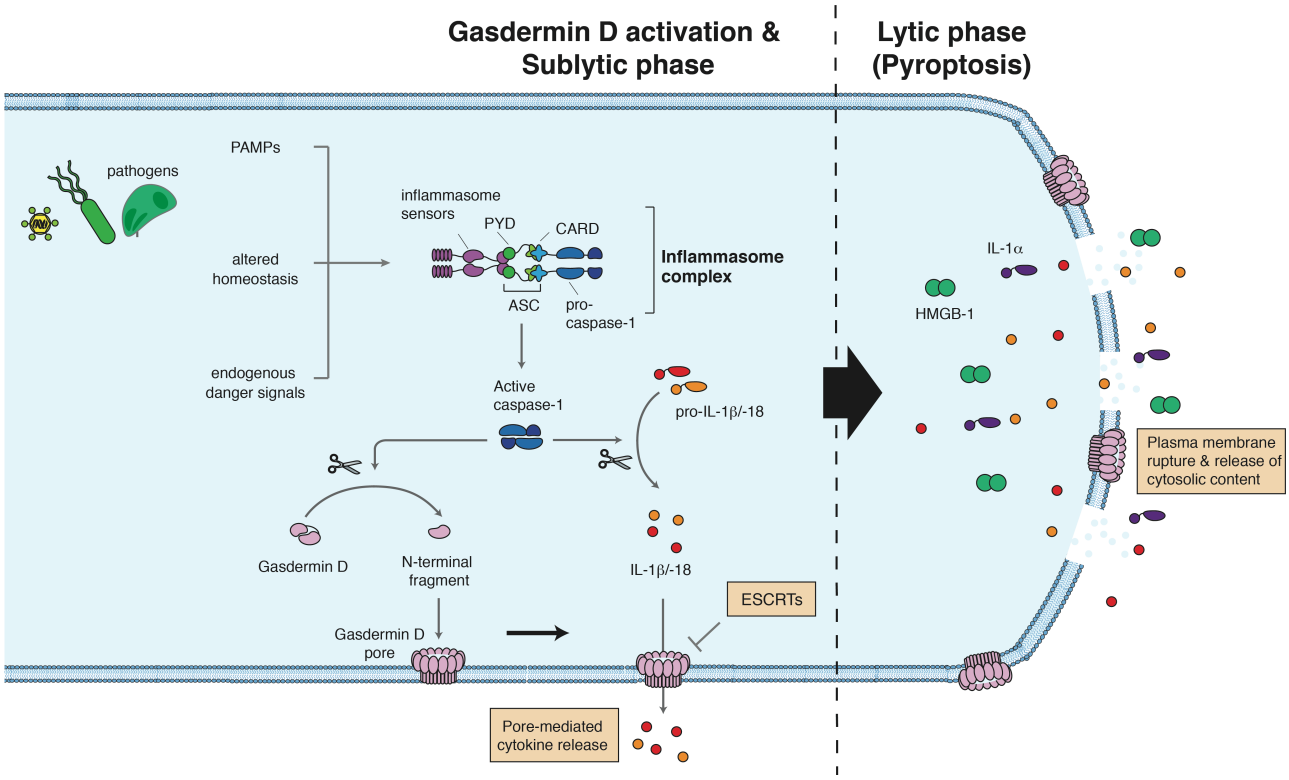
PJVK ------RFHFM-DEQNFKGRDKAIVFPAHTTIAFSVFELFIYLDGAFDLCVTSVSKGGFEREETATFALLYRL--RNILFER----------NRRVMDVISRSQLYLDDLF----------SDYYDKPLSMT---DI----------SLKEGTHIRVNLLNHNIPKGPCILCG

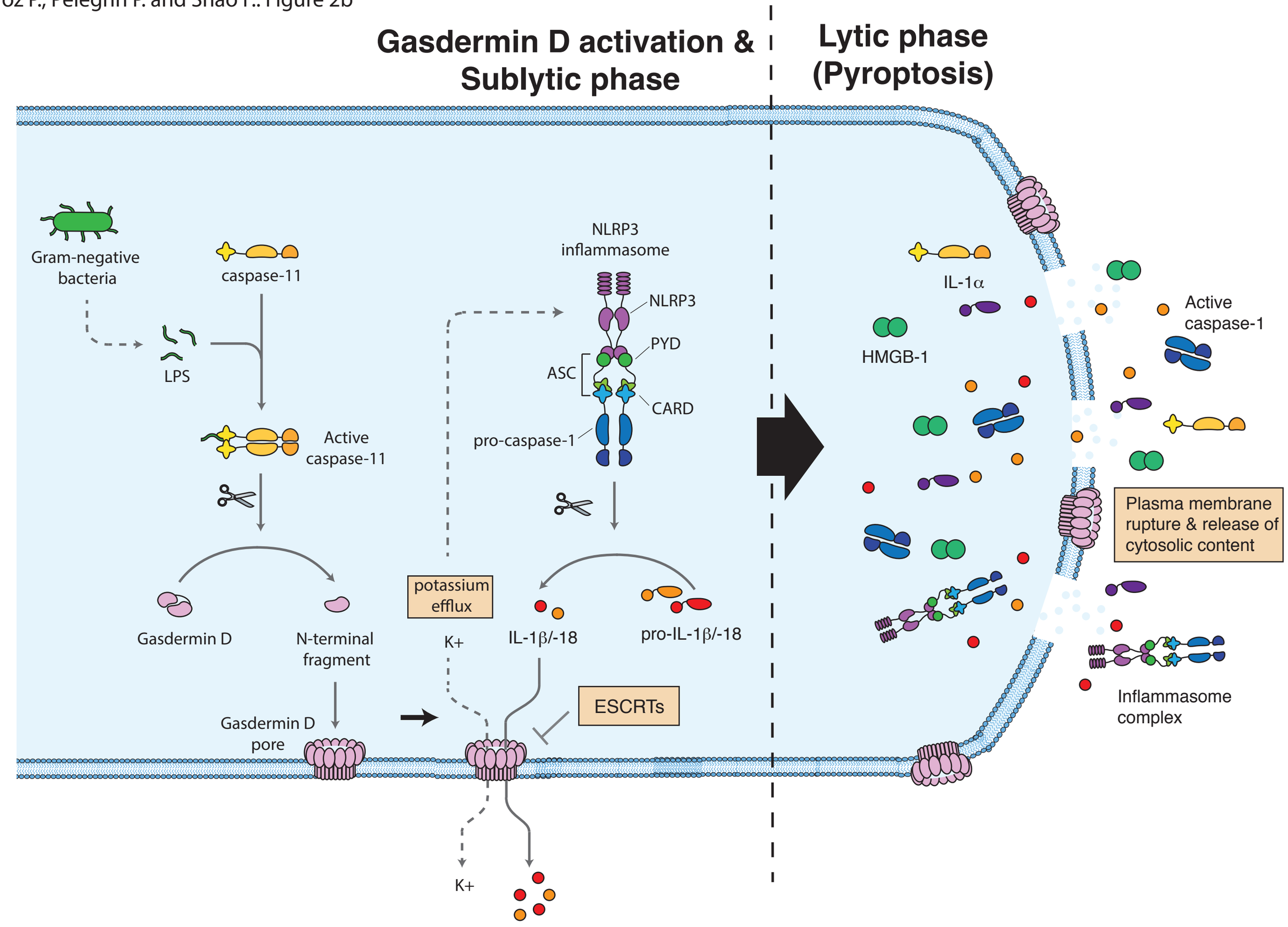
GSDMA ---------E----LALEGALDKGHEVTLEALPKDV-LLSKEAVGAILYFVGALTELSEAQQKLLVKSMEKKILPVQLKLVESTMEQNFLLDKE---------GVFPL----------QPELLSSLGDEELTLTEALVGLSGLEVQRSGPQYMWDPDTLPRLCALYAGLSLLQQLTKAS----- 445
GSDMB R-------VSE-VLISGELHMEDPDKPLLSSLFNAAGVLVEARAKAILDFLDALLELSEEQQ-FVAEALEKGTLPLLKDQVKSVMEQNWDE----------LASSPPDMDYDPEAR-ILCALYVVVSILLELAEGPTSVSS 411
GSDMC M-------LE----LDSSGHLDGPGGAILKKLQQDSNHAWFNPKDPILYLLEAIMVLSDFQHDLLACSMEKRILLQQQELVRSILEPNFRYPWS---------IPFTL----------KPELLAPLQSEGLAITYGLLEECGLRMELDNPRSTWDVEAKMPLSALYGTLSLLQQLAEA------ 508
GSDMD A-------LEQGQSLGPVEPLDGPAGAVLECLVLSSGMLVPELAIPVVYLLGALTMLSETQHKLLAEALESQTLLGPLELVGSLLEQSAPWQER---------STMSL----------PPGLLGNSWGEG-APAWVLLDECGLELGEDTPHVCWEPQAQGRMCALYASLALLSGLSQEPH---- 484
GSDME -------AFLQLVGCSLQGGCPGPEDAGSKQLFM----------TAYFLVSALAEMPDSAAALLGTCCKLQIIPTLCHLLRALSDDGVSDLEDPTLTPLKDTERFGIVQRLFASADISLERLKSSVKAVILKDSKVFPLLLCITLNGLCALGREHS---------- 496
PJVK MGNFKRETVYGCFQCSVDG---------QKYVRL----------H----------AVPCPDIWHKRMK---------- 352

Gasdermin-D function in canonical inflammasome activation
Broz P., Pelegrin P. and Shao F. Figure 2a



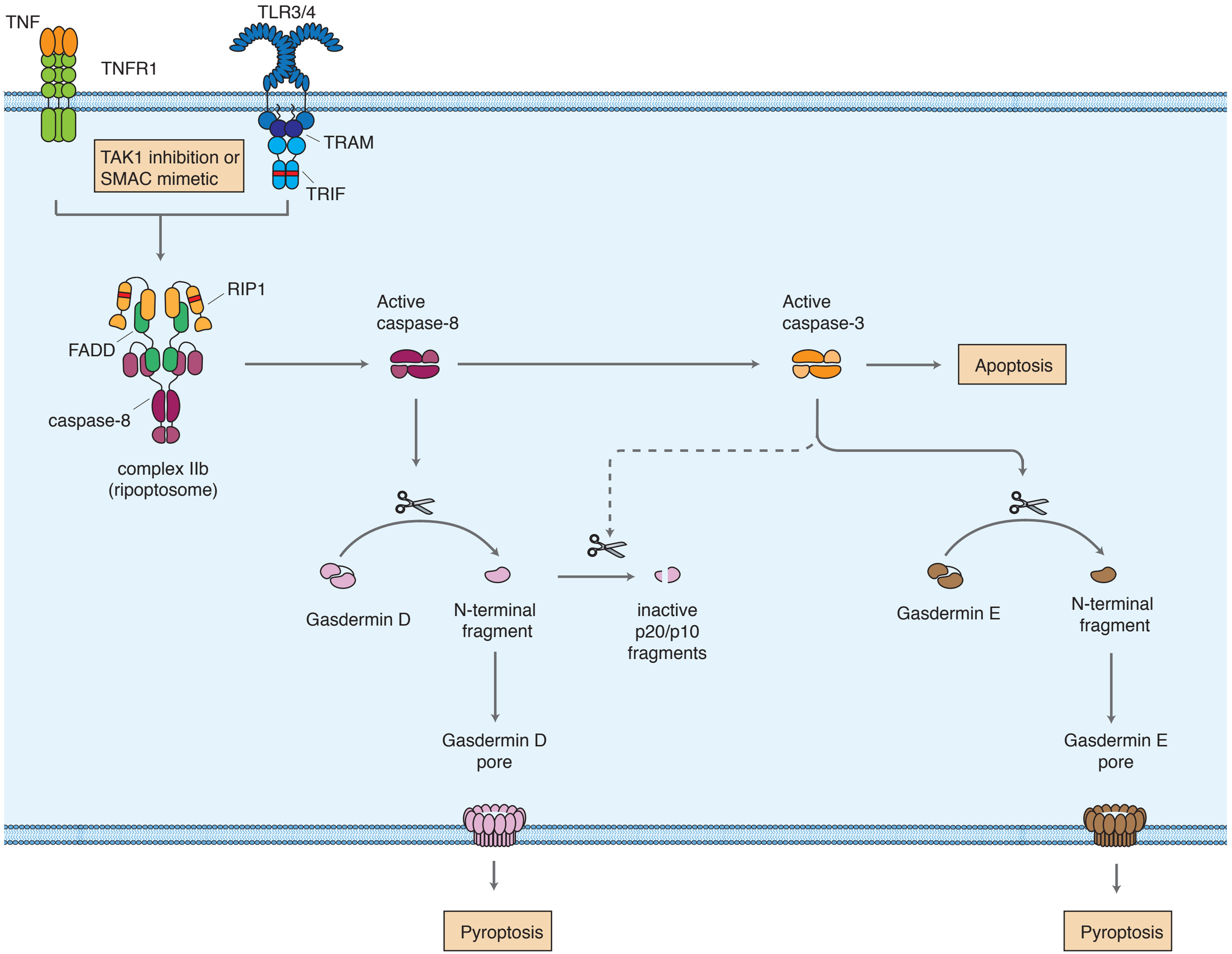
Gasdermin-D function in non-canonical inflammasome activation

Broz P., Pelegrin P. and Shao F.. Figure 2b



Function of gasdermins in canonical inflammasome activation

Broz P., Pelegrin P. and Shao F. Figure 3



Broz P., Pelegrin P. and Shao F. Figure 5