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Review

Biological mechanisms and therapeutic relevance of the gasdermin family

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ABSTRACT

Innate immunity enables host defense against pathogens and endogenous danger through inflammasomes, which are supramolecular complexes that recognize the threats and activate the immune response. Inflammasome activation often leads to pyroptosis, a highly inflammatory and lytic form of cell death, as a means of killing infected cells and releasing IL-1 family cytokines that communicate with other cells. Dysregulated inflammasome signaling results in a wide range of immune disorders including gout, sepsis, and hepatitis. Discovered as a direct killer molecule in pyroptosis, gasdermin D (GSDMD) is a pore-forming protein that represents a novel family with diverse cellular functions and pathological roles. This review summarizes current opinions in the biological mechanisms and therapeutic values of the GSDM family, particularly of GSDMD. Detailed mechanisms of auto-inhibition and pore formation by the GSDM family are presented, followed by a brief summary of the progress in the development of GSDM-targeting therapeutics.

1. Introduction

This review first gives a brief account of the discovery of gasdermin D (GSDMD), which represents a novel pore-forming protein family known as gasdermins (GSDMs). The GSDM family is predominantly expressed in the GI tract, skin, and immune cells and implicated in many diseases including inflammatory disorders and cancer (Table 1). The GSDM family was mentioned in literature two decades ago as a gene family associated with alopecia in mice and hearing loss in humans (Saeki et al., 2000a). The name "gasdermin" came from expression profiling of GSDMA in mice which showed predominant localization in the gastrointestinal (GI) tract and the dermis. In humans, the family contains six members, GSDMA through GSDMF. In mice, GSDMB is absent, and GSDMA and GSDMC have multiple isoforms, which are GSDMA1-3 and GSDMC1-4, respectively. As of now, the activating enzymes and biological functions have been elucidated for GSDMD, GSDME, and GSDMB. In this review, the functions, pore formation mechanism, and therapeutic potentials of GSDMs will be covered with an emphasis on insights from GSDM structures.

To understand GSDMs entails knowledge about inflammasomes, which are cytosolic protein complexes that function as signaling organelles in innate immunity (Lamkanfi and Dixit, 2014). In response to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs), sensors such as nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs) and AIM2-like receptors (ALRs) assemble into multimeric protein platforms (Hu et al., 2015; Tenthorey et al., 2017; Zhang et al., 2015), which in turn recruit adaptors such as apoptosis-associated, speck-like protein containing a caspase recruitment domain (ASC) (Lu et al., 2014) and effectors such as caspase-1 through homotypic death domain interactions (Shen et al., 2019). Caspase-1 promotes the proteolytic maturation of IL-1 family cytokines including IL-1β and IL-18. The NLR/ALR-containing sensor-adaptor--effector systems are often referred to as canonical inflammasomes, with the most studied example being the NLRP3 inflammasome. By contrast, the presence of oxidized lipids or exogeneous lipids such as lipopolysaccharides (LPS) from Gram-negative bacteria in the cytosol directly activates caspase-11 (mouse homolog of human caspase-4/5), which represents the non-canonical inflammasome (Kayagaki et al., 2013; Zanoni et al., 2016). Caspase-1 in the canonical inflammasomes and caspase-4, -5, and -11 in the noncanonical inflammasome are cysteine proteases collectively known as inflammatory caspases. The recruitment of these caspases to inflammasomes facilitates their oligomerization, higher-order assembly, and proximity-induced auto-activation (Wu, 2013). Direct downstream effects of active inflammatory caspases include IL-1 release and pyroptosis, a highly inflammatory and lytic form of programmed cell death. Yet, while these phenomena have long been observed, the direct executioner of IL-1 release and pyroptosis remained a mystery until very recently.

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2. Discovery and functions of the GSDM family

2.1. GSDMD, a direct executioner of pyroptosis

GSDMD, the first extensively studied member of the GSDM family, was discovered in the context of inflammasome biology and first functionally linked to pyroptosis. In 2015, an N-ethyl-N-nitrosourea (ENU)-based screen in mice identified mutations in the *Gsdmd* gene that impaired IL-1β secretion from macrophages following LPS challenge (Kayagaki et al., 2015). In line with this discovery, a CRISPR-Cas9-based screen in murine bone-marrow derived macrophages (BMDMs) identified GSDMD as involved in caspase-1- and caspase-11-dependent pyroptosis (Shi et al., 2015) (Table 1). Both studies reported that *Gsdmd*$^{-/-}$ BMDMs are defective in pyroptosis and IL-1β secretion in response to cytosolic LPS (Kayagaki et al., 2015; Shi et al., 2015). In addition, quantitative mass spectrometry revealed GSDMD as a molecule that interacts with inflammasomes (He et al., 2015). Together, these studies pointed that GSDMD is a direct substrate of inflammatory caspases and serves as the executioner for both IL-1β secretion and pyroptosis.

Later, researchers reported more detailed analyses of how GSDMD induces pyroptosis mechanistically. The expression of GSDMD is positively regulated by the transcription factors IRF1 and IRF2 (Benaoudia et al., 2019; Kayagaki et al., 2019). Upon inflammasome activation in host cells, inflammatory caspases cleave GSDMD at a flexible linker to release the functional N-terminal fragment of GSDMD (GSDMD-NT). Many GSDMD-NT subunits bind the inner leaflet of the plasma membrane and penetrate the membrane by forming pores. (Aglietti et al., 2016; Ding et al., 2016; Liu et al., 2016). Extensive pore formation compromises membrane integrity and leads to lytic cell death known as pyroptosis.

A systems-level effect of GSDMD-mediated pyroptosis is to cause blood coagulation and thrombosis (Wu et al., 2019). As bacterial infection, inflammasome activation, and pyroptosis occur in macrophages, cell membranes containing tissue factors (TFs) are excised to form microvesicles. Also, Ca^{2+} influx through GSDMD pore triggers the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane through the Ca^{2+}-dependent TMEM16F lipid scramblase (Yang et al., 2019), and TF activity is known to be promoted by PS exposure (Spronk et al., 2014). These active TFs eventually lead to disseminated intravascular coagulation (DIC), a life-threatening phenomenon due to the excessive activation of the coagulation cascade.

2.2. Other functions of GSDMD

The mechanisms for IL-1 secretion from macrophages have been intensively investigated over the past three decades. Unlike many secreted molecules, which contain an N-terminal secretion signal and are co-translationally translocated into the endoplasmic reticulum (ER) for later release through the conventional secretory pathway (Viotti, 2016), IL-1 family cytokines lack the secretion signal and are directly released from the cytosol to the extracellular space. The mechanism by which proteins are released from cells in an ER/Golgi-independent manner is generally referred to as unconventional protein secretion.

The GSDMD pore has emerged as the size-restricted conduit for IL-1 secretion from cells that are immunologically hyperactivated, which is defined to be a state where cells undergo inflammation but not pyroptosis (Evavold et al., 2018). This sub-lytic hyperactivated state could be achieved by the low abundance of GSDMD pores or active membrane repair mechanisms (Evavold et al., 2018; Ruhl et al., 2018). On the other hand, when pyroptosis and membrane rupture occur, intracellular contents are released regardless of the size restriction posed by the GSDMD pore. Therefore, GSDMD has attracted the spotlight as a regulator of cytokine release from living cells, and of cellular content release from pyroptotic cells (Evavold et al., 2018; Heilig et al., 2018).

In addition to IL-1 family cytokines, the large pores formed by GSDMD allow the flow of other cytosolic contents including ions. Potassium efflux will then further induce inflammasome activation through NLRP3, which senses membrane disruption and seeds the formation of a canonical inflammasome (Orning et al., 2018). This secondary inflammasome activation phenomenon supports a positive feedback loop and raises the possibility that GSDMD is upstream or downstream of inflammasomes, especially in light of recent discoveries that GSDMD activation can be independent of inflammatory caspases.

In neutrophils, GSDMD can initiate a different mode of cell death known as NETosis, the name of which originates from the formation of neutrophil extracellular traps (NETs) during this type of cell death (Chen et al., 2018; Sollberger et al., 2018). The NETs are composed of chromatin and proteins that possess antimicrobial properties. It has been demonstrated that in response to inflammasome stimuli, GSDMD pores form in neutrophils to facilitate cell rupture and NET extrusion into the extracellular space. GSDMD can also be detected on the extruded NETs, raising the possibility that GSDMD plays a role in directly clearing NET-trapped microbes, although bacteria trapped by GSDMD-pore induced intracellular pits (PITs) were shown to be predominantly killed by secondary phagocytes rather than GSDMD (Jorgensen et al., 2016). Therefore, in addition to executing pyroptosis, GSDMD is a critical mediator of NETosis and potentially other modes of cell death as well.

2.3. GSDME and GSDMB, drivers of anti-tumor immunity

Following the discovery of GSDMD as a critical player in cytokine release and pyroptosis, the functions and activating enzymes of GSDME and GSDMB have been uncovered. Rather than in phagocytes, the most well-characterized venue for GSDMD, GSDME and GSDMB appear to be major players in cancer cells. While inflammatory caspases do not directly interact with GSDME and GSDMB and their activation mechanisms appear inflammasome-independent, caspase-3, which is traditionally considered as an apoptotic caspase, cleaves GSDME after residue Asp270 and converts non-inflammatory apoptosis to pyroptosis in cancer cells (Rogers et al., 2017, 2019; Wang et al., 2017).

Table 1
GSDM family members, functions, and disease relevance.

Human GSDM (Other name)	Predominant expression	Biological function	Activating enzyme	Associated disease
GSDMA (GSDM1)	GI tract, skin	Mitochondrial homeostasis	Not known	Alopecia
GSDMB (GSDML)	Lungs, GI tract, liver, cancer cells	Pyroptosis, anti-tumor immunity	Granzyme A	Asthma
GSDMC (MLZE)	GI tract, skin, spleen, trachea	Not known	Not known	Not known
GSDMD (GSDMDC1)	GI tract, skin, monocytes, macrophages	Inflammation, pyroptosis, cytokine release, NETosis, bacteria killing	Caspase-1/4/5/11, caspase-8, neutrophil elastase, cathepsin G	Inflammatory disorders
GSDME (DFNA5)	GI tract, placenta, inner ear, brain, kidney, cancer cells	Pyroptosis, anti-tumor immunity	Caspase-3, granzyme B, caspase-8, caspase-7	Deafness Cancer
GSDMF (DFNB59, PJVK)	Inner ear	Not known	Not known	Deafness

Consistently, certain chemotherapeutics induce cancer cell pyroptosis, rather than commonly observed apoptosis, by activating the caspase-3-GSDME pathway (Wang et al., 2017). Therefore, GSDME has been proposed as a tumor suppressor by inducing pyroptosis in cancer cells. Consistently, cancer cells have evolved mechanisms to repress GSDME activity, such as by transcriptional silencing and loss-of-function mutations (Zhang et al., 2020).

Interestingly, GSDME can be activated via a cell-extrinsic mechanism through anti-tumor immunity. In one case, tumor suppression is mediated by tumor-infiltrating killer lymphocytes including NK cells and $CD8^+$ T cells. These killer cells release granzyme B into the tumor cells via a perforin-dependent manner, and granzyme B directly processes GSDME in addition to activating caspase-3 (Zhang et al., 2020) (Table 1, Fig. 2). In another case, researchers emphasized the ability of chimeric antigen receptor (CAR) T cells, rather than existing $CD8^+$ cells, to induce target cell pyroptosis also through perforin-dependent direct activation of GSDME by granzyme B (Liu et al., 2020). In the latter study, the target cells tested include B leukemic cells and solid tumor cells where GSDME is expressed. GSDME-induced target cell pyroptosis leads to the release of cellular factors that activate inflammasomes in macrophages. As a result, GSDMD is activated in macrophages and cytokines are secreted, which forms the basis for the cytokine release syndrome (CRS) commonly observed in CAR T cells therapy. Therefore, the safety of activating GSDME in cancer cells for immunotherapy or administering active GSDME as a tumor-killing agent (Wang et al., 2020) awaits further evaluation.

The recently discovered cellular activation mechanism for GSDMB is similar to that for GSDME (Zhou et al., 2020). In both cases, cytotoxic T lymphocytes and NK cells kill GSDME/GSDMB-positive cells through pyroptosis and the killing is dependent on perforin activity. A key difference is that granzyme A from the donor cells cleaves GSDMB in the target cells, whereas granzyme B cleaves GSDME, with no cross-reactivity observed so far. In addition to granzymes A and B, the granzyme family comprises granzymes H, K, and M in humans, and more variants in mouse. The potential interaction of these other granzymes with GSDMs and the structural basis for substrate specificity remain to be investigated.

3. Auto-inhibition, activation, and regulation of GSDMs

3.1. Auto-inhibition of GSDM-NT by GSDM-CT

GSDMs are neither strictly membrane proteins nor soluble proteins, and the solubility and localization of GSDMs are dependent on whether the proteins are proteolytically activated. While full-length GSDMs are auto-inhibited andsoluble, cleaved GSDMs bind lipids in the membranes to form pores and possess cytotoxic effects.

Prior to proteolytic activation, the GSDM family except for GSDMF feature a two-domain organization, with the functional, pore-forming fragment GSDM-NT kept inactive by the auto-inhibitory C-terminal domain (GSDM-CT) (Fig. 1A). Structurally, GSDM-NT contains predominantly loops and β-strands (β1-β11 in GSDMA3) besides several α

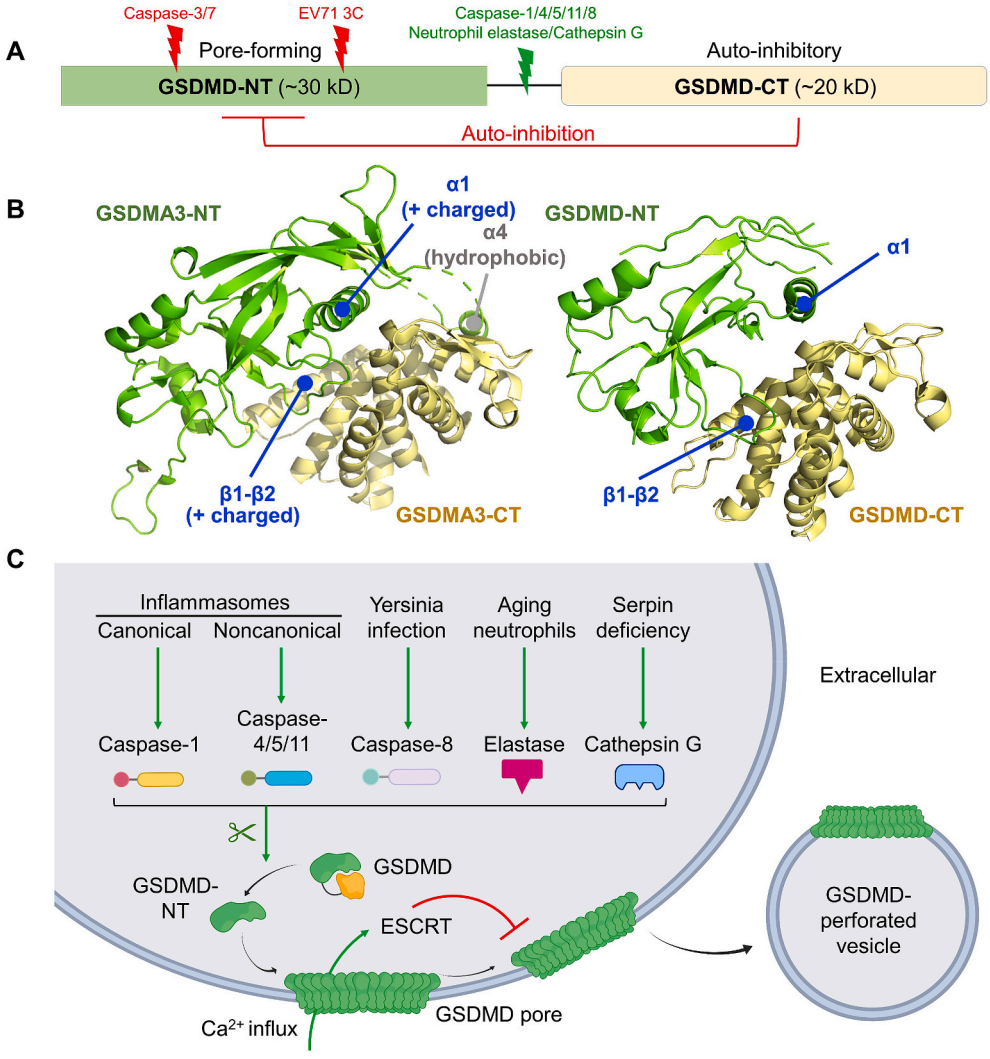

Fig. 1. Autoinhibition and biological pathways of GSDMD.
A) Two-domain architecture of the GSDM family. Prior to proteolytic activation, the pore-forming GSDM-NT is kept inactive by the auto-inhibitory GSDM-CT. The cleavage locations of activating (green) and deactivating (red) enzymes are marked above.
B) X-ray crystal structures of murine GSDMA3 (PDB: 5B5R) and human GSDMD (PDB: 6N9O). The structures show that GSDM-CT, colored in yellow, folds back on the functional GSDM-NT, colored in green, for auto-inhibition. Blue and gray: Predominantly positively charged and hydrophobic structural elements, respectively.
C) Activation and negative regulation of GSDMD. Collectively known as inflammatory caspases, canonical inflammasome-activated caspase-1 and non-canonical inflammasome-activated murine caspase-11 (and human homologs caspase-4/5) cleave GSDMD for pore formation. Other than inflammatory caspases, caspase-8 activates GSDMD during Yersinia infection. In aging neutrophils, an elastase released from cytoplasmic granules cleaves GSDMD, which induces neutrophil death. In neutrophils and monocytes, serpins inhibit cathepsin G which cleaves GSDMD to generate the pore-forming fragment. Ca^{2+} influx through the GSDMD pore catalyzes the assembly of the ESCRT-III complex, which repairs the membrane by removing the GSDMD pore through exocytosis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

helices (α1-α4 in GSDMA3) and undergoes dramatic conformational changes during pore formation (Ding et al., 2016). On the other hand, GSDM-CT is almost exclusively α-helical (α5-α12 in GSDMA3) and folded into a compact globular conformation, which partially explains why crystallization of full-length GSDMD requires significant construct engineering such as loop deletions whereas that of GSDMD-CT does not (Ding et al., 2016; Liu et al., 2019). The linker between GSDM-NT and GSDM-CT is long and flexible, consistent with its accessibility to inflammatory caspases and other activating enzymes. Of note, GSDMF has a relatively short CT region that lacks homology to other GSDM-CTs, suggesting evolutionary and mechanistic divergence within the GSDM family (Ruan, 2019).

The X-ray crystal structures of full-length GSDMD and mouse GSDMA3 provided insights into the molecular basis for the conserved auto-inhibition architecture (Ding et al., 2016; Liu et al., 2019). GSDM-CT folds back on GSDM-NT to form electrostatic, hydrophobic, and hydrogen bonding interactions at primarily three sites, which are contacts between GSDM-CT and the α1 helix (via predominantly charge-charge interactions), the α4 helix (via predominantly hydrophobic interactions), and the β1-β2 loop of GSDM-NT (via predominantly charge-charge interactions), respectively (Fig. 1B). It turns out that the α1 and α4 helices play essential roles in lipid binding and membrane insertion during GSDM-NT pore formation, and therefore GSDM-CT masks these helices to keep the full-length protein inactive (Ruan et al., 2018). It is currently unclear the role of the β1-β2 loop in pore formation, and why it needs to be masked by GSDM-CT to achieve auto-inhibition.

Interestingly, in vitro, GSDM-NT and GSDM-CT remain bound in the absence of lipids in the surrounding environment, regardless of whether the interdomain linker is cleaved (Ding et al., 2016). This observation suggests that a lipidic environment may be required for the dissociation of GSDM-NT from GSDM-CT. On the other hand, mutations of residues in GSDM-CT that contact GSDM-NT enable full-length GSDMs to form pores, suggesting that GSDM-CT does not need to be proteolytically removed for pore formation as long as it dissociates from GSDM-NT (Ding et al., 2016). The exact molecular mechanisms of how the GSDM-NT/CT non-covalent complex binds lipids, how GSDM-CT is displaced, and whether proteolysis is an absolute requirement for GSDM activation, await further elucidation. It is also possible that post-translational modifications of GSDMD, such as ubiquitination and phosphorylation (see PhosphoSitePlus database), may impact the auto-inhibition state and affect pore formation kinetics.

3.2. GSDMD activation by inflammatory caspases and other enzymes

GSDMD was discovered as a substrate of canonical inflammasome-activated caspase-1 and non-canonical inflammasome activated caspase-11 (murine homolog of human caspase-4/5). These inflammatory caspases cleave GSDMD at a disordered linker between GSDMD-NT and GSDMD-CT, more specifically after residue Asp276 in mouse GSDMD and after Asp275 in human GSDMD. Biochemical and cellular evidence demonstrated that this cleavage liberates GSDMD-NT and triggers its relocation from the cytoplasm to the plasma or mitochondrial membranes for pore formation (Aglietti et al., 2016; Ding et al., 2016; Liu et al., 2016).

Since inflammatory caspases were proved bona fide activators of GSDMD, several inflammatory caspase-independent pathways that activate GSDMD have been elucidated (Fig. 1C), which showed that the repertoire of GSDMD-activating pathways extends beyond inflammasome caspases and raised the possibility that GSDMD pore formation does not necessarily occur downstream of inflammasomes (Xia et al., 2019). When *Yersinia* infects murine macrophages, caspase-8 is activated in the context of TGFβ-activated kinase 1 (TAK1) inhibition by the effector molecule YopJ, results in the cleavage of GSDMD either directly or via other intermediates, and ultimately leads to pyroptosis (Orning et al., 2018; Sarhan et al., 2018) (Table 1). The site for caspase-8-directed GSDMD cleavage is the same as that for inflammatory caspases. It should be noted that although caspase-8-mediated GSDMD activation provides an example of GSDMD cleavage independent of inflammatory caspases, caspase-8 activation itself could be inflammasome-dependent (Antonopoulos et al., 2015; Mascarenhas et al., 2017; Pierini et al., 2012; Sagulenko et al., 2013) and TAK1 inhibition may lead to spontaneous inflammasome activation (Allam et al., 2014; Gurung et al., 2014; Malireddi et al., 2018, 2020), hence the possibility of a more intricate signaling network that intertwines inflammasome activation, caspase-8 activation, and GSDMD cleavage.

In addition to the caspases that mediate GSDMD activation, new activating enzymes that are serine, rather than cysteine, proteases have been discovered including the neutrophil elastase (ELANE) in aging neutrophils (Kambara et al., 2018) and cathepsin G, which is released from cytoplasmic granules in neutrophils and monocytes where serpins are deficient (Burgener et al., 2019) (Table 1). The cleavage sites for ELANE and cathepsin G, after Cys268 and Leu274 in GSDMD respectively, differ slightly from that for inflammatory caspases. Nonetheless, the approximately 30-kD GSDMD-NT fragment is capable of forming pores. Together, these studies highlight the intricate regulatory networks that modulate GSDMD activation in multiple cell types.

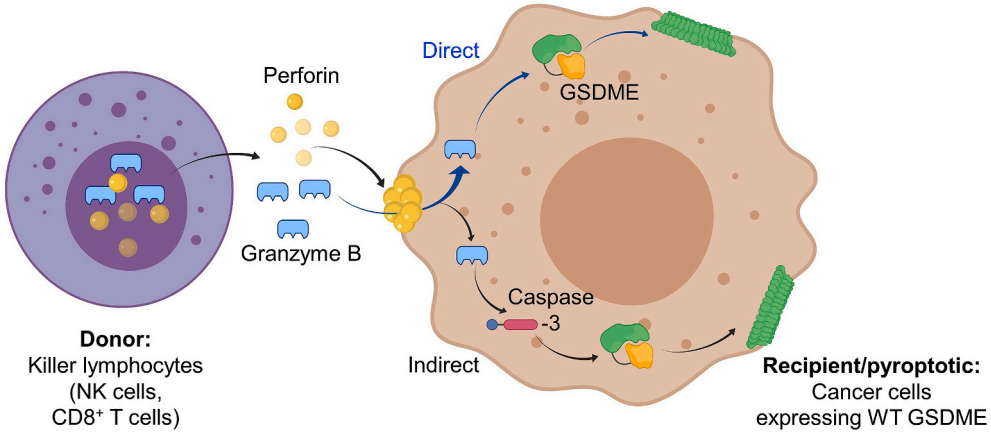

Fig. 2. GSDME as a tumor suppressor. Anti-tumor immunity is enabled by perforin-dependent delivery of granzyme B from killer lymphocytes to tumor cells, where granzyme B directly cleaves GSDME for cancer cell pyroptosis or indirectly via caspase-3, another activating enzyme for GSDME. GSDMB follows a similar activation mechanism mediated by granzyme A released from cytotoxic lymphocytes.

3.3. Repression of GSDMD activity

The non-pyroptotic functions of GSDMD hint at mechanisms for protection against GSDMD-induced cell death. Caspase-3 and caspase-7, traditionally defined as apoptotic caspases, have been shown as major and minor activators respectively for GSDME (Rogers et al., 2017, 2019; Sarhan et al., 2018; Wang et al., 2017) (Table 1). By contrast, the effects of caspase-3/7 on GSDMD are repression rather than activation (Taabazuing et al., 2017).

During apoptosis of murine monocytes and macrophages, caspase-3/7 cleaves GSDMD at a non-canonical site in GSDMD-NT, after residue Asp87, which is N-terminal to the inflammatory caspase cleavage site (Taabazuing et al., 2017). This cleavage results in fragmentation of GSDMD into nonfunctional pieces (residues 1–87 and 88–484), thereby rendering it unable to form pores. The proteolytic restriction of GSDMD by caspase-3/7 suggests interplay between apoptosis and pyroptosis, such that when apoptotic pathways are activated, cells try to prevent an inflammatory response by inactivating the executioner molecule of pyroptosis. A similar strategy to repress GSDMD pore formation may have evolved in pathogens as a mechanism to evade the innate immune response. For example, enterovirus (EV) 71, the causative agent of hand-foot-and-mouth disease, utilizes a 3C protease that cleaves GSDMD within GSDMD-NT, more specifically after residue Gln193 (Lei et al., 2017). The GSDMD 1–193 fragment lacks pore-forming ability and cannot cause pyroptosis in host cells.

Whether through the apoptotic caspases in hosts or enzymes in pathogens, GSDMD may be inactivated prior to pore formation. However, once GSDMD is proteolytically activated, it rapidly oligomerizes and inserts into the membrane, a process that is likely energetically downhill and irreversible. To counter GSDMD activity after the pores are formed and deter membrane rupture, cells have developed an active membrane repair mechanism that involves the endosomal sorting complex required for transport (ESCRT)-III (Ruhl et al., 2018). Catalyzed by the influx of Ca^{2+} through GSDMD pores, ESCRT-III forms membrane-localized puncta near the pores and mediates subsequent exocytosis of GSDMD-perforated membrane vesicles. Interestingly, ESCRT-III also governs the exocytosis of MLKL pores in cells undergoing necroptosis (Gong et al., 2017), and appears as a unified repair mechanism behind membrane pore-mediated cell death. The repair mechanism can be linked back to the observation that macrophages may achieve a hyperactive state characterized by prolonged inflammasome activation, IL-1 release through GSDMD pores, and the maintenance of cell survival. Other cellular mechanisms to disassemble or remove GSDMD pores await discovery.

4. Mechanism of GSDM pore formation

4.1. High-resolution snapshot of an active-state GSDM

While structures of full-length GSDMs provided valuable visualization of the auto-inhibition architecture of the family, the exact mechanism of GSDM pore formation requires the structural determination of GSDMs in their active states. However, even in the golden age of structural biology thanks to cryo-electron microscopy (cryo-EM), to solve the high-resolution structure of a GSDM pore is not an easy task. In crystallizing GSDMD, the loop-rich and flexible nature of GSDMD-NT necessitated multiple truncations, some of which led to the invisibility of several secondary elements such as the α4 helix (Liu et al., 2019). Despite ideal in vitro behaviors, it is unlikely that the crystallizable GSDMD constructs are useful for reconstituting the pores due to deletions of key residues that may render the protein nonfunctional. To date, protocols for reconstituting and purifying well-behaving GSDMD pores have not been established. On the other hand, full-length wildtype murine GSDMA3 was crystallized without further construct engineering and proved to form more homogeneous and less aggregated pores than GSDMD in vitro (Ruan et al., 2018). Even so, the GSDMA3 pores are large and heterogeneous assemblies that are unamenable to X-ray crystallography. Therefore, GSDMA3 was chosen as a promising candidate for determination of the structure of a GSDM pore using cryo-EM.

The cryo-EM structure of the GSDMA3 membrane pore, reconstituted on cardiolipin (CL)-containing liposomes and solubilized by detergent sodium cholate, revealed a striking β-barrel transmembrane (TM) region and a soluble rim adjacent to the β-barrel on the cytosolic side (Ruan et al., 2018) (Fig. 3A). 2D classification of projected particles from raw cryo-EM images revealed heterogeneity in oligomerization, which ranges from 26 to 28 subunits per pore. The dominant 27-subunit population is selected for 3D classification and subsequent refinement with C27 symmetry imposed, and a final resolution at 3.8 Å was achieved. For the 27-subunit GSDMA3 pore, the inner diameter is approximately 180 Å or 18 nm, larger than the hydrated diameter of about 40 Å for mature IL-1β. Given that IL-1β is more rapidly released through GSDMD pores than the much larger tetrameric lactate dehydrogenase (LDH), the structure supports a size restriction mechanism where molecules significantly smaller than the GSDM pore conduit are preferentially released (Evavold et al., 2018). Other than enabling intermolecular contacts for subunit oligomerization, the function of the cytosolic rim or globular region, which is 280 Å in diameter, merits further investigation.

4.2. Lipid binding and oligomerization

For illustration purposes, the process of GSDM pore formation is conceptually decomposed into three steps – lipid binding, oligomerization, and membrane insertion (Liu et al., 2019; Ruan et al., 2018), but the three steps may occur concomitantly. GSDMD-NT oligomers have not been observed in vitro in the absence of lipids, suggesting that oligomerization does not take place before lipid binding. Whether oligomerization precedes insertion or the two are coupled remains controversial.

In vitro lipid binding assays revealed that GSDMD-NT, but not full-length auto-inhibited GSDMD, selectively binds acidic lipids, which include phosphatidylinositol phosphates (PIPs), PS, and CL (Ding et al., 2016; Liu et al., 2016). While PIPs and PS are found at the inner leaflet of the plasma membrane in healthy cells, cardiolipin is exclusively in mitochondria and bacterial. The selectivity for acidic lipids underlies the ability of GSDMD to puncture the plasma and mitochondrial membranes (Lin et al., 2015; Rogers et al., 2019), and potentially directly kill bacteria to control infection (Liu et al., 2016). Consistent with the fact that PIPs and PS are at the inner but not outer leaflet of the plasma membrane, GSDMD only forms pores from within, and does not harm bystander cells when released outside (Liu et al., 2016). Importantly, the membrane attack complex/perforin/cholesterol-dependent cytolysin (MACPF/CDC) superfamily, which also form large β-barrel pores, require the presence of cholesterol or receptors in membranes for pore formation (Rosado et al., 2008). This difference in binding preference highlights the uniqueness of the GSDM family of pore-forming proteins.

The cryo-EM structure of the GSDMA3-NT pore identified the positively charged α1 helix, which contains three Arg residues, as a major membrane-binding element (Fig. 3A). In full-length GSDMA3, the α1 helix of GSDMA3-NT is masked by GSDMA3-CT, consistent with the observation that full-length GSDMs do not associate with acidic lipids. In line with the structural observation, Ala mutations of the basic residues in α1 compromised the pore-forming ability of GSDMA3 and GSDMD. A cryo-EM density blob was also observed adjacent to α1 and likely represented the density of the head of the acidic lipid cardiolipin.

While the cryo-EM structure clearly shows α1 as the membrane-binding helix, previous mutagenesis and lipid binding experiments proposed that basic residues around the α3 helix are potentially important for lipid interaction (Liu et al., 2016). The fact that mutation of α3 reduces GSDMD-lipid interaction is difficult to rationalize given that α3 is more membrane-distal than α1 and no lipid density was observed near α3 in the cryo-EM map. It turned out that α3 is located at a subunit oligomerization interface and mutations on α3 could result in a

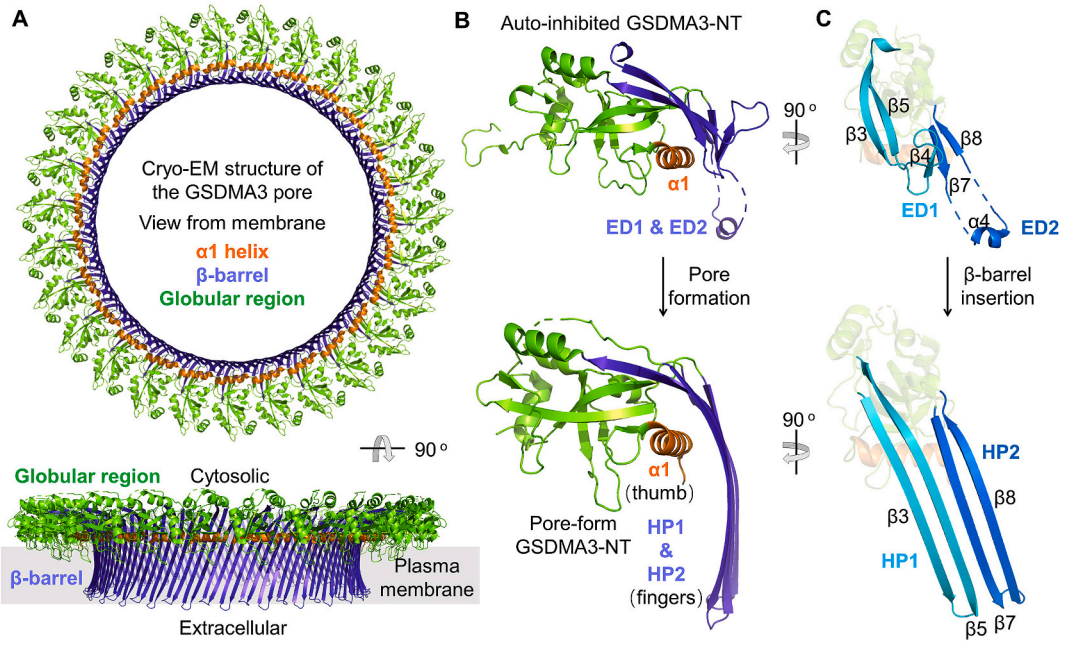

Fig. 3. Mechanism of GSDM pore formation.
A) The cryo-EM structure of the 27-subunit murine GSDMA3 pore (PDB: 6CB8). In addition to the 108-strand β-barrel that inserts into the membrane, each subunit contributes a basic α1 helix that interacts with acidic lipids, and a globular domain that caps the transmembrane (TM) region on the cytosolic side.
B) Conformational changes from auto-inhibited to membrane-inserted GSDMA3-NT. Purple: Extension domains ED1 and ED2, which transition into the finger-like hairpins HP1 and HP2, respectively. Orange: The membrane-binding helix α1 that resembles the thumb. Green: The globular region that resembles the palm.
C) Conformational changes with key secondary structures highlighted. ED1, the β3-β4-β5 region in the auto-inhibited structure, becomes HP1 upon membrane insertion, whereas the β7-α4-β8 region or ED2 becomes HP2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

failure to oligomerize (Ruan et al., 2018). Therefore, it is possible that there exists oligomerization-mediated cooperativity in lipid binding, such that monomeric GSDMD mutants bind less strongly to lipids than oligomeric GSDMD.

Regarding the mechanism of oligomerization, in the GSDMA3 pore, neighboring subunits form extensive inter-molecular contacts through predominantly hydrogen bonding and charged interactions. Please refer to the paper on the GSDMA3 pore structure for a detailed view of interfacial amino acids and intermolecular contacts (Ruan et al., 2018).

4.3. Membrane insertion through β-barrel formation

The 27-subunit GSDMA3 pore contains a striking anti-parallel β-barrel with 108 β-strands, with each subunit contributing four β-strands (Ruan et al., 2018). Along each β-strand, hydrophilic and hydrophobic amino acids are alternately arranged, such that the side of the β-barrel facing the conduit contains mostly polar amino acids whereas the side facing the fatty acid tails in the membrane is primarily non-polar.

Superimposition of auto-inhibited and membrane-inserted GSDMA3-NT structures revealed large conformational changes that accompany the formation of the four TM β-strands. On the other hand, the globular domain adjacent to the β-barrel on the cytoplasmic side as well as the α1 helix is relatively structurally unaltered throughout pore formation. To help to understand the mechanism of TM β-strand formation, we introduce the terms extension domain (ED) and hairpin (HP) as they were employed in the GSDMA3 pore structure study (Ruan et al., 2018) (Fig. 3B).

The four TM β-strands from each pore-form GSDMA3-NT subunit are two hairpins, HP1 and HP2, which are formed by extension domains ED1 and ED2 respectively in auto-inhibited GSDMA3-NT. ED1 is located at the β3-β4-β5 region, which inserts into the membrane to form HP1. During the insertion, β3 itself is elongated to form a TM β-strand and β4, β5, and the loop in between are integrated into the other TM β-strand of HP1. ED2 is located at the β7-α4-β8 region, which is masked by GSDMA3-CT in the auto-inhibited state. The α4 helix and the flanking loops are straightened and become continuous β-strands with β7 and β8, culminating in the formation of HP2 (Fig. 3C). Within and between HP1 and HP2, of a subunit or between neighboring subunits, backbone carbonyls and amides form an extended hydrogen bonding network to stabilize the β-barrel.

5. Pharmacological interventions

Physiological inflammation may recruit phagocytes cells to the site of infection for host defense, or directly kill compromised host cells in the case of pyroptosis. However, dysregulated inflammation can lead to severe pathologies, including inflammatory disorders such as gout, inflammatory bowel disease, sepsis, and alcoholic and non-alcoholic hepatitis (Khanova et al., 2018; Lieberman et al., 2019; So and Martinon, 2017; Xu et al., 2018) (Table 1). Precise control of the degree of inflammation is therefore vital to the health of living organisms. At the intersection of canonical and non-canonical inflammasome signaling pathways which respond to a wide array of endogenous and exogenous signals, and as a direct executioner of inflammatory cell death, GSDMD has emerged as a promising therapeutic target against inflammatory diseases.

Understanding of the pore formation mechanism of GSDMD opens the door to therapeutic development from a mechanistic perspective. For example, small molecules that target the GSDMD-NT-CT interface may help to lock the protein in its inactive conformation, thereby achieving an inhibitory effect. However, recently identified GSDMD inhibitors disulfiram (DSF), an FDA-approved drug for alcohol abuse, and necrosulfonamide (NSA) utilize a distinct mechanism (Hu et al., 2020; Rathkey et al., 2018) (Fig. 4). The discovery of NSA as a GSDMD inhibitor was based on the observation that it targets MLKL, the

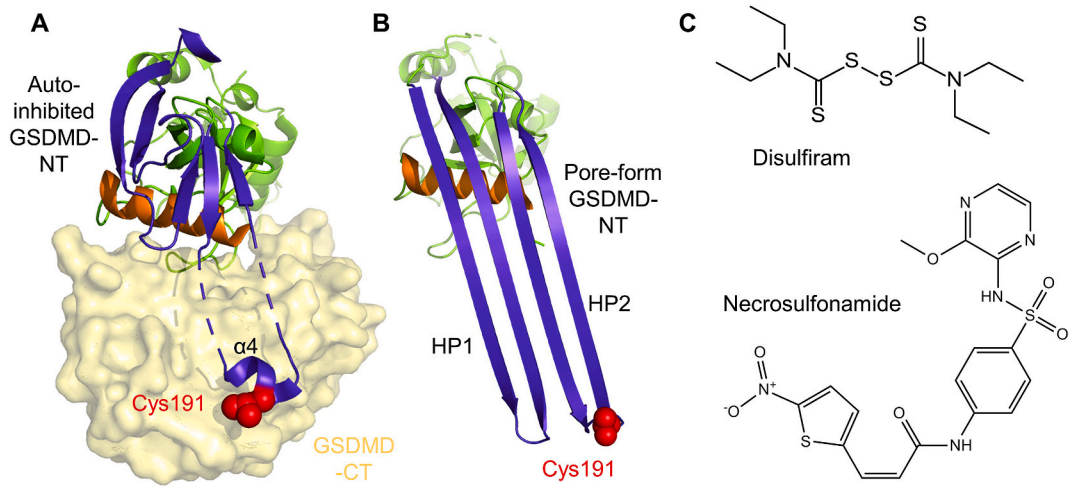

Fig. 4. Small-molecule inhibitors of GSDMD.
A) Cysteine 191 of GSDMD is located at the α4 helix in the auto-inhibited state. C191 is highlighted in red.
B) In the pore structure, C191 is located at the tip of HP2. Since HP2 is part of the transmembrane region, the inhibitors could potentially function by disrupting membrane insertion by GSDMD.
C) Chemical structures of small-molecule drugs DSF and NSA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

executioner molecule in necroptosis (Wang et al., 2014), whereas that of DSF was from a systematic liposome-based biochemical screen (Xia et al., 2019b). Both small molecules function by covalently modifying a reactive cysteine, more specifically Cys191 in human GSDMD-NT (Cys192 in mouse). According to models of auto-inhibited and membrane-inserted GSDMD-NT built based on the corresponding GSDMA3 structures, Cys191 is located at the α4 helix and at the tip of HP2 prior to and after pore formation, respectively (Fig. 4A–B). Therefore, DSF and NSA likely reduce the activity of GSDMD by preventing membrane insertion.

It was also reported that punicalagin (PUN), a phenolic compound commonly found in pomegranates, prevented membrane permeabilization and IL-1β release but not its processing upon canonical inflammasome activation (Martin-Sanchez et al., 2016). Although these observations are consistent with the speculation that PUN is a GSDMD inhibitor, further evidence is required to the direct interaction between PUN and GSDMD and to rule out the possibility that PUN functions by stabilizing the membrane. Another compound LDC7559 was reported as inhibitory to elastase-dependent and GSDMD-mediated NETosis in human neutrophils, also with unknown mechanism of action (Sollberger et al., 2018).

In light of recent discoveries that GSDME is tumor-suppressive, it may be an interesting future direction to identify small-molecule GSDM activators that may directly induce cancer cell death. In fact, despite their currently unclear roles in tumors, GSDMA, GSDMC, and GSDMD are frequently silenced in gastric cancer cells (Saeki et al., 2000b, 2007, 2009), suggesting that GSDME may not be the only tumor suppressor in the GSDM family. Other than small molecules, gene therapy methods may be employed to overexpress the pore-forming fragments of GSDMs to directly kill cancer cells.

6. Conclusions and future prospects

A rich body of research has begun over the past few years on the pyroptotic role of GSDMD, the first functionally elucidated member of the GSDM family. Adding to these insights are discoveries of the lysis-independent, non-pyroptotic functions of GSDMD, other regulators of GSDMD activation, and mechanisms for downregulating GSDMD developed by both host cells and pathogens. Clearly, there is still a long way ahead to elucidate the biological functions of the GSDM family, which extend far beyond inflammatory cell death.

Underlying the multiple functions of the GSDM family are many intriguing questions that remain to be answered. With advances in biological and structural knowledge, researchers have started to look at how GSDM activity explains pathological features, and how GSDMs may be utilized for therapeutic intervention. A bigger picture has emerged where several modes of cell deaths modulate one another and multiple GSDMs co-mediate intercellular and systems-level response. Further research will be conducted toward a goal to disentangle the complex signaling network of the GSDM family.

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Declaration of competing interest

The author confirms that there are no conflicts of interest.

Abbreviations

ALR	AIM2-like protein
ASC	Apoptosis-associated, speck-like protein containing a caspase recruitment domain
BMDM	Bone marrow-derived macrophage
CAR	Chimeric antigen receptor
Cryo-EM	Cryo-electron microscopy
CL	Cardiolipin
CRS	Cytokine release syndrome
DAMP	Damage-associated molecular pattern
DIC	Disseminated intravascular coagulation
DSF	Disulfiram
ED	Extension domain
ELANE	Neutrophil elastase
EV	Enterovirus
ESCRT	Endosomal sorting complex required for transport
GSDMA-F	Gasdermin A-F
GSDM-NT	Gasdermin N-terminal domain
GSDM-CT	Gasdermin C-terminal domain
HP	Hairpin
IL-1β/18	Interleukin-1β/18

LDH	Lactate dehyrogenase
LPS	Lipopolysaccharide.
NLR	Nucleotide-binding domain and leucine-rich repeat-containing protein
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NLR family PYD domain-containing protein 3
NSA	Necrosulfonamide
PAMP	Pathogen-associated molecular pattern
PIP	Phosphatidylinositol
PS	Phosphatidylserine
PUN	Punicalagin
TAK1	TGFβ-activated kinase 1
TM	Transmembrane

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mam.2020.100890.

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