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Gasdermin D activity in inflammation and host defense

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Abstract

The mechanisms underlying the release of interleukin-1 (IL-1) family cytokines from phagocytes have been the subject of intense investigations for more than 30 years. The absence of an N-terminal secretion signal from members of this family suggests a previously unknown mechanism of protein secretion that transfers cytosolic IL-1 directly across the plasma membrane into the extracellular space. The pore-forming protein gasdermin D (GSDMD) has emerged as the conduit for IL-1 secretion from the cytosol, serving to induce the release of IL-1 from living (hyperactive) or dead (pyroptotic) cells. In this Review, we discuss the mechanism by which GSDMD pore formation is regulated by the activity of inflammatory caspases, which are commonly associated with inflammasomes. We discuss how GSDMD promotes IL-1 release from hyperactive or pyroptotic cells, with a specific focus on defining how these distinct cell fates associated with GSDMD activity can be regulated. Last, the physiological consequences of GSDMD activity and therapeutic potential of targeting this pore-forming protein are discussed, which highlight the abundance of questions that remain to be answered by the community.

One sentence summary

This review summarizes our current understanding of the functions of Gasdermin D.

Introduction

The process by which mammalian cells express and secrete cytokines has long been the subject of investigation. Most cytokines are not present in resting cells and are encoded by genes that are inducible by inflammatory transcription factors, such as AP-1, nuclear factor–κB, and interferon regulatory factors (IRFs) (1). Consequently, it is generally accepted that various environmental stimuli induce intracellular signaling pathways that stimulate cytokine synthesis. Upon synthesis, most cytokines are translocated into the endoplasmic

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reticulum (ER), where transport through the biosynthetic pathway begins. Ultimately, these factors are released (secreted) into the extracellular space, where they bind cognate receptors and promote inflammatory and immunoregulatory activities. This general model applies to cytokines that contain N-terminal secretion sequences that mediate their cotranslational insertion into the ER.

One group of cytokines does not follow the path outlined above, members of which comprise some of the first cytokines identified: the interleukin-1 (IL-1) family (2). There are nine members of the IL-1 family, with the best characterized being IL-1α, IL-1β, and IL-18. These proteins do not contain N-terminal secretion signal sequences and are therefore not released into the extracellular space through the conventional secretory pathway. Rather, these proteins are secreted by a mechanism that (in many instances) depends on a pore-forming protein called gasdermin D (GSDMD). In this Review, we discuss the recent work on the biology of GSDMD and other GSDM family members, which all share the common ability to form pores within lipid bilayers (*3–5*). In the case of GSDMD, the pores formed operate as size-restricted conduits for the transport of IL-1 and other small molecules from the cytosolic space into the extracellular environment (6). If these GSDMD pores are formed in high abundance at the plasma membrane, a lytic form of cell death called pyroptosis occurs, which results in the release of numerous intracellular contents (including IL-1) (*3–5*). Pyroptosis should release intracellular (cytosolic) proteins regardless of whether they are small enough to pass through the pores formed by GSDMD. If GSDMD pores are formed in low abundance, plasma membrane pores form within living cells that serve as channels for IL-1 secretion (6). Thus, depending on context, GSDMD mediates the release of IL-1 from living or dead cells. In this Review, we describe recent cell biological, immunological, and structural analyses that have provided an increasingly clear view of the mechanisms and consequences of GSDMD activities in the immune system.

Genetic analysis of GSDMD and other GSDM family members

In 2015, three laboratories reported the identification of GSDMD as a regulator of IL-1 release from macrophages (*7–9*). All three studies focused their efforts on understanding inflammasome biology. Inflammasomes are members of a family of supramolecular organizing centers (SMOCs), which are receptor-proximal or cytosolic oligomeric protein complexes that operate as the principal subcellular sites of innate immune signal transduction (10). In this regard, SMOCs are considered the signaling organelles of the innate immune system. Of the known SMOCs, inflammasomes are unusual in their ability to drive immediate (transcription- independent) inflammatory responses because these organizing centers serve as the site of signals that induce inflammatory caspase activation, most notably caspase-1. Active caspase-1 can then cleave its cytosolic substrates, which include GSDMD and members of the IL-1 family. For the purpose of this Review, an extensive discussion of inflammasome biology is not required. We refer the reader to several recent reviews on this topic (*11–14*). However, some discussion of inflammasomes is necessary to best understand the regulation of GSDMD.

Like all SMOCs, inflammasomes are protein complexes that have two major components. One component is a “scaffold” protein with oligomerizing potential (classically referred to

as an adaptor) and a second component consisting of effector enzymes. Upstream of these proteins is a factor we refer to as a "seed." Interactions between the adaptor and the seed converts adaptor oligomerizing potential into activity, creating the scaffold for the recruitment and activation of effector enzymes within the inflammasome. The seed proteins are found in substoichiometric amounts within inflammasomes because a single seed can promote oligomerization of many adaptors, resulting in effector enzyme recruitment (*15, 16*). Numerous environmental stresses activate inflammasome seed proteins, such as microbial infections or cellular or subcellular injury. As such, proteins that seed inflammasome assembly include receptors that detect microbial products or other indicators of cellular dysfunction (11). The first studies that identified GSDMD as a regulator of inflammasome activities focused on cellular responses induced by cytosolic bacterial lipopolysaccharide (LPS) (*8, 9*), which binds directly to the mouse receptor caspase-11 (or human caspase-4 and −5) (17). After binding LPS, caspase-11 is activated to cleave GSDMD to form plasma membrane pores (3, 4). Disruption of the cell membrane then activates the protein NLRP3, which senses membrane disruption and serves as the seed to oligomerize the adaptor ASC. In this context, oligomerized ASC serves as the scaffold to recruit the downstream effector enzyme caspase-1 (18). This NLRP3-ASC– caspase-1 complex represents an inflammasome, which serves as the subcellular site of caspase-1 activation. Other inflammasomes activate caspase-1, with the best-defined being those seeded by the proteins NLRP1, AIM2, Pyrin, and several members of the NAIP family of NLRs (19). Different mechanisms drive activation of these receptors, but their activities culminate in a common outcome: the seeding of the oligomerizing unit that serves as a scaffold for caspase-1 recruitment, dimerization, and activation.

Caspase-1 has long been recognized as an enzyme that cleaves pro–IL-1β and other IL- 1 family members in the cytosol of mammalian cells and causes the release of IL-1 from cells. But the mechanism responsible for IL-1 release was perplexing until the discovery of GSDMD. Two independent forward genetic screens identified *Gsdmd* as a gene required for IL-1 release or pyroptosis in response to cytosolic LPS (8, 9). Shortly after that work was published, a third study identified GSDMD as a protein activated by the NLRP3 inflammasome that was necessary for IL-1 release and pyroptosis (7). Since these initial discoveries, GSDMD research has diverged into two areas: genetic analysis and mechanistic analysis. In the following sections, we will explore both of these areas of GSDMD biology.

Mechanisms of GSDMD activity

The human genome encodes six GSDM family members, whereas mice encode 10 members (9). Within these families, each member is ~45% homologous at the amino acid level. The best characterized and the subject of this Review is GSDMD, whose expression is positively regulated by the transcription factor interferon regulatory factor–2 (IRF2) (20). GSDMD is a 480–amino acid protein that contains two defined domains separated by a linker region. When inflammasomes are assembled, the GSDMD linker region is cleaved by caspase-1, which releases the N terminus from the C terminus (8, 9). Other enzymes can also cleave GSDMD, including mouse caspase-11, caspase-8, and human caspases-4 and −5 (Fig. 1) (8, 9, 21, 22). As will be described later, other enzymes can also cleave GSDMD. The cleaved N terminus is capable of auto-oligomerization on membranes when encountering

phosphoinositides or other acidic lipids, resulting in the formation of a large circular pore (*3, 4*). Interactions with these lipids are thought to promote the assembly and insertion of the pore into membranes, which creates a conduit through which small molecules can traverse a lipid bilayer. Of the lipids bound to the GSDMD N terminus, several are found at the inner leaflet of the plasma membrane, including phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] and phosphatidylserine (PS) (3, 4). None are found on the outer leaflet of the plasma membrane of live cells. Cardiolipin is also bound by the GSDMD N terminus; this lipid is located exclusively on mitochondrial and bacterial membranes. On the basis of these observations, a model emerged in which in resting cells, GSDMD is auto-inhibited and resident in the cytosol. Within cells that contain active inflammatory caspases, GSDMD is cleaved within the linker region to create a noncovalent complex of N terminus and the auto-inhibitory C terminus. Interactions between the N terminus and acidic phospholipids may then release the C terminus, promote N terminus oligomerization, and facilitate membrane insertion, resulting in a functional pore.

Evidence supporting this model is ample because caspase-dependent cleavage of GSDMD correlates with pore-forming activity within cells that contain active inflammatory caspases (8, 9). Moreover, cell-free systems demonstrated that full-length GSDMD can be cleaved by recombinant caspase-11 and that this cleavage event leads to the insertion of GSDMD pores into liposomes that contain acidic phospholipids (3, 4). Functionally, genetic ablation of inflammasome components renders macrophages unable to activate caspase-1 and therefore unable to cleave GSDMD (8, 9). Similarly, under conditions in which caspase-11 is the primary inflammatory caspase activated, genetic ablation of caspase-11 abolishes the cleavage of GSDMD (8, 9). Under these conditions, no plasma membrane pores are formed. Further support for the cleaved GSDMD pore model comes from an experiment in which the caspase-1 cleavage site within GSDMD was converted into a caspase-3 cleavage site (9). When this engineered GSDMD variant was expressed in cells, it was cleaved by caspase-3 in response to tumor necrosis factor receptor signaling, resulting in pore formation at the plasma membrane. Thus, cleavage of GSDMD is necessary and sufficient to stimulate the formation of plasma membrane pores that are classically associated with inflammasome activity. GSDMD can naturally be cleaved by caspase-3 within its N-terminal domain, an event that is thought to ablate its ability to form pores (23). In addition, the GSDMD homolog GSDME can be cleaved by caspase-3 to trigger pore formation (24). This process converts slow, noninflammatory apoptotic death to inflammatory pyroptosis (24, 25).

Structural perspectives of lipid binding, oligomerization, and pore formation

The auto-inhibited conformation of a full-length GSDM was revealed by the crystal structure of mouse GSDMDA3 (3), in which the C-terminal domain interacts closely with the N- terminal domain to suppress the toxic function of the N-terminal domain (Fig. 2A). When mapped to homologous structure models, many human genetic mutations in GSDM family members were shown to disrupt the auto-inhibited conformation, resulting in a gain of function (3). Recently, a crystal structure of human GSDMD was reported, confirming the overall structural architecture of GSDM proteins (26).

To reveal the structure of the pore form of a GSDM, the cleaved N-terminal domain of mouse GSDMA3 was reconstituted to form pores in cardiolipin-containing liposomes, which were subsequently solubilized in detergent and subjected to cryo–electron microscopy (cryo-EM) structure determination (27). Using data collected on a Titan Krios microscope and a K2 direct electron detector, the three-dimensional structure of GSDMA3 pore with 27-fold symmetry was reconstructed to 3.8-Å resolution (Fig. 2B). GSDMA3 pores with 26- and 28-fold symmetry were also observed, but the main population of the reconstituted pores had 27-fold symmetry. The size of the pore is large, ~180 Å in the inner diameter. This diameter should permit the release of IL-1 family cytokines and other small proteins but not the enzymatically active tetramer of lactate dehydrogenase (LDH), which is often used for detection of cell death. Therefore, IL-1 secretion may be separated from cell death, with the former involving pore formation on the cytoplasmic membrane and the latter also requiring bursting of the cytoplasmic membrane after pore formation (6).

The subunit structure of the GSDMA3 N-terminal domain in the pore form resembles the shape of a left hand, with a globular palm domain, a positively charged thumb helix (α1), and four membrane-inserted fingers from two pairs of β-hairpins (Fig. 2C). The active conformation of the N-terminal domain displays prominent conformational changes in comparison with the auto-inhibited conformation, especially at the β-hairpin region (Fig. 2, D and E). By contrast, the palm and the thumb regions of the structures mainly retain their conformations during membrane insertion. The cryo-EM density revealed the bound cardiolipin head group, which is negatively charged and situated adjacent to the positively charged α1 thumb helix. In a full-length GSDM structure, this helix is buried at the interface with the C-terminal domain and not exposed to acidic lipids, explaining at least part of the mechanism of auto-inhibition. The interface for subunit interaction and oligomerization involves the palm region, the thumb helix, and the inserted β-hairpins.

Consequences of GSDMD activity within cells

The consequences of GSDMD activity were originally suggested to be terminal, resulting in pyroptosis or the lysis of bacterial cells containing exposed cardiolipin (8, 9). In mammalian cells, this conclusion was derived from studies that used stimuli that represent a variety of threats to the host. These threats can be of microbial or host origin. Well-characterized threats that stimulate GSDMD cleavage activate proteins that seed the assembly of inflammasomes. These stimuli include (i) ionic imbalances or organelle disruption (which activate NLRP3), (ii) bacterial proteases that induce the functional degradation of NLRP1, (iii) cytosolic double-stranded DNA (which stimulates AIM2 in mice and NLRP3 in humans), and bacterial toxins that alter Rho guanosine triphosphatase activity (which stimulate Pyrin) (*11–13*). Each of these threats leads to the assembly of an inflammasome and the consequential caspase-1–dependent pore formation. The cleavage of GSDMD results in pore formation at the cell surface and the death of the cell by means of pyroptosis (*4, 5, 8, 9*). For this reason, pyroptosis can be defined as GSDM-mediated lytic cell death (28). Because these stimuli were known to promote pyroptosis, the genetic identification of GSDMD as a regulator of pyroptosis led to the belief that pore formation led to the immediate lysis of the cell. However, subsequent work demonstrated that GSDMD pore

formation does not necessarily lead to death (6, 29). Cell death after GSDMD pore formation is regulated and can be delayed or even avoided.

Evidence that suggests that pore-forming activity after inflammasome activation may not immediately kill cells derived from work that predated the discovery of the GSDMD pore. These studies were focused on defining how cytosolic LPS can promote pyroptosis and IL-1 release (18). Experimentally, there are several means by which LPS is delivered into the cytosol, with the simplest models being those that transfect or electroporate cells with this bacterial product (30, 31). Exposure to living gram-negative bacteria or bacteria-derived outer-membrane vesicles also delivers LPS to the cytosol (32, 33). Additional strategies of LPS delivery to the cytosol include infection with virulent gram-negative bacteria that encode type III or type IV secretion systems (30). All of these approaches are now recognized to activate the mouse LPS receptor caspase-11 (or caspase-4 and −5 in humans), resulting in GSDMD cleavage, pore formation, and pyroptosis (34). However, this minimal pathway of LPS–caspase-11–GSDMD does not involve caspase-1. This point is critical because caspase-11 cannot cleave pro–IL-1β (35). In fact, caspase-11 has no other known substrates besides GSDMD. Caspase-1, by contrast, can cleave GSDMD (8, 9) and pro–IL-1β (36, 37). Moreover, pro-IL-1β is cleaved by caspase-1 within cells transfected with LPS (30, 31), suggesting a mechanism to somehow activate caspase-1 after LPS detection by caspase-11. Caspase-11–dependent pores cause an efflux of K^+ from the cell (18), an ionic imbalance that promotes the assembly of an NLRP3 inflammasome that activates caspase-1 (38). These conditions place GSDMD at two critical places: at the end of a short LPS-triggered pathway (LPS sensing activates caspase-11, −4, and −5 to cleave GSDMD) that directly causes membrane permeabilization and subsequent NLRP3 inflammasome assembly. The NLRP3 inflammasome stimulates caspase-1 to unleash a second wave of GSDMD activity and the sole wave of pro-IL1 processing. It is likely that NLRP3 activation occurs secondarily as a consequence of caspase-11–dependent K^+ efflux when the membrane is damaged by GSDMD pores. The fact that wild-type cells release active IL-1 after detection of cytosolic LPS suggests that these cells must survive for at least a short time after pore formation in order to stimulate NLRP3. Thus, GSDMD pore formation does not instantaneously kill all cells.

In response to certain stimuli, phagocytes can tolerate the presence of active inflammasomes and release IL-1 while maintaining viability for extended periods of time (*39–41*). The ability to add IL-1 to the repertoire of cytokines secreted renders these cells immunologically “hyperactive” (41). The term hyperactive is used to distinguish this activation state from that of traditionally activated macrophages (such as those stimulated with extracellular LPS), which cannot release IL-1. This distinction is physiologically important because immunizations that hyperactivate dendritic cells (DCs) stimulate a more robust T helper cell type I (T_H1) adaptive immune response than immunizations that merely activate DCs (41). Cells that die from pyroptosis after inflammasome activation are poor stimulators of $T_H1$immunity but induce strong local inflammatory responses (Fig. 3) (42). Recent work has demonstrated that GSDMD pores serve as conduits for the release of IL-1 from living (hyperactive) cells (6). This finding raises the question of how cells can tolerate the presence of GSDMD pores while maintaining viability.

When the plasma membrane is damaged by mechanical disruption or formation of large non–ion-selective pores, the concentration of ions that normally differs between the cytosol and extracellular fluids rapidly equalizes; not only is K^+ released, which activates the NLRP3 inflammasome, but there is also an influx of Ca^{++} and Na^+. All cells have the capacity to trigger a rapid mechanism to repair plasma membrane damage; this repair process is initiated when intracellular Ca^{++} levels rise above ~100 μM (43). This response has been called the “cellular wound-healing response” because it is activated by mechanical trauma to the cell membrane. However, it is also activated in response to bacterial pore-forming toxins and immune pore- forming proteins, including the killer lymphocyte cytotoxic granule protein, perforin, the pore- forming protein that delivers the granzymes into target cells to induce noninflammatory programmed cell death, and MLKL, the pore-forming protein responsible for necroptosis (*44–46*). Perforin forms a non–ion-selective β-barrel shaped pore of similar size and structure as GSDMD (~160-Å inner diameter) (47). The membrane repair response involves three processes: (i) Intracellular vesicles, including endosomes, lysosomes, and multivesicular bodies, are mobilized to the damaged membrane to donate their membranes to patch and reseal the damaged membrane; (ii) accelerated endocytosis pinches off and internalizes the damaged membrane; and (iii) the damaged membrane is removed into extracellular blebs or vesicles (43, 44, 48). Many of the proteins that target to membranes contain Ca^{++}-sensing C2 domains, which regulate their trafficking to membranes. Consequently, cellular membrane repair can be inhibited by incubating cells with a cell-permeable Ca^{++}-chelator. Rapid cell-membrane repair also depends on the endosomal sorting complex required for transport (ESCRT), particularly ESCRT-III, which is recruited to the damaged membrane (49). Knockdown of its components, such as CHMP4B and Vps4, can also be used to investigate whether membrane repair occurs.

Membrane repair plays a critical role in regulating cell death caused by immune pore-forming proteins. In killer cell cytotoxic granule-mediated cytotoxicity, repair of perforin plasma membrane damage is critical for preventing lytic cell death upon perforin-mediated delivery of the granzymes into cells, which is intended to activate noninflammatory death, so that only the targeted infected or cancerous cell is harmed without damaging bystander cells (*45, 50*). In necroptosis, activation of MLKL can be slowed down or averted by shedding damaged membrane bubbles, which can give the damaged cell time to function (present antigen and secrete chemokines) and even survive (46). Recently, Ca^{++} and ESCRT-III–dependent cell membrane repair was shown to be mobilized in response to GSDMD pores to reduce pyroptotic cell death by bone marrow–derived macrophages that were activated by *Salmonella enterica* serovar typhimurium infection or LPS transfection (51). Further work is needed to identify under what circumstances membrane repair is able to prevent GSDMD-mediated cell death and how these events influence the release of IL-1β and other inflammatory mediators. In some cells, the levels of GSDMD or the levels or activation of the inflammatory caspases that cleave it may be low enough that membrane repair overcomes membrane damage, whereas in other cells, membrane damage is too severe to be repaired (Fig. 3). So far, we do not know of cellular mechanisms that regulate how cleaved GSDMD forms pores, but phosphorylation of GSDME is a mechanism that influences pore-forming activity (52). Discovery of processes that regulate pore formation will undoubtedly shed light on the situations in which pyroptosis is triggered, but cell death is averted through

membrane repair and other types of regulation. A recent study implicated the Toll–IL-1R protein SARM in macrophages in regulating how much pyroptosis versus IL-1 release occurs after NLRP3 inflammasome activation of macrophages (53). SARM deficiency increased IL-1β production and release but reduced pyroptosis, and increasing SARM had the opposite effect. SARM suppressed IL-1β by directly restraining the NLRP3 inflammasome and, hence, caspase-1 activation. It is likely that membrane repair is also triggered when other gasdermins are activated and regulates whether cells survive, although this has not been shown. Activation of membrane repair is likely the most rapid and immediate consequence of GSDMD pore formation at the cell surface.

Although studies of pyroptosis have focused on plasma membrane damage by activated GSDMD, GSDMD (and GSDME and possibly other gasdermins) bind avidly to cardiolipin that is on mitochondrial membranes (*3, 4, 25*). Although most cardiolipin is on the inner mitochondrial membrane, which is not accessible to cytosolic GSDMD, cardiolipin also shuttles to the outer membrane, where it could serve as a docking site for pore formation. Moreover, other acidic phospholipids that N-terminal GSDMD bind in the inner leaflet of the plasma membrane are also contained on the outer leaflet of endosomes, phagosomes, and lysosomes (54); these membranes could potentially be damaged during pyroptosis. Recent studies suggest that activated gasdermins induce mitochondrial damage, which amplifies pyroptosis. Single-cell imaging of macrophages undergoing pyroptosis showed that activated GSDMD causes loss of mitochondrial transmembrane potential and lysosomal disruption before the plasma membrane is permeabilized (55). This study also showed that cells undergoing pyroptosis release into their culture supernatants proteins normally found within mitochondria (cytochrome c), lysosomes (cathepsin B), and nuclei (HMGB1), suggesting that organelle membranes are also damaged. Mitochondrial reactive oxygen species (ROS) has been shown to be induced by Shiga toxin 2 plus LPS in a caspase-4– and GSDMD-dependent, but NLRP3-independent, manner in THP-1 cells (56). Moreover, in this system, ROS scavengers suppress IL-1 release and pyroptosis. SARM, which increases pyroptosis, localizes to mitochondria and, after LPS priming and nigericin treatment of macrophages, clusters on mitochondria (53). Treatment of macrophages with peptidoglycan, which causes IL-1 release without pyroptosis (“hyperactivation”) (6, 57), did not cause SARM clustering or much mitochondrial depolarization (53). How SARM does this and whether SARM plays a role in other inflammasome-mediated pathways is unclear. Taken together, these studies suggest that activated GSDMD might bind rapidly to mitochondrial membranes to damage them and cause mitochondrial ROS and loss of transmembrane potential that promote the commitment of cells to pyroptosis. However, further studies are needed to understand how mitochondria are damaged, how mitochondrial damage is regulated, and how it drives pyroptosis. In a recent study, an ectopically expressed N-terminal GSDME fusion protein also appeared to localize to mitochondria and cause mitochondrial release of mitochondrial intermembrane space proteins (cytochrome c and HtrA2) (52), suggesting that other gasdermins also trigger mitochondrial damage and possibly activate apoptotic caspases.

The scenarios described above indicate that pore formation at the cell surface may not be sufficient for cells to commit to pyroptosis. In some settings, mitochondrial disruption may be needed for pyroptosis to occur, and/or membrane damage may be repaired to allow the cell to survive. Cells that lack mitochondrial damage, with low levels of GSDMD pores or

with exuberant membrane repair, may avoid pyroptosis but rather use plasma membrane–localized GSDMD pores as conduits for the secretion of IL-1 family cytokines while maintaining viability (Fig. 3).

An increasing diversity of contexts have been identified in which living cells release IL-1. In most instances in which the function of GSDMD has been examined, this pore-forming protein is necessary for the rapid release of IL-1 (6, 29). However, even in cells where GSDMD is necessary for the rapid release of IL-1, a delayed release of this cytokine can occur in the absence of GSDMD (58). GSDMD is therefore considered a regulator of cell hyperactivation as well as pyroptosis. Evidence supporting this claim derives from studies that have demonstrated the correlation between GSDMD pore formation in cellular or liposomal membranes and the release of IL-1 across these same lipid bilayers. This correlation has been observed with several stimuli that hyperactivate macrophages, including the oxidized phospholipids PGPC or POVPC, bacterial peptidoglycan or its N-Acetylglucosamine (NAG) fragment, and mutant *Staphylococcus aureus* that contain easily degradable peptidoglycans (6, 29). There is no evidence to support the idea that IL-1 is specifically selected as cargo for release by means of GSDMD pores. The release of IL-1 may simply be because (i) its small size (diameter 4.5 nm), which would increase its diffusion rate and ability to translocate through the GSDMD pore; (ii) its abundance in cells after TLR activation; and (iii) its proximity to the pore itself. On this latter point, a recent study demonstrated that cleaved IL-1 localizes to the plasma membrane (58), perhaps through interaction with lipids, poising this cytokine for rapid secretion upon pore formation. The full spectrum of factors released by GSDMD pores from living cells in unclear, but K^+ ions represent factors of interest. Immediately after pore formation occurs, K^+ efflux via GSDMD can occur, which (as described above) can drive NLRP3 inflammasome assembly. In addition, the efflux of K^+ inactivates the interferon-inducing DNA sensor cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS) (59).

Within neutrophils, GSDMD appears to have additional activities. Like DCs and macrophages, neutrophils can be induced to release IL-1 while maintaining viability (39). This resistance to cell death is likely linked to cell type–specific differences in the kinetics and robustness of caspase-1 activity (60). When using stimuli that promote pyroptosis in macrophages, robust caspase-1 activity is observed within minutes of inflammasome assembly. Side-by-side stimulations of neutrophils revealed that caspase-1 activity occurs less robustly and with slower kinetics (60). Consequently, the rates of GSDMD pore formation differs between these cell types, which may result in macrophages experiencing an abundance of GSDMD pores that overwhelm the repair machinery and result in pyroptosis. Neutrophils, by contrast, may be able to repair the smaller pool of GSDMD pores before lysis occurs, allowing these cells to achieve a hyperactive state in which IL-1 is released from viable cells.

In aged neutrophils, caspase-1 is not responsible for GSDMD cleavage. Rather, neutrophil elastase (ELANE) and cathepsin G are reportedly capable of GSDMD cleavage (61, 62). These two studies differ in their conclusion on the ability of ELANE to cleave GSDMD, suggesting that further work is necessary to clarify the role these enzymes in GSDMD

activities in neutrophils. Neutrophils produce neutrophil extracellular traps (NETs), which are antibacterial webs of nuclear DNA that are released from these cells during various infections (63). GSDMD pores appear to play a critical role in NET formation and release because GSDMD appears to be necessary for nuclear envelope disruption and genomic release into the cytosol and for plasma membrane disruption that releases DNA into the extracellular environment (64, 65). The necessity of GSDMD for disruption of the nuclear envelope provides additional evidence that the plasma membrane is not the only site of pore formation.

Consequences of GSDMD activity within mice

Although much of our mechanistic insight into GSDMD activities has been obtained from in vitro studies, several functions of GSDMD in mice have been identified. GSDMD-deficient mice are more susceptible than their wild-type counterparts to a variety of infections, such as *Burkholderia thailandensis* and *Francisella tularensis* subspecies *novicida* (66, 67).

GSDMD has also been implicated in the regulation of the inflammatory responses within mice that harbor active alleles of NLRP3 or Pyrin. These NLRP3 alleles are associated with neonatal-onset multisystem inflammatory disease (NOMID) and result in IL-1–dependent inflammation (68). GSDMD is necessary for the inflammatory responses in mice that harbor NOMID-associated NLRP3 mutants (69). Similar findings were made with mice that harbor active alleles of Pyrin, which also induce inflammasome-dependent IL-1–associated inflammation (70). In these mice, which contain Pyrin mutants associated with the human disease Familial Mediterranean Fever (FMF), GSDMD is necessary for inflammation-associated pathology. Whether the GSDMD-dependent IL-1 that is released from these patients (or mice) occurs from pyroptotic or hyperactive cells is unknown, but FMF mice do not have reduced numbers of inflammatory phagocytes (71).

GSDMD deficiency is not uniformly associated with increased susceptibility to infection because the lethality associated with mouse norovirus infections is delayed in the absence of this pore-forming protein (72). The reason for this delay is likely linked to a decrease in intestinal inflammation in GSDMD-deficient mice. In the absence of GSDMD-dependent inflammation, mice live longer and have fewer infection-associated symptoms (72). Studies of how the NAIP-NLRC4 inflammasome induces pyroptosis of intestinal epithelial cells have also implicated GSDMD in this process because GSDMD-deficient small intestinal epithelia are resistant to inflammasome-induced pyroptosis (73). *Gsdmd*$^{-/-}$ mice also control *Escherichia coli* better than wild-type mice (61). In this case, the paradoxical improvement in host defense was linked to more neutrophils at the site of infection and a longer neutrophil half-life because there was no ELANE-triggered pyroptosis.

Because of the increasing importance of GSDMD-dependent pore formation in inflammation, pharmacological inhibitors of this activity have begun to emerge. For example, necrosulpfonamide was identified as a molecule that bind directly to GSDMD and prevents pyroptosis (74). Consequently, this compound suppresses inflammatory responses associated with murine models of sepsis. Genetic GSDMD deficiency also protects mice from LPS-induced or polymicrobial sepsis-induced inflammation (75).

Perspectives

The discussions offered above were designed to offer a perspective on the mechanisms and consequences of GSDMD activity. Although the inflammatory functions for GSDMD were necrosulfonamide was identified as a molecule that binds directly to GSDMD and prevents pyroptosis (74). Consequently, this compound suppresses inflammatory responses associated with mouse models of sepsis. Genetic GSDMD deficiency also protects mice from LPS-induced or polymicrobial sepsis–induced inflammation (75).

Only recently defined, research into this protein has proceeded at a rapid pace. Today, we have some understanding of how GSDMD pores are regulated and the importance of this regulation for a variety of physiological responses. However, large gaps remain in our understanding of GSDMD and the pathways that regulate this protein. For example, why some cells and stimuli use GSDMD pores to promote pyroptosis or cell hyperactivation is still not completely understood, yet the physiological distinction between these two cell fates (life verses death) is huge. The upstream regulators of inflammasome activity that influence GSDMD cleavage are only beginning to be defined, as well as the cell types that naturally use GSDMD activities. The spectrum of factors that can be secreted by means of GSDMD pores also remains undefined.

When the discussion shifts to the in vivo consequences and therapeutic potential of GSDMD activity and manipulations, even more questions arise. What is the fate of GSDMD pores once assembled in the plasma membrane? The N-terminal domain is released into culture supernatants in vitro and is active (4), but is it released in membrane bound vesicles and active in vivo, and is it degraded in the extracellular space or in lysosomes? Does the ability of GSDMD to add IL-1 to the repertoire of factors secreted by DCs render these hyperactive cells more potent stimulators of protective immunity? oxPAPC has been shown to synergize with LPS to promote stronger antigen-specific T cell responses in mice than does LPS alone, but the role of GSDMD in these activities is undefined. Last, the emerging focus on the development of GSDMD inhibitors raises the question of whether these therapeutics will provide different benefits than current those of strategies that block IL-1. We envision a bright future for GSDMD research, which will be focused on answering the questions outlined above and raising new ones.

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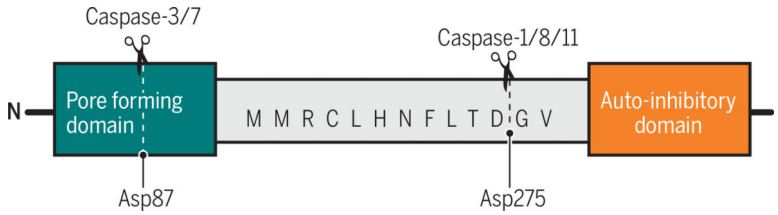

Fig. 1. Activating and inhibitory cleavage sites within GSDMD.

Schematic of the domain structure of human GSDMD. The N-terminal pore-forming and C-terminal auto-inhibitory domains are indicated, along with an intervening linker region. The arrows above the schematic indicate the enzyme that cleaves GSDMD, and the arrows below the schematic indicate the amino acid that is targeted by each enzyme. The cleavage events mediated by caspase-3 and −7 are inactivating, which prevent pore formation. The cleavage mediated by ELANE (neutrophil elastase) and caspase-1, −8, and −11 promote pore-forming activity. Credit: A. Kitterman/*Science Immunology*

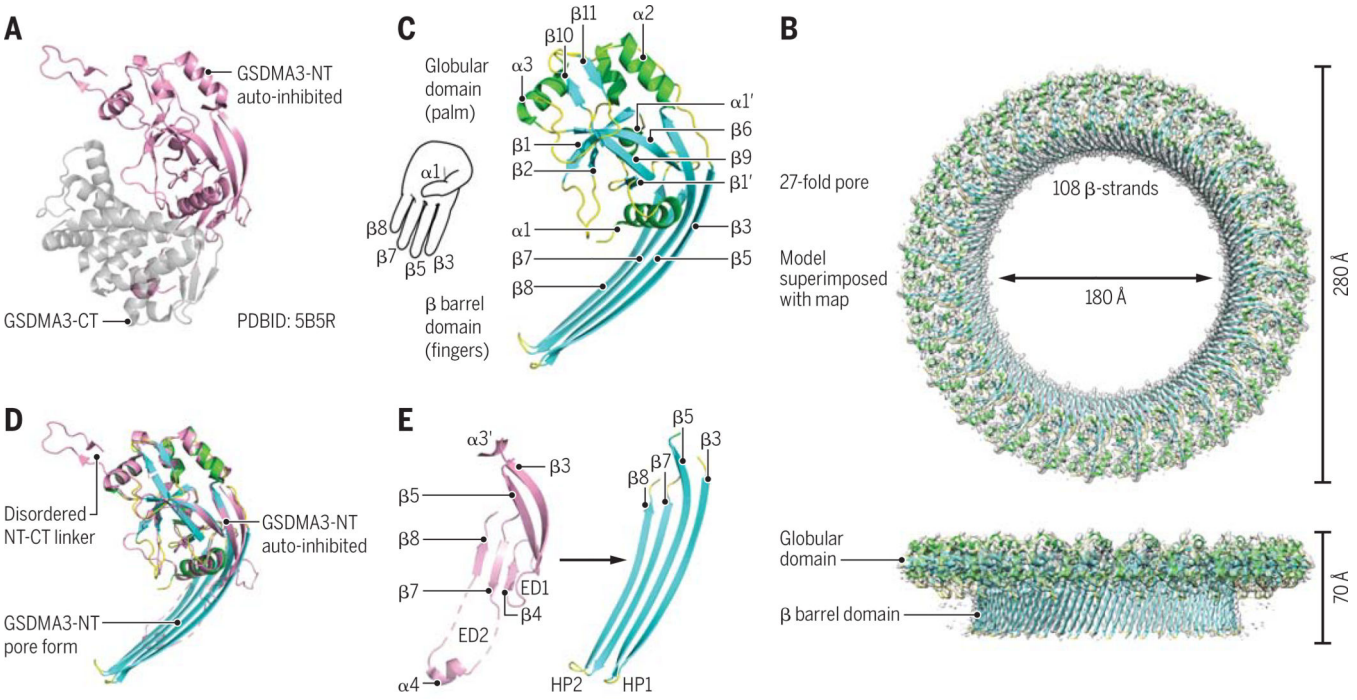

Fig. 2. GSDM structures and mechanisms.

(**A**) Crystal structure of auto-inhibited GSDMA3. (**B**) Cryo-EM map (gray) superimposed onto the atomic model of the 27-fold symmetric GSDMA3 N-terminal domain pore at 3.8-Å resolution. (**C**) Ribbon diagram of GSDMA3 N-terminal domain in the pore conformation. (**D**) Superposition of the auto-inhibited form and the pore form of GSDMA3 N-terminal domain. (**E**) Structural transitions that accompany the formation of the two β-hairpins HP1 and HP2 in the pore conformation. ED1 and ED2, extension domains 1 and 2, respectively. [Adapted from (27).] Credit: A. Kitterman/*Science Immunology*

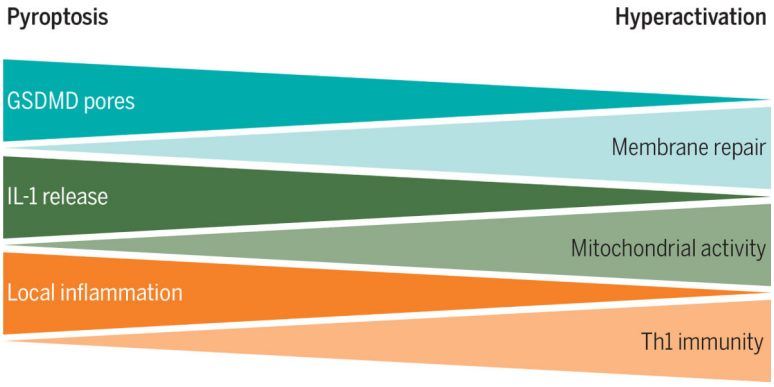

Fig. 3. A working model to explain the cellular and physiological consequences of inflammasome-dependent GSDMD activity

Within cells that contain inflammasomes, GSDMD is cleaved to form membrane pores. If these pores are formed in high abundance, membrane repair pathways are unable to repair them, and pyroptosis results. Pyroptotic cells release a large bolus of IL-1β at the time of lysis, which results in a strong local inflammatory response. However, the death of the responding cell limits its ability to participate in later immunological events, and consequently, pyroptosis-inducing stimuli are poor at inducing T cell–based adaptive immunity. When GSDMD pores are formed in low abundance, membrane repair pathways can remove them through exocytosis, endocytosis, or both. Some pores persist over time, resulting in the long-term release of IL-1β and the viability of the responding cell. The ability to survive GSDMD pores renders responding cells capable of participating in subsequent immunological events, which render these cells hyperactive, in terms of the ability to stimulate adaptive immunity. Credit: A. Kitterman/*Science Immunology*