Growing a gasdermin pore in membranes of pyroptotic cells

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Inflammasome-activated caspase-1, caspase-11, caspase-4, and caspase-5 cleave GSDMD to unleash its N-terminal gasdermin-N domain (GSDMD^{Nterm}) that perforates the plasma membrane to execute pyroptosis and stimulate inflammation. The mechanism underlying GSDMD^{Nterm} pore formation is unclear. Mulvihill *et al* use high-resolution atomic force microscopy (AFM) to analyze the dynamic pore formation process of GSDMD^{Nterm}. GSDMD^{Nterm} protomers are inserted into the lipid membrane to assemble arc- or slit-shaped oligomers that can incorporate additional protomers and grow into large and stable ring-shaped oligomers to form pores.

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See also: E Mulvihill *et al* (July 2018)

One of the most important immune responses to infection and danger signal is pyroptosis. Pyroptosis is a lytic form of programmed cell death that can destruct the pathogen-residing niche, release cytosolic contents, and induce strong inflammation. Historically, pyroptosis refers to caspase-1-mediated monocyte death. Caspase-1 is activated by the canonical inflammasome complex scaffolded by a pattern recognition receptor that detects a pathogen-associated molecular pattern or an endogenous danger signal. Murine caspase-11 and its human orthologs caspase-4 and caspase-5, upon activation by direct binding to bacterial lipopolysaccharide (LPS), can also induce pyroptosis. A recent breakthrough in pyroptosis and innate immunity researches is the identification of gasdermin-D (GSDMD) protein, the key executioner of

pyroptotic cell death (Kayagaki *et al*, 2015; Shi *et al*, 2015). GSDMD contains an N-terminal gasdermin-N and a C-terminal gasdermin-C domain linked by a loop. The gasdermin-N domain has a potent pore-forming activity that interacts with and is inhibited by the gasdermin-C domain. Activated caspase-1 and caspase-11/4/5 cleave the linking loop in GSDMD, thereby unlocking the autoinhibition on the gasdermin-N domain. The unleashed gasdermin-N domain translocates to plasma membrane through binding to membrane phosphoinositides to perforate the membrane, causing cell swelling and ultimately osmotic lysis (Aglietti *et al*, 2016; Ding *et al*, 2016; Liu *et al*, 2016; Sborgi *et al*, 2016). The GSDMD pore releases cellular contents including the inflammatory cytokines IL-1 β and IL-18 (Kayagaki *et al*, 2015; Shi *et al*, 2015), the other substrates of caspase-1.

GSDMD belongs to a gasdermin family that contains six members in human and ten in mouse (Shi *et al*, 2017). All the family members (except for DFNB59) share the two-domain architecture, and their gasdermin-N domains are capable of inducing pyroptosis (Shi *et al*, 2015; Ding *et al*, 2016). Natural GSDMA3 mutants losing the autoinhibition are constitutively pro-pyroptotic and cause alopecia with skin inflammation in mice (Shi *et al*, 2015; Ding *et al*, 2016). Two recent studies show that GSDME is cleaved and activated by caspase-3 and its expression confers pyroptotic cell death upon caspase-3 activation by TNF α or DNA damage (Rogers *et al*, 2017; Wang *et al*, 2017). The gasdermin-N domains of GSDME, GSDMA, and GSDMA3 are also capable of forming pores on liposomes or

monolayer membranes *in vitro* (Shi *et al*, 2015; Ding *et al*, 2016). These suggest a common pore-forming mechanism adopted by the gasdermin family to lyse the cell and execute pyroptosis, which redefines pyroptosis as gasdermin-mediated programmed necrotic cell death (Shi *et al*, 2017). However, the mechanism by which the gasdermin-N domain assembles pores to perforate the lipid membrane is unknown.

To answer this question, Mulvihill *et al* applied high-resolution and time-lapse atomic force microscopy (AFM) and directly imaged the pore-forming process of GSDMD gasdermin-N domain (GSDMD^{Nterm}) (Mulvihill *et al*, 2018). Under the force-distance curve-based AFM, GSDMD^{Nterm}, released from caspase-1 cleavage of full-length GSDMD, was observed to assemble arc-, slit-, and ring-shaped oligomers that could form transmembrane pores in muscovite mica-supported membranes made of artificially mixed lipids or *E. coli* polar lipid extracts (Fig 1). The slit- and ring-shaped oligomers protruded about 3.6 nm from the lipid surface, suggesting a nearly full insertion into the membrane. The ring-shaped oligomers have an average diameter of 22.6 nm, agreeing with the previous study (Ding *et al*, 2016). These observations were confirmed in liposome membranes by negative-stain transmission electron microscopy. Mulvihill *et al* also checked the effects of lipid composition on GSDMD^{Nterm} membrane binding and pore formation. Consistent with the previous studies (Aglietti *et al*, 2016; Ding *et al*, 2016; Liu *et al*, 2016; Sborgi *et al*, 2016), GSDMD^{Nterm} assembled arc-, slit-, and ring-like oligomers in the presence of phosphoinositide (PI(4,5)P2) but not

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Figure 1. Schematic of the GSDMD^{Nterm} pore formation process.

In pyroptosis, site-specific cleavage of GSDMD by activated caspase-1 or caspase-11/4/5 unlocks the autoinhibition on GSDMD^{Nterm}, which allows the GSDMD^{Nterm} to bind to membrane lipids (specifically phosphoinositides) and translocates to the membrane. The GSDMD^{Nterm} protomers are inserted into the lipid membrane and form arc- or slit-shaped transmembrane oligomers. The arc- and slit-shaped oligomers can incorporate additional protomers or oligomers, thereby growing into large and stable ring-shaped oligomers to assemble complete pores. Remnants GSDMD^{Nterm} oligomers are occasionally present in the pores, resulting from the fusion process, and eventually disappear in the final ring-shaped pores. Formation of the GSDMD^{Nterm} pores in the plasma membrane causes cell swelling and ultimately osmotic lysis to execute pyroptotic cell death.

phosphatidylinositol; PI(4,5)P₂ did not alter the size of the ring-shaped oligomers, but greatly facilitated membrane binding of GSDMD^{Nterm} with reduced occurrence of arc- and slit-like oligomers and much increased ring-like oligomers. Notably, cholesterol

failed to support GSDMD^{Nterm} oligomer formation and even inhibited GSDMD^{Nterm} binding to PI(4,5)P₂-containing membrane and oligomer formation. These findings substantiate that GSDMD can only trigger pyroptosis from the inside of mammalian

cells as the outer leaflet of plasma membrane contains cholesterol but not phosphoinositides (Ding *et al*, 2016).

GSDMD can also be cleaved by caspase-11/4/5. The cleavage occurs at the same site as that by caspase-1, generating the same GSDMD^{Nterm} fragment. Not surprisingly, Mulvihill *et al* found that caspase-4/5-cleaved GSDMD formed similar arc-, slit-, and ring-shaped oligomers and its lipid binding and pore formation properties resembled those of caspase-1-cleaved GSDMD. Mulvihill *et al* further snapshotted GSDMD^{Nterm} oligomer formation process by time-lapse AFM analyses. The arc-, slit-, and ring-shaped transmembrane GSDMD^{Nterm} oligomers appeared within 10 min after incubation of GSDMD with caspase-1. Over time, the arc- and slit-shaped oligomers grew from one or both ends with addition of more monomers or oligomers, and the two ends eventually fused with each other to generate a larger and stable ring-shaped transmembrane oligomer (Fig 1). The majority of ring-shaped oligomers assembled from the slit-like oligomers. These suggest that the arc- and slit-shaped GSDMD^{Nterm} oligomers represent intermediate states of transmembrane pore formation. The authors also found that the lipid composition did not evidently affect the dynamic fusion process during the ring-shaped oligomer formation. Previous studies of other pore-forming proteins suggest two modes of pore formation, pore growing directly in the membrane (without vertical collapse of pore-forming proteins) or a mechanism involving prepore-to-pore transitions. In the time-lapse AFM topographs, Mulvihill *et al* observed no considerable changes of the heights of GSDMD^{Nterm} oligomers during formation of the ring-shaped oligomers. The absence of a vertical collapse event favors the notion that GSDMD^{Nterm} pore formation is initiated in lipid membranes and features a continuous growing process without prepore-to-pore transitions. Lastly, the authors recorded AFM topographs of the membrane-bound oligomers at the sub-nanometer resolution and analyzed the oligomerization of GSDMD^{Nterm} pores, which revealed an average of 30 GSDMD^{Nterm} monomers (range from 15 to 45) in each ring-shaped oligomer.

The findings made by Mulvihill *et al* provide detailed mechanistic insights into transmembrane pore assembly by GSDMD^{Nterm}. Given the conserved pore-forming activity in the entire gasdermin

family, the Mulvihill study (Mulvihill *et al.*, 2018), together with the recently determined Cryo-EM structure of GSDMA3 pores (Ruan *et al.*, 2018), builds an elegant framework for understanding membrane pore formation during other gasdermin-mediated pyroptosis that are associated with diverse pathophysiological processes.

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