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News and Commentary

How ICE lights the pyroptosis fire

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Immune antigen-presenting cells and skin and mucosal epithelia vigorously respond to invasive microbial infection. When NOD-like receptors (NLRs) or AIM2-like receptors (ALRs) in the immune cell cytosol sense microbial products, they assemble the canonical inflammasome, which activates caspase-1 (initially called ICE).[1] Caspases-4/5 in humans and caspase-11 in mice, expressed by immune cells and epithelial barrier cells, directly sense cytosolic lipopolysaccharide (LPS) from Gram – bacterial cell walls.[2] These caspases are directly activated in an LPS–inflammatory caspase complex (the noncanonical inflammasome).[3,4] Inflammatory caspase activation causes a 'fiery' death, pyroptosis, that is morphologically and functionally distinct from apoptosis or necroptosis.[5,6] Caspase-1, but not other inflammatory caspases, also process and cause release of inflammatory cytokines (including IL-1β, IL-18) that cause fever. Triggering the noncanonical inflammasome indirectly activates the canonical inflammasome.[3]

Inflammasome activation protects against invasive pathogens by recruiting immune cells to infection sites and expelling microbes from favorable intracellular niches that promote their replication.[7] However, this protective response causes sepsis —a vascular leak, multi-organ failure syndrome—if the infection is poorly controlled. Humans are particularly susceptible to sepsis. Sepsis is the leading cause of childhood death worldwide and contributes to a high proportion of hospitalized patient deaths.[8] Hundreds of clinical studies that mostly inhibit proinflammatory cytokines have failed to improve treatment of sepsis, suggesting that cytokines are not the major mediators of septic death. These failures highlight our poor understanding of pyroptosis.

Indeed, the recently discovered noncanonical inflammasome likely has a major role in sepsis. It is more widely expressed than the canonical inflammasome and is standing ready—in humans, caspases-4/5 are constitutively expressed, unlike the canonical inflammasome, whose components need to be induced by danger. Many effects previously attributed to caspase-1 may actually be partly due to caspase-11, as the original *Casp1*$^{-/-}$ strain is deficient in both.[3] In fact, mice deficient only in caspase-11 (or its main known substrate, gasdermin D (GSDMD, see below)), but not caspase-1, survive a lethal LPS challenge.[3,9]

Until now, the molecular basis of pyroptosis was unknown. Cells undergoing pyroptosis lose plasma membrane integrity and release inflammatory intracellular contents, including ATP, proinflammatory cytokines and HMGB1.[10] Inflammatory caspase cleavage of GSDMD, a protein expressed in the cells that undergo pyroptosis, after Asp276(mouse)/275 (human) was recently identified as the critical pyroptosis initiator.[9,11] Cleavage removes the C-terminal fragment, which is believed to fold back on the N-terminal fragment (GSDMD-NT) to keep it inactive. Although caspase-1 has multiple substrates, caspases-4/5/11 have few that are known, other than GSDMD and themselves (and possibly caspase-1 and -3).

Five recent papers describe how GSDMD-NT causes pyroptosis.[6,12–15] GSDMD-NT shares structural homology with the membrane attack complex perforin-like domain of the killer lymphocyte pore-forming protein, perforin (unpublished data). In particular, the two perforin regions predicted to unwind into amphipathic β-strands for membrane insertion have structurally homologous regions in GSDMD-NT, suggesting that GSDMD-NT might cause pyroptosis by forming membrane pores. In fact, mutation of four basic residues on these strands in GSDMD-NT completely blocks pyroptosis.[12] Indeed, GSDMD-NT migrates as oligomers on non-reducing or native gels, while GSDMD is monomeric.[6,12] Full-length GSDMD is distributed in the cytosol, while the GSDMD-NT oligomer translocates to the plasma membrane.[6,12,13,14,15] Although neither full-length nor C-terminal GSDMD binds lipids, GSDMD-NT binds immobilized phosphatidylinositol phosphates, phosphatidic acid and phosphatidylserine, all on the inner leaflet of mammalian cell membranes.[6,12,13] GSDMD-NT also strongly binds to the mitochondrial and bacterial lipid, cardiolipin.[6,12,13] GSDMD-NT binds selectively to liposomes reconstituted with the phospholipids it binds on membrane strips and causes them to leak.[12,13,14,15] Negative staining electron microscopy of liposomes treated with GSDMD-NT or full-length GSDMD and caspase-11 shows homogeneous ring-like pores with ~15 nm inner diameters, composed of approximately 16 monomers.[12,13,14] Thus inflammatory caspase cleavage of GSDMD causes pyroptosis by triggering pore formation (Figure 1). The GSDMD-NT pore is big enough to allow small molecules such as the pro-inflammatory cytokines to pass through. These pores explain the 'unconventional protein secretion' of the leaderless proinflammatory cytokines after inflammasome activation. How the noncanonical inflammasome causes

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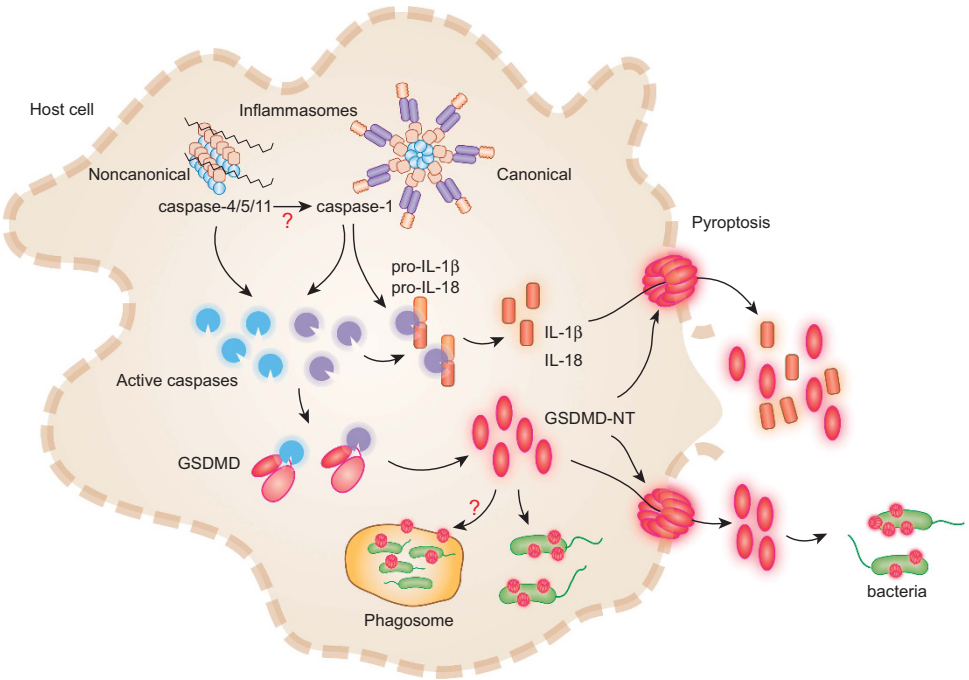

Figure 1 GSDMD cleavage by the inflammatory caspases leads to pyroptosis. GSDMD-NT induces mammalian cell pyroptosis and bacterial death by punching membrane holes. In response to microbial infection and danger signals, inflammatory caspases (caspase-1 (purple) and caspase-4/5/11 (blue) are activated via canonical or non-canonical inflammasomes, respectively. In turn, active inflammatory caspases target and cleave GSDMD, releasing its N-terminal fragment GSDMD-NT. Activated caspase-1 processes pro-IL-1β and pro-IL-18, but the other inflammatory caspases do not do this directly but are thought to do it indirectly by causing caspase-1 activation. GSDMD-NT forms pores in the plasma membrane of the infected cell, resulting in pyroptotic cell death and release of bacteria and cytokines that alarm the immune system. GSDMD-NT also directly kills the bacteria that trigger inflammasome activation. It is not known whether GSDMD-NT also permeabilizes vesicular membranes, such as the phagosome, in cells

proinflammatory cytokine processing is not completely clear. Potential mechanisms include caspase-4/5/11 cleavage of caspase-1 and/or activation of the NLRP3 inflammasome[3] when the plasma membrane is permeabilized. Careful kinetics experiments in inflammasome component-deficient cells will answer this question.

GSDMD-NT (30 kD) itself is released from pyroptotic cells. Because GSDMD-NT does not bind any outer leaflet lipids, it does not damage non-pyroptotic bystander cells,[12,13] limiting the inflammation and cellular damage caused by leaked GSDMD-NT. However, released GSDMD-NT also binds to, permeabilizes and kills Gram– and Gram+ bacteria.[12] In addition, GSDMD-NT overexpression or knockdown of endogenous GSDMD inhibits or promotes, respectively, intracellular Listeria replication, suggesting that GSDMD-NT directly kills the bacteria that trigger the inflammasome.[12] GSDMD-NT binding and bacterial death is probably mediated by bacterial cardiolipin, but that remains to be shown. How and whether GSDMD-NT passes through the bacterial cell wall and outer membrane of Gram– bacteria to permeabilize the bacterial inner membrane needs to be demonstrated. Although the antibacterial effect of pyroptosis was formerly attributed to phagocytic cell recruitment and expulsion of bacteria from their intracellular niche,[7] GSDMD-NT may more potently suppress infection by direct killing. Indeed a recent study shows that bacterial replication is inhibited by inflammasome activation independently of mammalian cell death.[16] However, inhibition occurred in both *Gsdmd*$^{-/-}$ and wild-type cells, suggesting that the activated caspases have other antibacterial effects. There is currently no way to separate GSDMD-NT membrane damage to bacteria and host cells, making it difficult to assess the relative importance of direct bacterial killing *versus* host cell pyroptosis in suppressing infection.

Many questions remain. GSDMD-NT is a poorly behaved protein for structural studies. Neither crystal structure nor high-resolution cryoEM of GSDMD-NT and its pore have so far been possible. Thus the conformational changes that monomeric GSDMD undergoes after cleavage to form the pore and pore characteristics are not yet well defined. So far, no antibodies recognize the intact pore. The outer leaflet of some intracellular organelles, especially endosomes and phagosomes, contain the same phospholipids as the inner leaflet of the plasma membrane. Does GSDMD-NT bind to these membranes and damage these organelles during pyroptosis, releasing bacteria to the cytosol, as another mechanism to control infection? Although cardiolipin is only on the inner membrane of intact mitochondria and presumably inaccessible, are mitochondria damaged during pyroptosis?

GSDMD belongs to a family of poorly characterized gasdermins (6 in humans, 10 in mice) expressed in immune cells, the skin and mucosal epithelia, suggesting that they may have an important role in the initial immune response to infection.[17] Not much is known about what cells express the

gasdermins, how expression is regulated and how they function. There is no evidence that other gasdermins are inflammatory caspase substrates. However, N-terminal fragments of most of them cause pore formation and pyroptosis.[13] Mutations that disrupt the N- and C-terminal Gsdma3 interaction cause pyroptosis and inflammatory alopecia in mice.[11,18] Genetic alterations of *GSDMB*, highly expressed in the lung bronchi, are linked to asthma.[19] However, the link may be independent of pyroptosis as GSDMB appears to be mostly a nuclear protein that activates gene transcription to induce airway hyper-responsiveness without inflammation.[19]

Perhaps the most important unanswered question, given the toll from sepsis, is whether our new understanding of pyroptosis will lead to effective therapies. If GSDMD-NT pores both directly kill bacteria, contributing to bacterial control, and cause inflammation that leads to sepsis, therapeutic manipulation of this pathway may be challenging.

Conflict of Interest

The authors declare no conflict of interest.

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