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Pyroptosis in defense against intracellular bacteria

Lupeng Li[1,2,3,4], **Mary S Dickinson**[2], **Jörn Coers**[1,2], **Edward A Miao**[1,2,3,4]

[1]Department of Integrative Immunobiology, Duke University School of Medicine, Durham, NC, USA

[2]Department of Molecular Genetics and Microbiology, Duke University School of Medicine, Durham, NC, USA

[3]Department of Cell Biology, Duke University School of Medicine, Durham, NC, USA

[4]Department of Pathology, Duke University School of Medicine, Durham, NC, USA

Abstract

Pathogenic microbes invade the human body and trigger a host immune response to defend against the infection. In response, host-adapted pathogens employ numerous virulence strategies to overcome host defense mechanisms. As a result, the interaction between the host and pathogen is a dynamic process that shapes the evolution of the host's immune response. Among the immune responses against intracellular bacteria, pyroptosis, a lytic form of cell death, is a crucial mechanism that eliminates replicative niches for intracellular pathogens and modulates the immune system by releasing danger signals. This review focuses on the role of pyroptosis in combating intracellular bacterial infection. We examine the cell type specific roles of pyroptosis in neutrophils and intestinal epithelial cells. We discuss the regulatory mechanisms of pyroptosis, including its modulation by autophagy and interferon-inducible GTPases. Furthermore, we highlight that while host-adapted pathogens can often subvert pyroptosis, environmental microbes are effectively eliminated by pyroptosis.

Keywords

pyroptosis; intracellular bacteria; host-adapted pathogen; environmental pathogen; autophagy; guanylate-binding protein

1. Introduction

Pathogens are categorized into two main groups according to their dominant location relative to host cells: extracellular and intracellular[1]. Further classification of intracellular pathogens results in two subtypes: vacuolar and cytosolic[2]. Intracellular bacterial pathogens

Correspondence to: Edward A. Miao, edward.miao@duke.edu, 919-668-7555.

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invade host cells to facilitate their replication and spread. This location offers intracellular bacterial pathogens advantages, such as protection from the host's humoral immunity (e.g., complement, secreted antimicrobial peptides, antibodies), sequestration from neutrophils, and access to nutrients that may be scarce extracellularly[2]. As a result, intracellular bacterial pathogens manipulate host cells to access their preferred niches within targeted cells. After invasion, bacteria are contained within a plasma membrane-derived vacuole in host cells, such as phagosomes or endosomes. Vacuolar bacteria, such as *Salmonella enterica* serovar Typhimurium, remain within the vacuole, while cytosolic bacteria, for example, *Listeria monocytogenes*, rupture the vacuole and reside within the host cytosol. Notably, recent studies suggested that whether a bacterium is vacuolar or cytosolic is more context-dependent, such as the host cell types, tissue, metabolic status, and cellular environment[2–4].

The virulence strategies of host-adapted pathogens exert selective pressure on the host immune system, leading to the evolution of new host defense mechanisms. In turn, host defense mechanisms impose evolutionary pressure on the virulence strategies of host-adapted pathogens. Together, these selective pressures create a continuous cycle of adaptation and counter-adaptation, resulting in a constant arms race between the host immune system and pathogens' virulence strategies that shapes the evolution of both host and pathogens[5,6]. As a result, both hosts and host-adapted pathogens are constantly evolving to maintain their respective advantages in the arms race, an example of the Red Queen hypothesis[7]. The net result is that the host-pathogen interface is maintained, preserving the ability of host-adapted pathogens to survive, replicate, and transmit to new hosts. In contrast, environmental (opportunist) pathogens, are microorganisms commonly found in a habitat that are typically avirulent to immunocompetent individuals despite encoding potent virulence factors. Environmental pathogens often fail to evade the host's innate immune defenses and are therefore efficiently eliminated[8].

Regulated cell death pathways, including apoptosis, pyroptosis, and necroptosis, are innate immune defense mechanisms that remove the replicative niche of intracellular pathogens while simultaneously recruiting immune cells to the site of infection[9,10]. Pyroptosis, a type of cell death characterized by cell lysis, is typically initiated by the pyroptotic caspases (caspase-1/4/5/11). Among these, caspase-4/5 (human) and caspase-11 (mouse) are directly activated by their LPS ligand. Activated caspase-4/5/11 cleave the linker region of gasdermin D (GSDMD) to release its N-terminal pore-forming domain, which oligomerizes and forms pores in the plasma membrane, ultimately leading to pyroptosis[11–14]. In parallel, caspase-1 is activated by inflammasomes, which are cytosolic multiprotein complexes made up of an inflammasome sensor and often an adaptor protein called ASC[15]. Inflammasomes, including NLRPs, NLRC4, AIM2, and pyrin, detect the presence of pathogen-associated molecular patterns (PAMPs) or other patterns of pathogenesis, leading to the activation of caspase-1[16]. Once activated, caspase-1 also cleaves and activates GSDMD[11–14]. It should be noted that many genes in these pathways are often duplicated or contracted between humans and mice, however the core functions that we cover in this review are all conserved.

Here, we describe the mechanisms of pyroptosis in defending against intracellular bacterial pathogens. In general, host-adapted bacterial pathogens often have evolved strategies to evade or suppress pyroptotic cell death. We therefore contrast muted host defenses against

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host-adapted bacterial pathogens with the effective clearance of environmental bacterial pathogens by pyroptosis.

2. Pyroptosis

Regulated cell death pathways are critical for defending against intracellular infections by eliminating the replicative niche of pathogens[17]. These pathways, including apoptosis and lytic cell death, are distinguished by their morphological and immunological features[18]. Apoptosis is characterized by DNA fragmentation, cell blebbing, and the formation of apoptotic bodies. The apoptotic bodies are rapidly cleared by phagocytes through efferocytosis, which is not intrinsically inflammatory[19]. In contrast, regulated lytic cell death, including pyroptosis and necroptosis, leads to the release of cellular contents that cause a pro-inflammatory response. The utility of different cell death pathways can depend upon the cell type that is infected as well as the nature of the infecting pathogen. Sometimes regulated cell death pathways act redundantly, whereas in other cases one specific pathway is required to mediate clearance of a pathogen[20,21].

Pyroptosis is typically induced by the activation of caspase-1/4/5/11[10] (Figure 1). The signals that activate cytosolic sensors called inflammasomes upstream of caspase-1 or the nature of direct activation of caspase-4/5/11 by LPS are reviewed elsewhere[10]. Pyroptosis is characterized by the rupture of the plasma membrane, which is facilitated by gasdermins, a family of pore-forming proteins. Among the gasdermins, GSDMD is considered the prototype[22]. Recent studies have shown that other members of the gasdermin family, including gasdermin A, B, C, and E, also possess pore-forming activities (note that mice lack GSDMB and have expanded *Gsdma* and *Gsdmc* genes). These proteins consist of two domains that are connected by a cleavable linker region: an N-terminal pore-forming domain and a C-terminal autoinhibitory domain. Upon cleavage of the linker region, the activated N-terminal domain recognizes membrane lipids and undergoes oligomerization, resulting in the formation of a soluble prepore. This prepore subsequently undergoes a conformational change as it inserts into the membrane, forming large pores with an approximate diameter of 21 nm[23,24]. These pores allow cellular contents to exit the cell, including proinflammatory cytokines (e.g., IL-1β and IL-18), ATP, and other alarmins[11,13,14,24,25]. The full dispersion of any particular molecule probably takes several minutes to reach equilibrium. GSDMD-independent release of the proinflammatory cytokines has also been reported[26,27].

After the GSDMD pore opens, the subsequent membrane rupture is an active process mediated by NINJ1[28]. The mechanism by which NINJ1 is activated downstream of the gasdermin pore remains unclear, but once activated, NINJ1 polymerizes in the membrane resulting in rupture of the membrane that immediately disperses all soluble cytosolic molecules, including large proteins such as the lactate dehydrogenase tetramer that is often used as a marker for cell lysis. One possible clue to the NINJ1 activation mechanism is the long-known property of extracellularly applied glycine (or alanine) to inhibit pyroptosis[29]. Glycine does not inhibit the opening of the gasdermin pore, but does significantly delay rupture of the membrane by several hours, which is similar to the phenotype of a *Ninj1*-knockout cell. High extracellular glycine inhibits NINJ1 clustering[30], which was hypothesized to explain the mechanism of glycine cytoprotection. Interestingly, the very

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high 5-10 mM concentrations of glycine that must be applied to cells matches the cytosolic concentration of glycine[31], suggesting a possible mechanism whereby NINJ1 activates in response to the loss of cytosolic glycine. Although speculative, this would provide a common mechanism whereby NINJ1 can activate in response to multiple forms of cell death that all converge upon a common cytosolic event.

2.1 The role of cell lysis in intracellular bacteria-induced pyroptosis

Pyroptosis plays a crucial role in fighting intracellular bacterial infections, and it has been extensively studied in macrophages using both host-adapted and environmental pathogens[8]. This lytic form of cell death removes the replicative niche for intracellular pathogens by inducing the formation of large pores in the plasma membrane. This process releases highly inflammatory cytoplasmic contents, including proinflammatory cytokines and damage-associated molecular patterns (DAMPs), which recruit and activate immune cells to combat the infection. In addition, pyroptosis also helps combat bacterial infections by converting the pyroptotic cells into pore-induced intracellular traps (PITs)[32]. These PITs trap bacteria inside the dead cells, and simultaneously facilitate the recruitment and efferocytic properties of neutrophils through complement and scavenger receptors. These neutrophils then efferocytose the trapped bacteria and subsequently kill the bacteria. It has also been shown that GSDMD can forms pores on the bacterial membrane and directly kill bacteria[33], however, intracellular bacteria do survive pyroptosis in macrophages in vitro[32]. We previously speculated that pyroptosis damages bacteria, which may involve GSDMD pores or mitochondrial ROS[34]. Indeed, caspase-1 activation results in mitochondrial ROS production[35,36], which could explain how intracellular bacteria trapped in PITs become damaged during the process of pyroptosis. Release of bacterial damaging mitochondrial ROS has also been demonstrated after inhibition of TAK1 that results in apoptotic/necroptotic cell death[37], suggesting a generalized defense mechanism during regulated cell death.

Ninj1-deficient mice were found to be more susceptible to *Citrobacter rodentium* infection[28]. Additionally, the expression of NINJ1 was shown to be beneficial to the host during *Y. pseudotuberculosis* infection[38]. Although the exact mechanism of NINJ1-mediated protection during bacterial infection remains unclear, it was speculated that DAMPs released by NINJ1-mediated plasma membrane rupture play a role in defending against bacterial infection[28]. Furthermore, whether NINJ1-driven membrane rupture will be generally required downstream of GSDMD remains to be established.

2.2 Pyroptosis in neutrophils

Pyroptosis has been predominantly studied in macrophages, but recent research is revealing its role in other cell types during bacterial infection. Neutrophils, for example, are among the first immune cells to respond to infection and accumulate in large numbers at the site of infection. Although neutrophils express inflammasome sensors, inflammatory caspases (caspase-1/4/5/11), and GSDMD[39,40], their capacity to undergo pyroptosis seems to be more tightly regulated than that of macrophages. Neutrophils can release bio-active IL-1β upon inflammasome activation[39,41–43], but the release of IL-1β in some contexts occurs without membrane rupture[39,41], possibly to preserve the antibacterial function of neutrophils.

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For instance, neutrophils infected with *Burkholderia thailandensis* undergo caspase-11 dependent pyroptosis[44]. In contrast, NLRC4 and NLRP3 inflammasome activation in neutrophils did not induce pyroptosis[39,45]. However, recent studies using extracellular bacterial infections showed that NLRC4 and pyrin inflammasome activation could induce pyroptosis in neutrophils depending upon the nature of the agonist[46,47]. The difference between lysis or non-lytic caspase activation was caused by the cellular location through which inflammasome activating stimuli entered the cytosol – across the plasma membrane compared to endosomal, however, the mechanism by which this is achieved remains unknown. Additionally, GSDMD activation in neutrophils induces NETosis[48,49], which is the release of decondensed nuclear DNA into extracellular compartments. Specifically, cytosolic Gram-negative bacteria, such as *Salmonella ΔsifA*[50] and *Citrobacter rodentium*, active caspase-11 and GSDMD. Caspase-11 and GSDMD coordinately facilitate nuclear membrane permeabilization and histone degradation in neutrophils, ultimately leading to NETosis. This response contributes to defense against *Salmonella ΔsifA* infection in mouse models[48].

2.3 Pyroptotic caspase-driven extrusion in intestinal epithelial cells

Pyroptosis is not limited to immune cells and can also be activated in epithelial cells to combat bacterial infections[51]. The invasion of the intestinal epithelium by *Salmonella* drives its pathogenesis in the gut. The host has evolved mechanisms to fight back against invasion of intestinal epithelial cells (IECs) by extruding the infected IECs into the intestinal lumen. Extrusion can be triggered via several pathways, including by activation of the NAIP-NLRC4 inflammasome; thus pyroptotic signaling causes IEC extrusion. The mechanisms by which NLRC4 activation causes extrusion are still being elucidated, but they include redundant pathways via GSDMD or the ASC to caspase-8 backup pathway[51–55]. The extrusion of infected IECs removes infected cells from the tissue and prevents further spread of the pathogen to deeper sites[56]. Apoptotic signaling also causes IEC extrusion, and also limits bacterial burdens in the intestine[21]. Successfully completed extrusion also limits tissue damage caused by *Salmonella* infection[57]. We recently proposed that extrusion is one of the specific effector programs that a cell must execute before the cell loses all functional capacity. These effector programs can be thought of as a “bucket list” of tasks that must be completed before the cell dies[58].

The discovery that the host successfully detects the *S.* Typhimurium SPI1 T3SS as it attempts to invade IECs, and successfully extrudes IECs in response appears to contradict the idea that host-adapted pathogens should evade innate defenses. This is particularly confounding in the case of *S.* Typhimurium, where the primary intestinal virulence strategy seems to be invasion of IECs, yet the host seems to have a potent and direct countermeasure to extrude the invaded cells. This raises the possibility that the “failure” of *S.* Typhimurium to evade IECs extrusion may be a deliberate attempt by the bacteria to provoke an inflammatory response. Indeed, *S.* Typhimurium benefits from the inflammatory response that generates molecules that the bacteria use as alternate electron acceptors in respiration[59]. It could be that the bacteria “have their cake and eat it too” by simultaneously benefiting from triggering wide scale IEC extrusion that causes inflammation while the few IECs that fail to extrude become replicative niches for the bacteria and promote their dissemination

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to macrophages in the lamina propria or draining lymph nodes. We propose that these phenotypes be considered carefully in the context of the Red Queen's race, where *S.* Typhimurium and NLRC4 compete, sometimes resulting in a victory for *Salmonella* and invasion, and sometimes in a victory for host and extrusion (but a victory that the bacteria may simultaneously exploit).

2.4 Host-adapted bacterial pathogens subvert pyroptosis

As an innate defense mechanism, pyroptosis exerts selective pressure on pathogens. To enhance their own survival, replication, and transmission, these pathogens are compelled to develop strategies to evade, suppress or even exploit the pyroptotic pathway at all levels, including evading detection or inhibiting inflammasomes, caspases, and gasdermins[60,61]. For example, during their intracellular phases, *S.* Typhimurium and *L. monocytogenes* evade NLRC4 inflammasome by suppressing flagellin expression[8]. In other pathogens, variant LPS structures allow bacteria to evade cytosolic LPS detection. For example, *Francisella* species have tetraacylated LPS allowing evasion of murine caspase-11[62,63], but not human casapse-4[64]. *Shigella* species overcome cytosolic LPS detection by direct inhibition, translocating the T3SS effector OspC3, which inactivates human caspase-4 and murine caspase-11 by covalently modifying the caspases via ADP riboxanation. Consequently, *S. flexneri ospC3* mutants are recognized and cleared by caspase-11 in a mouse infection model[65–67]. *S. flexneri* can also target the executioners of pyroptosis, the gasdermins. The *S. flexneri* ubiquitin-ligase virulence factor IpaH7.8 targets human GSDMD for proteasome destruction. Interestingly, IpaH7.8 cannot target mouse GSDMD due to amino acid substitutions[68–71], which could partially explain the natural resistance of mice to *S. flexneri* infection. *S. flexneri* also uses IpaH7.8 to target GSDMB for proteasome destruction (discuss further below)[69–72]. *Mycobacterium tuberculosis* has also developed strategies to overcome pyroptosis[73]. The pore-forming domain of GSDMD has a strong affinity for cell membrane lipids. This allows GSDMD to bind to the inner leaflet of plasma membrane to form pores and induce pyroptosis[13,74]. *M. tuberculosis* effector protein PtpB, a phospholipid phosphatase, hijacks ubiquitin to mediate PtbB activation. Activated PtbB dephosphorylates plasma membrane lipids that are targets of the pore-forming domain of GSDMD to disrupt GSDMD membrane localization and inhibit pyroptosis[75]. *M. tuberculosis* also inhibits inflammasome detection via PknF through mechanisms that remain to be fully elucidated[76].

While inflammasomes are generally believed to be beneficial during bacterial infection, in vivo studies have shown incremental rather than definitive differences in phenotype between wild-type and inflammasome-deficient mice infected with host-adapted bacteria. For example, *S.* Typhimurium has been extensively studied in the inflammasome field[53,77–84]. Studies on *S.* Typhimurium have shown that although WT mice have lower bacterial burdens compared to inflammasome-deficient mice, they still succumb to infection, albeit more slowly. As a result, the lethal dose difference (ΔLD100) between the two types of mice remains unchanged (ΔLD100 = 1-fold), indicating that inflammasomes are unable to prevent death during *S.* Typhimurium infection. The mild phenotype observed in inflammasome-deficient mice infected with *S.* Typhimurium may be due to the pathogen's ability to evade inflammasome detection. Our previous research has demonstrated that *S.* Typhimurium can evade detection by the NLRC4 inflammasome during systemic infection by repressing

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flagellin[83] and expressing a SPI2 version of the T3SS rod protein that carries evasive point mutations[84]. However, when these evasion strategies are eliminated in engineered *S.* Typhimurium, inflammasomes efficiently and completely eradicate the pathogen[84].

2.5 Environmental pathogens are efficiently cleared by pyroptosis

Environmental pathogens that are unable to evade or inhibit inflammasomes are usually cleared effectively via pyroptosis[8]. In the case of *B. thailandensis*, its T3SS apparatus rod/needle proteins and LPS are recognized by the NAIP-NLCR4 inflammasome and caspase-11, respectively[85]. Notably, WT mice survived infection by $2x10^7$ CFU of *B. thailandensis*, while inflammasome-deficient mice were susceptible to as few as 100 CFU[85]. These findings suggest that inflammasomes changed the lethal dose by 2,000,000-fold during *B. thailandensis* infection. Mechanistically, *B. thailandensis* is recognized by the NLRC4 inflammasome in macrophages, leading to the secretion of IL-18. This pro-inflammatory cytokine, in turn, stimulates natural killer (NK) cells and T cells to produce interferon gamma (IFN-γ). IFN-γ then primes caspase-11 in neutrophils, which is subsequently activated by LPS release by the cytosol-invasive *B. thailandensis*, leading to pyroptosis. Notably, pyroptosis is critical for the efficient clearance of B. thailandensis[44,85,86].

Chromobacterium violaceum, a Gram-negative bacterium, is widely distributed in freshwater sediment within tropical and subtropical regions[87]. This environmental organism is known to only infect individuals with compromised immune systems, especially those suffering from chronic granulomatous disease[88,89]. *C. violaceum* encodes a T3SS that is similar to SPI1 in *S.* Typhimurium[90]. NLRC4 inflammasome detects the *C. violaceum* T3SS apparatus and drives bacterial clearance in vivo[91]. While WT mice were able to survive infection with 10^6 CFU of *C. violaceum*, inflammasome-deficient mice died after only 100 CFU infection. Therefore, the difference in ΔLD100 between WT and inflammasome-deficient mice was estimated to be 100,000-fold.

The dramatic lethal dose change between *Salmonella* (1-fold) and these two environmental pathogens (more than 100,000-fold) leads us to further investigate the ΔLD100 between WT and inflammasome-deficient mice during infection by host-adapted pathogens. To accomplish this, we extensively reviewed published literature on infection studies that utilized inflammasome-deficient mice for a lethal challenge. We then estimated the change in lethal dose comparing WT to inflammasome-deficient mice[8,9]. Our analysis revealed that nearly all host-adapted infection models had a ΔLD100 of less than 8-fold. However, we observed a significant contrast with environmental pathogens such as *B. thailandensis* and *C. violaceum*, where inflammasomes provided potent and fully penetrant protection (Table 1). While WT mice survived the challenge by $2x10^7$ and 10^6 CFU, respectively, inflammasome-deficient mice succumbed to challenge with as few as 100 CFU.

The discrepancies in the lethal dose change between host-adapted (less than 8-fold) and environmental pathogens (more than 100,000-fold) have led us to suggest that the evolutionary role of inflammasomes is to defend against pathogens that typically infect lower hosts, where inflammasomes are not present. For example, NOD-like receptors are absent in insects[92]. Interestingly, species from the genus *Chromobacterium* exhibit toxicity

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towards insects, such as southern green stink bug and corn rootworm[93,94]. Further research is needed to identify additional environmental pathogens in which inflammasomes are essential for host defense.

3. Interplay of autophagy and pyroptosis

Autophagy is a cellular process of capturing and degrading cytoplasmic materials. Xenophagy refers to the targeting and elimination of invading pathogens by autophagy machinery. Therefore, xenophagy is an important arm of cell-autonomous immunity, which refers to the ability of an individual cell to defend itself against invading pathogens, and to survive the encounter. Essentially every cell in the body is equipped with this ancient defense mechanism[95]. Autophagy inhibits inflammasome activation and regulates inflammatory responses[96–99] (Figure 2A). The first in vivo evidence of autophagy inhibiting inflammasome activation came from studies with mice lacking an autophagy-related protein ATG16L1. *Atg16l*-deficient mice produced higher amounts IL-1β compared to WT mice in a colitis model[100]. In vitro studies showed that autophagy inhibits inflammasome signaling by degrading assembled inflammasomes[101] or by removing damaged mitochondria[102–104]. Autophagy also clears cytosolic PAMPs, such as LPS, to reduce inflammasome activation[105,106]. Besides removing DAMPs and PAMPs, it was also proposed that autophagy directly targets inflammasome pathway components to inhibit inflammasome activation, such as AIM2, NLRP3, and caspase-1[101,107,108].

Moreover, the relationship between autophagy and inflammasome activation is complex and multifaceted, with evidence suggesting that in certain situations, inflammasome activation can also promote xenophagy. For instance, in macrophages responding to *Burkholderia cenocepacia* infection, an opportunistic bacterium that causes infections in immunocompromised individuals, caspase-11 activation promotes xenophagy, and caspase-11-deficient macrophages exhibit a defect in xenophagy[109,110]. Notably, a subsequent investigation demonstrated that upon exposure to *B. cenocepacia* in macrophages, GSDMD activation led to mitochondrial damage and the release of mitochondrial ROS (mtROS). Subsequently, mtROS facilitated xenophagy, which in turn promoted the elimination of the bacterial pathogen[34] (Figure 2B). These findings highlight the intricate mechanisms underlying cell-autonomous immunity and the interconnectedness of cellular processes such as autophagy and inflammasome activation. We recently speculated that successful xenophagy of intracellular bacteria eliminates the need for pyroptosis, which could explain how these mechanisms work in sequence during infection[111].

4. Regulation of pyroptosis by interferon-inducible GTPases

Interferon-inducible guanosine triphosphate hydrolyzing enzymes (GTPases) belong to the dynamin-like protein family and play a critical role in cell-autonomous immunity to defend against bacterial infections[112,113]. Interferon-inducible GTPases are classified into four subfamilies, which include immunity-related GTPases (IRGs), guanylate-binding proteins (GBPs), myxoma resistance proteins, and very large inducible GTPases[114]. Here, we discuss the role of GBPs and IRGs in regulating pyroptosis.

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GBPs promote inflammasome activation through several different strategies depending on the type of bacteria present (Figure 3). They can act on bacteria-containing vacuoles, directly bind to cytosolic bacteria, or bind LPS released by bacteria, either in the form of free LPS micelles or packaged in outer membrane vesicles (OMVs). The diverse actions of GBPs allow activation of several different inflammasomes in response to a variety of bacterial pathogens. By targeting either bacteria themselves, pathogen containing vacuoles, or PAMPs released by bacteria, GBPs provide a multifaceted defense against intracellular invasion.

GBPs are required for efficient caspase-4/11-dependent pyroptosis in IFN-γ primed cells[106,115–124]. This was first demonstrated in mouse macrophages, where GBPs were important for caspase-11-dependent pyroptosis induced by intracellular Gram-negative bacteria *Legionella pneumophila* and *S*. Typhimurium[115]. The main clue to GBPs' role in caspase-11 activation came from an experiment showing GBPs were also able to promote pyroptosis when purified LPS was transfected into the cytosol[115]. Subsequent studies further demonstrated that in the absence of infection, both free LPS and OMVs containing LPS were able to induce GBP-dependent caspase-11 activation both in macrophages and in an *in vivo* sepsis model[117,125]. Three of the seven human GBPs (and 3 of 11 mouse GBPs) contain C-terminal prenylation motifs that direct the attachment of lipid farnesyl or geranylgeranyl groups. The prenylated human GBP1 and GBP2 proteins directly bind LPS and promote caspase-4 activation[118,123,124]. It is likely, but has not yet been proven, that mouse GBP2, the closest ortholog of human GBP1 and GBP2, also directly binds LPS. Recombinant GBP1 or GBP2 cluster LPS micelles into larger aggregates and enhance the LPS-dependent activity of caspase-4 in vitro, suggesting that GBP-mediated LPS aggregation is responsible for GBP-dependent caspase-4 activation[123,124]. GBP1 binds to LPS on the surface of Gram-negative bacteria, coating the bacterium with a dense layer of GBP protein[118,119,121,124,126,127]. This GBP coatomer has direct consequences for the health of the bacterium and can disrupt bacterial virulence traits. GBPs act as a surfactant that extracts LPS molecules from the bacterial surface into the host cell cytosol[128] and exposes the lipid A portion of bacterial membrane-embedded LPS for improved cytosolic access[124]. Consequently, caspase-4 is recruited to the surface of GBP1-coated bacteria[118,119,121,124]. However, this GBP1-dependent caspase-4 recruitment is dispensable for the induction of pyroptosis[123,124]. Thus, GBPs likely promote caspase-4/11 activation through multiple mechanisms by allowing caspase-4/11 to deposit upon the bacterial surface, by promoting the release of LPS from the bacterial surface, and by acting directly upon LPS that has been released from the bacterium into the cytosol.

GBPs, in concert with IRG family member IRGB10, also promote activation of the DNA sensing AIM2 inflammasome[129–133]. Bacterial DNA is normally sequestered within the bacterial cell, shielded from cytosolic detection by AIM2. GBPs recruit IRGB10 to the surface of cytosolic *Francisella novicida*, resulting in bacterial lysis and liberation of bacterial DNA that then is detected by the AIM2 inflammasome[129–132]. GBPs similarly facilitate the liberation of DNA from cytosol-exposed *Legionella pneumophila*, thereby mediating AIM2 inflammasome activation[133].

GBPs were also shown to promote NLRP3 inflammasome activation[134]. Human GBP1 was found to activate the NLRP3 inflammasome during *C. trachomatis* infection.

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Mechanistically, hGBP1 is responsible for the hydrolysis of guanosine triphosphate (GTP) into guanosine monophosphate (GMP). The resulting GMP is then catabolized to uric acid, which activates the NLRP3 inflammasome[134]. Monosodium urate (MSU) crystals are a long-established activator of NLRP3 after macrophages phagocytose the crystal[135]. Whether uric acid produced by GBP enzymatic activity is in high enough concentration to crystalize within the *C. trachomatis*-infected cell was not yet determined.

While GBPs promote pyroptosis, conversely, IRGs within the IRGM subset impede pyroptosis. IRGM2 cooperates with GATE16 to inhibit caspase-11 activation in response to various Gram-negative bacteria[136,137]. Interestingly, extracellular LPS, which does not typically reach the cytosol to activate caspase-11, also triggered inflammasome activation in *Irgm2*-knockout macrophages[137]. IRGM2 did not affect caspase-11 activation when LPS was electroporated or transfected into the cytosol. This suggests IRGM2 and GATE16 may suppress caspase-11 activation by preventing aberrant vacuolar instability that could release LPS in the cytosol. The knockout of the sole human *IRGM* did not phenocopy *Irgm2*-knockout in mice, but *GATE16* knockout in human cells did enhance pyroptosis, suggesting a similar pathway exists in humans though it is likely regulated by different proteins[136].

5. Inflammasome-independent activation of pyroptosis

While pyroptosis is frequently associated with inflammasome activation, recent studies have revealed mechanisms of inflammasome-independent activation of pyroptosis (Figure 4). For example, GSDMB, expressed in humans but not in mice, is activated by granzyme A from cytotoxic lymphocytes (i.e., cytotoxic T cells and NK cells) to trigger pyroptosis in target cells[138]. Although GSDMB has been explored as a potential target for cancer immunotherapy, further investigation is required to understand its role in antibacterial infection. This will be complicated by the natural absence of GSDMB from the mouse genome. Intriguingly, GSDMB can be degraded by IpaH7.8, a virulence factor of *Shigella*[69–72]. This suggests the possibility of GSDMB-driven pyroptosis playing a role in defending against bacterial infections.

Apoptotic signaling pathways can be diverted towards pyroptosis through the cleavage of gasdermins. One such gasdermin, GSDME, undergoes cleavage by caspase-3, the primary executor of apoptosis, resulting in the transition from caspase-3-mediated apoptosis to pyroptosis[139,140]. Unlike pyroptosis, apoptosis is typically non-inflammatory and does not elicit an immune response. Consequently, the activation of GSDME by caspase-3 must be tightly controlled to ensure normal apoptosis can occur. The expression level of GSDME may play a role in determining whether cells undergo apoptosis or pyroptosis upon caspase-3 activation. Furthermore, in caspase-3-activated cells, any low-level gasdermin pores that form are likely to be promptly repaired to facilitate the progression of apoptosis[58]. Granzyme B could also achieve GSDME activation indirectly by activating caspase-3[141]. However, GSDME was cleaved even in caspase-3 deficient HeLa cells when co-cultured with NK cells. Furthermore, in a cell-free assay, recombinant granzyme B but not granzyme A cleaved GSDME. These findings indicate that granzyme B can directly cleave and activate GSDME[142].

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Caspase-8, traditionally known as an initiator of apoptosis, exhibits the ability to trigger pyroptosis as well. This might occur when caspase-8 activates caspase-3 in settings of high GSDME. Additionally, this can occur through the cleavage of GSDMD in response to *Yersinia* infection or TNF-α signaling combined with the inhibition of transforming growth factor beta-activated kinase (TAK1)[143–145]. We presume that this caspase-8-mediated pathway involving GSDMD either operates at a slower pace compared to typical apoptotic signaling or is only licensed in a specific manner within cells experiencing TAK1 inhibition.

Caspase-8 can also induce pyroptosis through the cleavage of GSDMC[146,147]. For example, caspase-8 cleaves GSDMC in response to a metabolite known as α-ketoglutarate (α-KG)[147]. This process is initiated by α-KG, which induces an elevation in reactive oxygen species (ROS), leading to the oxidation of the death receptor DR6 present on the plasma membrane. Consequently, DR6 is internalized through endocytosis. Upon entry into the cell, DR6 recruits both pro-caspase-8 and GSDMC, resulting in the cleavage of GSDMC by caspase-8, ultimately culminating in pyroptosis. It is important to note that the expression of GSDMC is primarily limited to epithelial tissues, such as stomach and intestine[22]. As a result, the activation of GSDMC by caspase-8 could be confined by the expression pattern of GSDMC.

In addition to host-derived proteases, pathogen-derived factors can also activate gasdermins[148–150]. For instance, in keratinocytes, pyroptosis can be induced by SpeB, a cysteine protease virulence factor produced by *Streptococcus pyogenes*, which cleaves GSDMA and possible other GSDMs[148,149]. Notably, the expression of GSDMA is restricted to stratified epithelial cells, including skin keratinocytes[151,152]. In a skin infection model, GSDMA restricted the dissemination of *S. pyogenes* into inner organs[148]. SpeB accessed the cytosol during a cytosolic-invasive phase of the bacterial infection. However, GSDMA is not involved in skin infection by *Staphylococcus aureus*[148] or herpes simplex virus type 1[152]. These studies highlight the dual role of GSDMA as both a direct sensor of pathogen-derived proteases and an executor of pyroptosis.

6. Conclusions

The immune response to intracellular bacteria involves a complex interplay between host and pathogen. Host-adapted pathogens can evade host immune defenses, whereas environmental microbes are effectively eliminated by host defense mechanisms. As their first line of defense, most cells rely on cell-autonomous immunity (including xenophagy), in which the infected host cell eliminates the pathogen, survives, and returns to homeostasis. Pyroptosis is another mechanism used to combat intracellular bacteria, as it eliminates replication niches for these pathogens and releases danger signals that activate subsequent inflammatory responses. However, for certain intracellular pathogens that cannot be cleared by cell-autonomous immunity or cell death, the granuloma response has been suggested as a solution. This response involves the formation of an organized structure, by various cell types, to wall off such bacteria[153].

This review emphasizes the critical role of pyroptosis in combating intracellular bacterial infections, particularly environmental pathogens. The interaction between host and pathogen is dynamic and shapes the evolution of the host's immune response. To fully understand

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these immune responses, further studies are needed to unravel the complex molecular mechanisms involved. Additionally, how the multiple gasdermin family members act either together or distinctly in defending against intracellular infection warrants further investigation. Moreover, investigating the crosstalk between regulated cell death pathways in the context of bacterial infection can also shed light on the immune response to these pathogens. To address these questions, the discovery of new environmental pathogens that are unable to inhibit or evade the host immune responses can provide valuable tools for understanding the optimal functioning of our immune system.

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Highlights:

- Pyroptosis combats intracellular bacterial infection.
- The role of pyroptosis is cell-type dependent.
- Host-adapted pathogens evade or inhibit pyroptosis.
- Environmental pathogens are efficiently cleared by pyroptosis.
- Pyroptosis is regulated by autophagy and interferon-inducible GTPases.

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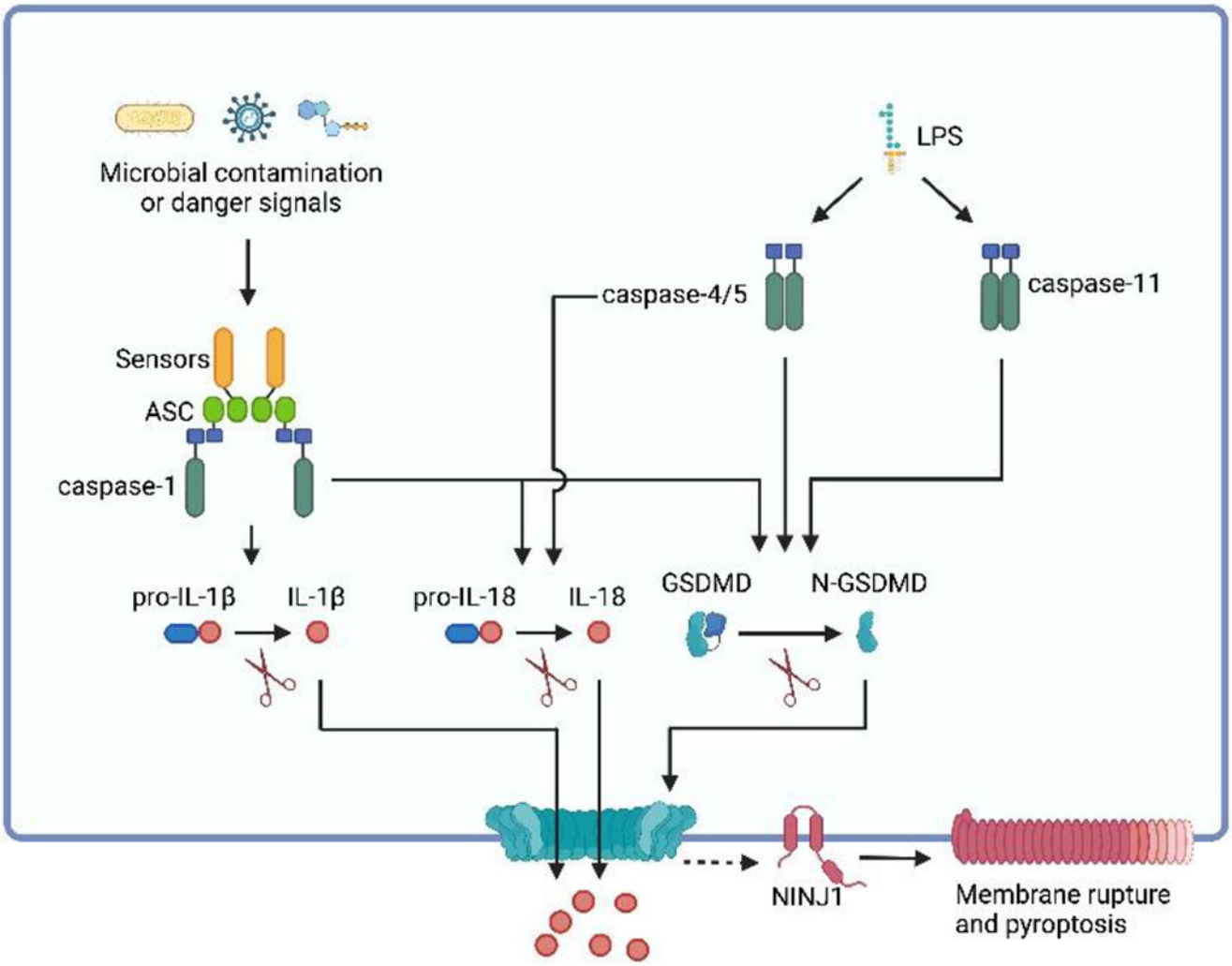

Figure 1. Inflammasome activation induces pyroptosis.

Inflammasomes are multiprotein complexes located in the cytosol that trigger the activation of caspase-1. Typically, an inflammasome comprises an inflammasome sensor and often the protein adaptor ASC. Inflammasome sensors are responsible for detecting various cellular disturbances, including microbial contaminants or danger signals. Upon polymerization, inflammasome sensors often recruit ASC, leading to the formation of ASC specks. Inflammasomes either directly or via ASC recruit pro-caspase-1 and trigger its activation. Once activated, caspase-1 cleaves GSDMD into its active form, N-terminal GSDMD (N-GSDMD). N-GSDMD polymerizes and forms pores in the plasma membrane, resulting in pyroptosis. NINJ1, a transmembrane protein, facilitates plasma membrane rupture in pyroptosis and other types of lytic cell death. Furthermore, caspase-1 also cleaves pro-IL-1β and pro-IL-18, generating active IL-1β and IL-18, respectively. These pro-inflammatory cytokines can be released from the GSDMD pores, along with other inflammatory cellular components. In parallel, caspase-4/5/11, are activated by cytosolic LPS. This activation leads to the cleavage of GSDMD, ultimately inducing pyroptosis. Notably, caspase-4/5 but not caspasae-11 can cleave pro-IL-18 into its mature form[156].

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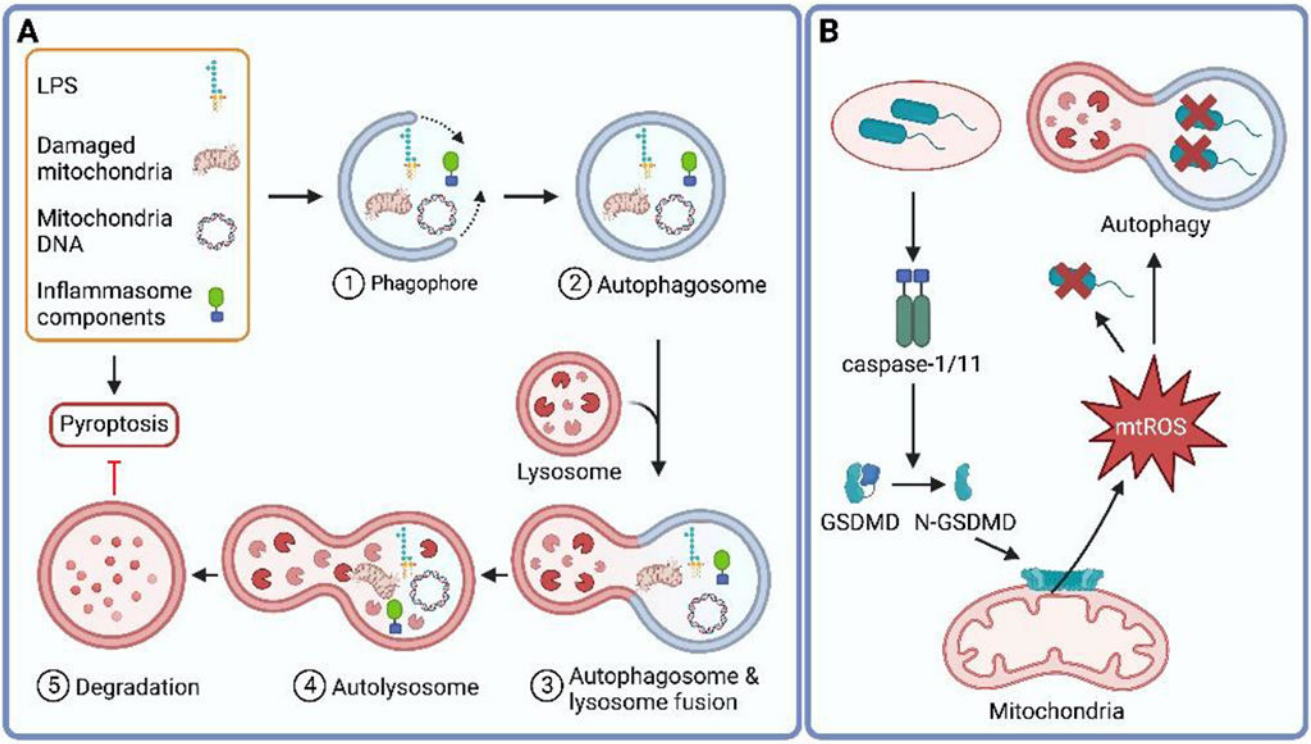

Figure 2. Interplay of autophagy and pyroptosis.

A. Autophagy is a cellular process that captures and degrades a variety of cellular materials, including damaged organelles, self-proteins, and pathogen-derived molecules. This process is initiated by the formation of a double-membrane structure called a phagophore, which expands and engulfs the targeted material to form an autophagosome. The autophagosome fuses with a lysosome to form an autolysosome, where the materials are degraded. Autophagy plays a role in inhibiting inflammasome activation by removing damaged mitochondria, mitochondrial DNA, cytosolic PAMPs, and inflammasome pathway components. **B.** In response to *Burkholderia cenocepacia* infection, the inflammasome can be activated by bacterial effectors that are secreted through the bacterial secretion system. Once activated, the inflammasome cleaves GSDMD, which then targets mitochondria to mediate the release of mitochondrial ROS (mtROS). mtROS can directly target cytosolic bacteria or promote the clearance of bacteria through xenophagy, a selective form of autophagy.

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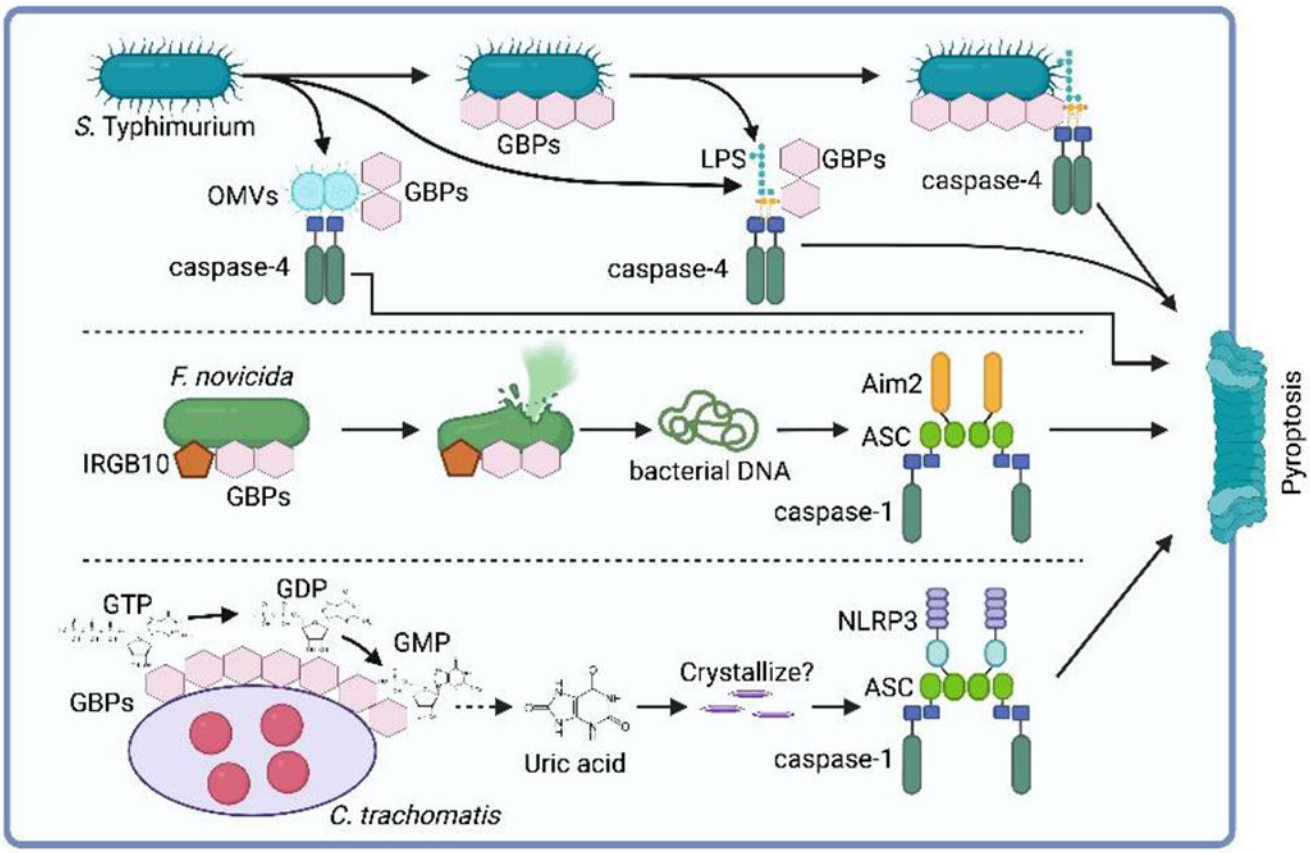

Figure 3. Regulation of pyroptosis by GBPs.

GBPs exhibit diverse mechanisms to activate inflammasomes in response to bacterial infections. GBPs can target bacteria in vacuoles or cytosol, as well as bind to released PAMPs such as LPS. GPBs facilitate caspase-4 activation in response to cytosolic free LPS or LPS packaged in outer membrane vesicles (OMVs). GBP-mediated caspase-4 activation can also occur by recruiting the caspase to the bacterial surface or by promoting the release of LPS into the cytosol. Additionally, GBPs, in conjunction with IRGB10, promote AIM2 inflammasome activation by facilitating bacterial lysis and liberation of bacterial DNA. Furthermore, GBP1 can activate the NLRP3 inflammasome during *C. trachomatis* infection by hydrolyzing GTP into GMP, which is then catabolized to uric acid that activates the NLRP3 inflammasome. However, it is unclear whether the uric acid concentration is sufficient for crystallization within the infected cell.

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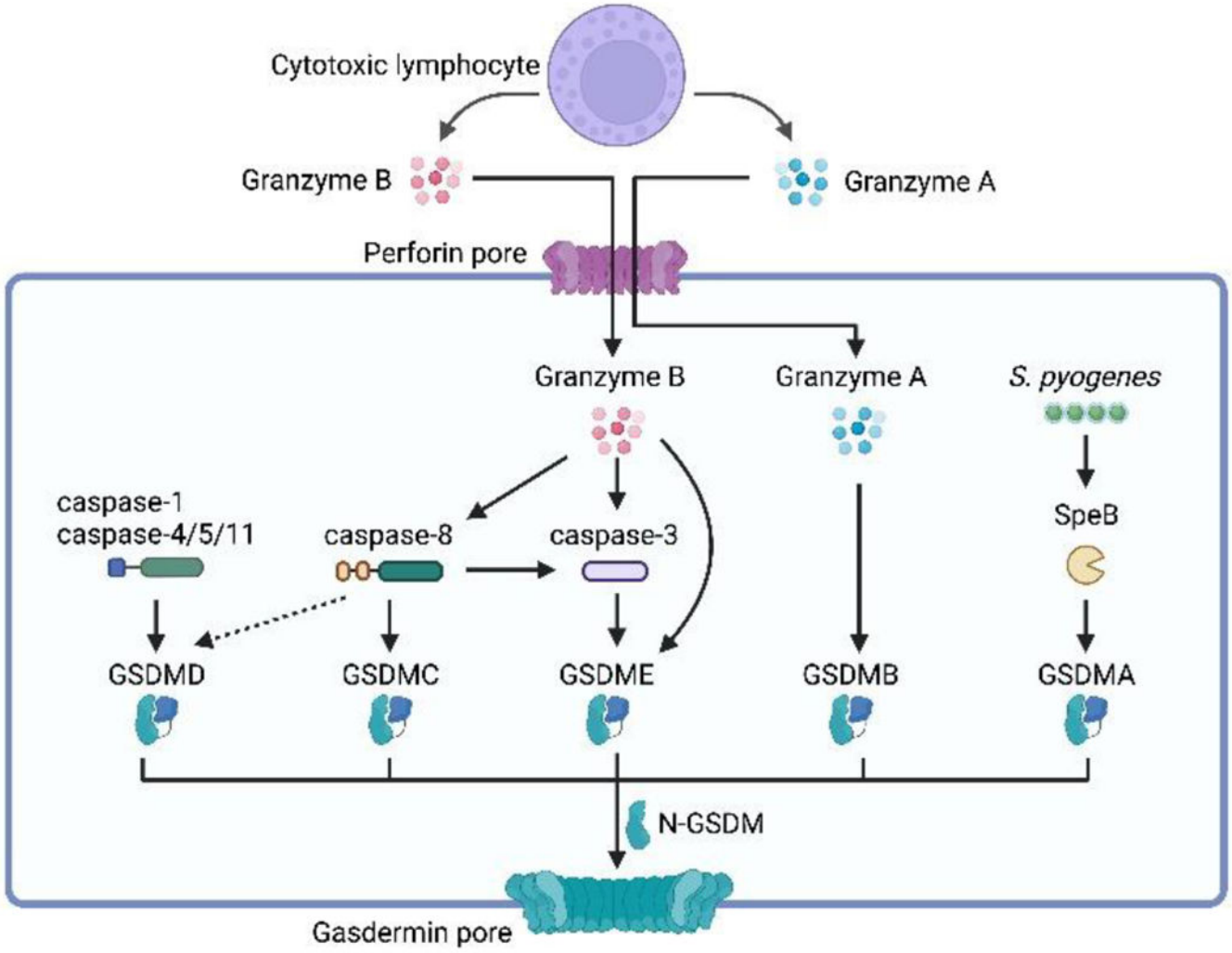

Figure 4. Activation mechanisms of gasdermins.

Pyroptosis is triggered through the cleavage of gasdermin proteins, which involves both inflammasome-dependent and inflammasome-independent pathways. Gasdermin D (GSDMD), the prototype of gasdermins, can be activated by inflammatory caspases, including caspase-1/4/5/11, through the inflammasome-dependent pathway. Gasdermins can also divert apoptotic signaling towards pyroptosis through their cleavage. Caspase-8, traditionally known as an initiator of apoptosis, can induce pyroptosis by cleaving GSDMD under specific conditions. Caspase-8 can also cleave GSDMC, which is primarily expressed in epithelial tissues, leading to pyroptosis. Caspase-3 cleaves GSDME, transitioning from caspase-3-mediated apoptosis to pyroptosis. Furthermore, granzyme B, produced by cytotoxic lymphocytes, can directly cleave GSDME, resulting in pyroptosis independent of caspase-3. Granzyme A, another protease produced by cytotoxic lymphocytes, activates GSDMB, triggering pyroptosis in target cells. In addition to host-derived proteases, pathogen-derived factors can also activate gasdermins. For instance, the cysteine protease virulence factor SpeB produced by *Streptococcus pyogenes* cleaves GSDMA, inducing pyroptosis in keratinocytes.

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Table 1.

ΔLD100 in inflammasome-deficient mice.

The calculation of ΔLD100 requires publications that investigate WT and inflammasome-deficient mice and utilize multiple doses within a single study[155]. In cases where only a single dose is administered, we rely on estimations based on studies conducted by the Re lab. Specifically, during *Burkholderia pseudomallei* infection, the Re lab demonstrated that 100% of WT mice survived a very low dose challenge, whereas 0% of inflammasome-deficient mice survived. Notably, when the Re lab increased the dose from 25 CFU to 200 CFU, 0% of WT mice survived. This data indicates an 8-fold change in the lethal dose. Therefore, if a single dose is used and all WT mice survive while all inflammasome-deficient mice succumb, it can only be interpreted as an eight-fold or greater change in the lethal dose. Notably, most published infection studies utilize only a single infectious dose[8,9]. Consequently, the full extent of the effect of inflammasomes in preventing lethal infection remains to be fully quantitated.

	Pathogens	ΔLD100	References
Host-adapted	*Salmonella* Typhimurium	1	[77,78]
	Listeria monocytogenes	>2	[154]
	other examples	>8	[8,9]
Environmental	*Burkholderia thailandensis*	2,000,000	[85]
	Chromobacterium violaceum	100,000	[91]