Activation and manipulation of inflammasomes and pyroptosis during bacterial infections

Elliott M. Bernard[1], Petr Broz[1*]

1: Department of Biochemistry, University of Lausanne, Epalinges, Switzerland
* Corresponding author: petr.broz@unil.ch

Abstract

Following detection of pathogen infection and disrupted cellular homeostasis, cells can activate a range of cell death pathways, such as apoptosis, necroptosis and pyroptosis, as part of their defence strategy. The initiation of pro-inflammatory, lytic pyroptosis is controlled by inflammasomes, which respond to a range of cellular perturbations. As is true for many host defence pathways, pathogens have evolved multiple mechanisms to subvert this pathway, many of which have only recently been described. Herein, we will discuss the mechanisms by which inflammasomes sense pathogen invasion and initiate pyroptosis and the effector mechanisms used by pathogens to supress this pathway and preserve their niche.

Introduction

Throughout human evolution our bodies have been challenged by infections with a wide range of bacterial, viral, fungal, and parasitic pathogens. As a result of this selection pressure, we have evolved multiple mechanisms for detecting these invading pathogens and defending ourselves against them. From cell autonomous processes such as selective targeting of cytosol-invading bacterial pathogens to autophagy [1] and induction of cell death [2], to the development of the innate and adaptive immune systems, humans, and indeed all mammals, have a well-stocked arsenal to tackle infectious challenge.

One key effector of the cell autonomous and innate immune response is the ability of infected cells to undergo cell death through a range of modalities including apoptosis, necroptosis, autophagic cell death and pyroptosis [2]. Whilst apoptosis is widely considered an anti-inflammatory and immunologically silent event, both pyroptosis and necroptosis are forms of necrotic, inflammatory cell death associated with cellular lysis and the release of cellular debris and cytokines into the tissue microenvironment. How these different cell death pathways are initiated in the context of pathogen infection, and what role they play in organismal defence has been a field of intense study over recent decades.

In order to initiate pyroptosis the presence of intracellular pathogens must be sensed, which is achieved through the recognition of pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) or perturbations to cellular homeostasis by a wide range of inflammasome sensor proteins. The currently known canonical inflammasome sensors comprise PYRIN, members of the nucleotide-binding oligomerisation domain and leucine rich repeat-containing (NLR) family, the pyrin and HIN (PYHIN) domain-containing protein absent in melanoma 2 (AIM2) [3], and caspase activation and recruitment domain 8 (CARD8) [4,5]. Whilst the PYHIN domain-containing protein IFI16 has been reported to form an inflammasome during Kaposi sarcoma-associated herpesvirus infection [6], it has been demonstrated that IFI16 dimerisation is unable to drive cell death, unlike what is observed for AIM2, suggesting that IFI16 cannot form a functional inflammasome [7]. Between them, these sensors are able to recognise PAMPs including nucleic acids such as DNA and RNA, cell wall components such as lipoteichoic acid (LTA), and the activity of secreted bacterial effectors and toxins (**Table 1**) [2,8,9]. Once activated, these sensor proteins initiate the formation of the inflammasome, a large multiprotein assembly consisting of the receptors, the adaptor protein ASC, and the effector protease caspase-1 (**Figure 1**) [3]. In addition, a non-canonical inflammasome, consisting of caspase-11 in mice and caspase-4/5 in humans, has been described to sense lipopolysaccharide (LPS) from Gram-negative bacteria and directly initiate pyroptosis without the need for ASC or caspase-1 [10–13]. Following inflammasome assembly, the caspases undergo auto-processing into their active forms, which is essential for their ability to bind and cleave their downstream substrates [14–16].

Inflammasome	Activator(s)	Example bacterial pathogens	*In vivo* knockout phenotype
NLRC4	Flagellin T3SS components	*S.* Typhimurium *S. flexneri* (Mouse only)	Increased susceptibility to infection by *S.* Typhimurium (Oral), *K. pneumoniae*, *C. rodentium*, *S. flexneri*
NLRP1	*B. anthracis* Lethal Factor Viral proteases dsRNA	*B. anthracis* *S. flexneri*	Increased susceptibility to infection by *B. anthracis*
NLRP3	K^+ efflux Mitochondrial ROS Lysosomal damage Pore forming toxins (e.g., LLO, SLO)	*M. tuberculosis* *L. monocytogenes* *S. aureus*	No effect during *M. tuberculosis infection* Variable effect during *S. aureus* infection
NLRP6	dsRNA LTA	*L. monocytogenes* *C. rodentium*	Enhanced resistance to *L. monocytogenes* and *C. rodentium*
AIM2	dsDNA	*L. monocytogenes* *M. tuberculosis* *F. novicida*	More susceptible to *F. novicida* and *M. tuberculosis* infection
PYRIN	RHOA inactivation Steroid catabolites	*Y. pestis* *C. difficile* *H. somni* *V. parahaemolyticus* *B. cenocepacia*	Increased susceptibility to *Y. pestis, Y. pseudotuberculosis* and *B. cenocepacia*
Caspase-4 (-11)	LPS	*S.* Typhimurium *B. thailandensis* *S. flexneri*	Resistant to LPS induced sepsis

Table 1: Inflammasomes, their activation signals, and relevant pathogens.

Once activated, caspase-1 cleaves its substrates, including the pro-forms of interleukin (IL)-1 family cytokines, IL-1β and IL-18, and the pore-forming protein gasdermin-D (GSDMD). Following proteolytic cleavage, the N terminus of GSDMD can homo-oligomerise into a large pore with an inner diameter of around 21.4 nm in the plasma membrane [17]. The formation of these pores disrupts cellular ion homeostasis and osmotic balance leading to ballooning of the cells, as well as facilitating the rapid secretion of IL-1β [18]. In the final steps of pyroptotic cell death, and indeed other forms of lytic cell death, ninjurin-1 (NINJ1), is recruited to the plasma membrane, where it is hypothesised to assemble oligomers that lead to complete cellular rupture and, in the context of pyroptosis but not necroptosis, the release of larger cytosolic content and DAMPs (**Figure 1**) [19]. It is worth noting that while *NINJ1*-deficieny blocks DAMP release it does not protect inflammasome activated cells from dying, as the accumulation of GSDMD pores in cellular membranes will eventually be lethal. How NINJ1 becomes activated downstream of GSDMD during pyroptosis, and indeed other lytic cell death

modalities, remains unknown. Given that NINJ1 appears to be the final effector of cellular lysis downstream of multiple pathways it is likely a common mechanism of activation exists; the currently favoured model is that changes in plasma membrane tension, curvature, or composition as a result of swelling facilitate NINJ1 activation.

The efficient and controlled induction of pyroptosis is a critical event in the control of many intracellular bacterial pathogens, as evidenced by the hyper-susceptibility of mice with genetic defects in inflammasome activation to multiple pathogens [20–26]. The protective effect of pyroptosis is likely a result of multiple mechanisms, more thoroughly discussed in [2]. Through destroying infected cells, pyroptosis eliminates the intracellular replicative niche harnessed by these pathogens as a strategy to utilise host nutrients and escape detection by the innate immune system. Moreover, as a result of pyroptosis, there is a massive release of cytokines and DAMPs, which serve to recruit and activate further cellular components of the innate and adaptive immune response, for example IL-1 family cytokines have wide ranging effects on cells of both the innate and adaptive immune system [27]. Additionally, pyroptotic cells infected with *Salmonella* Typhimurium or *Shigella flexneri* are extruded from epithelial monolayers thus restoring barrier integrity, for example in the gut [28,29]. Bacterial pathogens within pyroptotic cells fail to escape the corpses and are trapped within pore-induced intracellular traps (PITs). These PITs are then efficiently cleared following efferocytosis by neutrophils with ROS mediated killing of the pathogen [30]. In neutrophils, inflammasome activation and cell death are linked with the production of neutrophil extracellular traps (NETs) to restrict bacteria [31]. The effect of pyroptosis on neighbouring bystander cells is just beginning to be explored and is likely to provide novel insights into the protective role of pyroptosis [32]. Furthermore, active GSDMD has been shown to directly target bacteria, including *E. coli* and *Burkholderia thailendensis, in vitro* however the relevance and importance of this *in cellulo* and *in vivo* remains to be determined [33,34]. While inflammasomes are important for host defence, over- or mis-activation of inflammasomes can also be detrimental to the organism. For example, LPS-induced lethality, a murine model for septic shock syndrome, is driven by caspase-11 activation [10,35], and patients with activating mutations of inflammasome components suffer from autoinflammatory conditions [36–38].

In this review we will discuss recent advances in the identification of bacterial ligands for inflammasome activation and how they can be liberated by the host and sensed by a range of inflammasome sensor proteins. Furthermore, we will explore emerging ways in which bacterial pathogens seek to subvert inflammasome activation to prevent pyroptosis and destruction of their intracellular niches.

NLRs

The NLR family of inflammasome sensors were some of the first to be discovered and described. All those that are currently known to be competent at forming an inflammasome contain a leucine rich repeat (LRR) domain and a nucleotide binding domain (NBD). The NBD is important for oligomerisation of the NLR, an essential step in inflammasome activation. The LRR most often confers autoinhibition to prevent activation of the inflammasome without a stimulus, defects in which cause autoinflammatory conditions. The family can be split into two smaller groups: those with a pyrin domain are known as NLRPs and those with a caspase activation and recruitment domain (CARD) as NLRCs.

NLRC4. The best studied member of the NLRC family is NLRC4, which is activated by a range of proteins only found in bacteria**.** Many pathogens encode secretion systems to translocate effector proteins into the host cell. For example, the type 3 secretion systems (T3SS) of Gram-negative pathogens such as *Salmonella enterica* serovar Typhimurium form a needle like complex to translocate effectors essential for virulence [39]. Components of these T3SSs can be detected by NLR family, apoptosis inhibitory proteins (NAIPs), with murine NAIP2 binding the rod protein and NAIP1 binding the needle protein, which facilitate NLRC4 activation [40–43]. Furthermore, flagellin, the protein component of flagella that allow bacterial motility, can bind to murine NAIP5/6 to activate NLRC4 [40,41]. In humans, where there is a single NAIP gene, one isoform is able to bind to all three of these ligands from pathogens including *S.* Typhimurium*, S. flexneri* to activate NLRC4 [44].

Nlrc4$^{-/-}$ mice are more susceptible to infection with a range of Gram-negative bacteria, including *S.* Typhimurium [21], *Klebsiella pneumoniae* [45], and *Citrobacter rodentium* [46]. The route of infection is important in determining the importance of NLRC4, during intraperitoneal infection of *S.* Typhimurium *Nlrc4*$^{-/-}$ mice show no difference to wild type controls but following oral challenge *Naip*$^{-/-}$ or *Nlrc4*$^{-/-}$ mice are more susceptible and display higher bacterial burdens [20,47], likely to due to differential expression of flagellin and T3SS components during intracellular versus extracellular infection. Moreover, NLRC4 activation in intestinal epithelial cells is important for restricting migration of *S.* Typhimurium from the gut to draining lymph nodes as well as in facilitating extrusion of infected cells [29,47,48]. Wild type mice are highly resistant to the development of shigellosis during *S. flexneri* infection; however, it was recently shown that mice deficient in NAIP or NLRC4 develop shigellosis. This inflammasome is not activated by *S. flexneri* in human infection, and in mice the activation of NLRC4 in intestinal epithelial cells is sufficient to protect against disease [49], Disruption of the NLRC4 inflammasome or IL-1 signalling provides enhanced protection against pneumonia during *P. aeruginosa* infection [50].

NLRP1. The human genome encodes for only one isoform of NLRP1, whereas in mice three paralogs exist, NLRP1a-c, with NLRP1b being the most studied and most likely the functional homolog of human NLRP1. It was initially shown that *Bacillus anthracis* lethal factor (LF) cleaves NLRP1b at its N-terminus leading to pyroptosis [51–53]. Mechanistically, the N-terminus is then ubiquitinated, following the N-end rule, and degraded by the proteasome. As NLRP1 is auto processed within its function to find (FIIND) domain, the C-terminus, containing the CARD, is released upon N-terminal degradation and can oligomerise and engage caspase-1 to form the active inflammasome complex [54,55]. *S. flexneri* also activates NLRP1 through ubiquitination by the effector E3 ubiquitin ligase IpaH7.8, leading to proteasomal degradation of the N-terminus [54]. Human NLRP1 is also activated during infection with viruses such as human rhinovirus and other picornaviruses, whereby viral proteases cleave NLRP1, in a manner akin to that of LF, in airway epithelia [56,57]. Non-proteolytic activation of human NLRP1 has also been reported, whereby long double stranded RNA (dsRNA) binds to NLRP1 to bring about ATP hydrolysis by the NACHT domain of NLRP1 lead to activation and pyroptosis [58]; this is important during RNA virus infection, where large amounts of long dsRNA are generated in the cytoplasm. Importantly, murine NLRP1b is not activated by dsRNA but is activated by viral proteases [57,58].

NLRP1 activation is negatively regulated through an interaction with the dipeptidyl peptidases DPP8 and DPP9. Recent studies elucidated the structure of NLRP1 in complex with DPP9 and revealed that DPP9 forms a ternary complex with one copy of the C terminus of NLRP1 and one copy of full length, autoinhibited NLRP1 [59,60]. The formation of this complex prevents the accumulation of sufficient quantities of free C-terminal NLRP1 to oligomerise and drive inflammasome activation. Small molecular inhibitors of DPP8/9, such as Val-Boro-Pro, activate NLRP1, however how DPP8/9 are inhibited during activation of NLRP1 by the signals described above remains unknown.

In vivo, mice expressing a mutant of NLRP1b that cannot be cleaved by LF are hypersusceptible to infection with *B. anthracis* spores, demonstrating the importance of activation of this inflammasome in defence at the organismal level [61,62].

NLRP3. The activation of the NLRP3 inflammasome requires a two-step process. Firstly, a priming signal, such as signalling through TLRs, is needed to both transcriptionally upregulate NLRP3 as well as prime it for activation by post-translational modifications, such as altering the phosphorylation and ubiquitination state [63]. Subsequently, a second, activation signal, is required for the assembly of an inflammasome complex and induction of pyroptosis. A wide

range of activation signals have been described, including disruption of the trans-Golgi network (TGN) [64], disrupted cellular ion homeostasis, especially K^+ efflux [65], cathepsin leakage from damaged lysosomes [66], and mitochondrial dysfunction and ROS production. However, whilst several of these triggers converge on K^+ efflux, a unifying mechanism linking these triggers and those that are independent of K^+ efflux remains elusive [63,67,68]. The ability of cathepsin leakage to trigger NLRP3 activation remains debated, as studies have demonstrated that knockout or knockdown of individual cathepsins does not lead to a defect in NLRP3 activation following lysosomal damage [69]. This could be explained through redundancy with other cathepsins activating NLRP3, for example knockout or knockdown of several cathepsins in parallel has been demonstrated to be required to impact IL-1β secretion following NLRP3 activation by sterile triggers such as silica crystals [69]. The role of the TGN in NLRP3 activation was further elucidated in a recent structural study [70]. In the resting state, NLRP3 localises to the TGN where it forms an inactive oligomeric cage structure that sequesters the pyrin domains inside the cage to prevent activation. This localisation and cage formation is critical to allow the dispersal of the TGN that occurs downstream of several NLRP3 activating stimuli and is crucial for NLRP3 transport to the centrosome for activation [64,70,71]. Based on these most recent data, the current model for NLRP3 activation suggests that downstream of either K^+ efflux dependent or independent signals the TGN becomes dispersed to allow NLRP3 trafficking for assembly into an active inflammasome. How these diverse signals lead to TGN dispersal is a major question remaining in the field. Given that TGN dispersal requires NLRP3 [64,70], this could imply that NLRP3 must still sense these diverse signals to bring about TGN vesicle formation and how this might happen is unknown.

In the context of bacterial infection, direct activation of NLRP3, as opposed to through the non-canonical inflammasome (discussed later), is most often mediated by plasma membrane damage resulting in disrupted ion homeostasis. Pore forming toxins, such as listeriolysin O (LLO) [72,73], streptolysin O (SLO) [74], and α-hemolysin [75] from *Listeria monocytogenes, Streptococcal* species, and *Staphylococcus aureus* respectively, among others [76], form pores in the plasma membrane leading to K^+ efflux thus activating NLRP3. LLO is also able to release Cathepsin B from *L. monocytogenes* containing phagolysosomes to activate NLRP3 [77]. Furthermore, *Mycobacterium tuberculosis* damages the plasma membrane of infected macrophages through a mechanism potentially involving its T7SS ESX-1, which also results in activation of NLRP3 and pyroptotic death [78,79].

The importance of NLRP3 *in vivo* is best demonstrated in the context of the non-canonical inflammasome and will be discussed later. However, in the context of *M. tuberculosis* infection, *Nlrp3*$^{-/-}$ mice show no increased susceptibility, potentially due to IL-1β release that is NLRP3

independent [80]. Meanwhile, during *S. aureus* infection both protective and detrimental effects of NLRP3 activation by α-hemolysin have been observed, likely due to differences in infection routes [81,82]. During skin infection, neutrophil derived IL-1β is important for host defence [81] whereas during pulmonary infection *Nlrp3*$^{-/-}$ mice developed less severe pneumonia [82].

NLRP6. At the organismal level, NLRP6 is most highly expressed in the intestine [83], whilst at the cellular level both immune and epithelial cells express *Nlrp6* [84], and thus this inflammasome been linked with the outcome of infections with enteric pathogens [85]. Currently, NLRP6 has been described to be activated in the context of both Gram-positive and Gram-negative bacterial infection, as well as during RNA virus infection [8,9,86]. During *L. monocytogenes* infection of mouse macrophages it has been proposed that NLRP6 recognises LTA in the bacterial cell wall through its LRR domain, leading to an unconventional activation of caspase-11 via the recruitment to ASC specks, and pyroptosis [8]. The activation of caspase-11 by NLRP6 leads to downstream activation of caspase-1 for IL-18 secretion, although the mechanism for this is not discussed it may be through NLRP3 activation as seen in the non-canonical inflammasome, discussed later. During viral infection of primary mouse hepatocytes, for example with mouse hepatitis virus, binding of long double-stranded RNA to the NACHT-LRR region of NLRP6 induces liquid-liquid phase separation to drive inflammasome assembly and caspase-1 activation [9]. NLRP6 also dimerises upon binding of *E. coli* LPS and is able to recruit ASC following addition of ATP, however whether this is sufficient to drive inflammasome activation remains unknown [87]. *Nlrp6*$^{-/-}$ mice also show reduced inflammasome activation by the Gram-negative pathogen *Citrobacter rodentium* [88].

In vivo, NLRP6 activation is detrimental to mice during challenge with intravenous (IV) *L. monocytogenes* or *S. aureus*, as *Nlrp6*$^{-/-}$ mice show reduced bacterial burdens and better survival, which was mediated by loss of IL-18 production [8]. This data is further supported by data showing that *Nlrp6*$^{-/-}$ mice are resistant to *L. monocytogenes* infection by the intraperitoneal (IP) route, potentially as a result of increased NFκB dependent cytokine production by circulating monocytes [84]. However, during *Citrobacter* infection through oral gavage, *Nlrp6*$^{-/-}$ mice show increased body weight loss, diarrhoea and inflammation [88]. Interestingly, knockout of the deubiquitinase CYLD enhances NLRP6 activation and also leads to worse disease outcomes than in wild type controls, highlighting the importance of balanced inflammasome activation to prevent excess tissue damage and inflammation [88]. This difference in the role of NLRP6 with *L. monocytogenes* and *C. rodentium* could be due to the difference between Gram-positive and Gram-negative pathogens but could also be a result of

the different infection routes used. Infection via the oral route, as was performed for *C. rodentium*, will lead to delivery to the intestinal lumen where NLRP6 is likely activated in intestinal epithelial cells. On the other hand, IV or IP infection will lead to a more systemic infection without the need to infect intestinal epithelial cells or disrupt the intestinal barrier.

The AIM2 inflammasome responds to double stranded DNA

Due to compartmentalisation, double stranded DNA (dsDNA) in eukaryotic cells is maintained with the nucleus or mitochondria and is not exposed to the cytosol. Therefore, detection of dsDNA in the cytosol serves as an alarm signal for disrupted cellular homeostasis due to pathogen infection, such as DNA viral infection, or breakdown in nuclear membrane or mitochondrial integrity. In the context of inflammasomes, AIM2 is the sensor of dsDNA, which binds to the HIN200 domain of AIM2. DNA binding leads to AIM2 clustering, recruitment of ASC and activation of caspase-1 driven pyroptosis.

Bacterial pathogens including *Francisella novicida*, *L. monocytogenes*, and *M. tuberculosis* have all been shown to activate the AIM2 inflammasome [79,89,90]. The localization of bacterial DNA to the host cell cytosol requires bacteriolysis. Indeed, mutations affecting the integrity of the bacterial cell wall that lead to more frequent spontaneous bacteriolysis lead to increased AIM2 activation by both *F. novicida* and *L. monocytogenes* [90,91].

During the infection of mouse macrophages with *F. novicida* the bacteria rapidly enter the cytosol where they are targeted by interferon-inducible guanylate binding proteins (GBPs) [92]. GBP recruitment to *F. novicida* facilitates the recruitment of another interferon inducible GTPase, IRGB10. IRGB10 is able to access the inner bacterial membrane where it brings about bacteriolysis releasing dsDNA into the cytosol for detection by AIM2 [93]. How the DNA of *L. monocytogenes* and *M. tuberculosis* access the cytosol is less well understood. Whilst it was initially proposed that spontaneous lysis of *L. monocytogenes* is the cause of DNA release [90,94], it was later proposed that lysozyme may play a role here [94]. For *M. tuberculosis*, the release of DNA requires the ESX-1 type 7 secretion system [79]. However, as this system is required for phagosomal rupture and access of the bacteria to the cytosol [95,96] it is possible that other host factors are required for the release of mycobacterial DNA, rather than ESX-1 directly secreting DNA into the cytosol. Further studies to identify novel host proteins capable of directly lysing bacteria, especially in human cells where IRGB10 is not found, are required to more completely understand how different pathogens trigger AIM2 activation.

Not only is AIM2 important for the induction of pyroptosis during *F. novicida* infection by directly nucleating ASC specks and activating caspase-1, but it is also required for the activation of

other inflammasomes and cell death modalities. It was recently demonstrated that following AIM2 activation both PYRIN and ZBP1 are recruited into the same ASC speck as AIM2 and knockout of either PYRIN or ZBP1 reduces cell death. Not only was pyroptosis affected, but the processing of the apoptotic caspases and activation of the necroptosis proteins RIPK3 and MLKL were also reduced in *Aim2*$^{-/-}$ macrophages. Thus, AIM2 serves as an initiator for inducing multiple cell death pathways in parallel [97]. How broadly applicable this pathway is, and whether similar pathways exist during infection with other pathogens remains to be tested.

The role of AIM2 *in vivo* during bacterial infection has only been minimally studied. In the context of *F. novicida* infection, *Aim2*$^{-/-}$ mice have higher bacterial burdens in the liver and spleen, as well as decreased survival [22,23]. During intratracheal infection with *M. tuberculosis Aim2*$^{-/-}$ mice demonstrate increased lethality and high bacterial burdens [24].

PYRIN responds to inactivation of the small GTPase RHOA

The protein PYRIN, encoded by the *MEFV* gene, forms an inflammasome and is activated by infection with multiple bacterial pathogens including *Clostridium difficile, C. botulinum, Yersinia pestis, Y. pseudotuberculosis, F. novicida, B. cenocepacia, Vibrio parahaemolyticus* and *Histophilus somni* [98–101]. Toxins secreted by these bacteria activate the PYRIN inflammasome by inhibiting the activity of the small GTPase RHOA. The most well described PYRIN activating toxins include: TcdB (*C. difficile*), C3 (*C. botulinum*), TecA (*B. cenocepacia*), IbpA (*H. somni*), VopS (*V. parahaemolyticus*), and YopE and YopT (*Y. pestis and Y. pseudotuberculosis*) [99–102]. These toxins employ a range of mechanisms including chemically modifying residues in the Switch-I region of the GTP binding domain, acting as a GTPase-activating protein, and cleaving RHOA from the plasma membrane, to inactivate RHOA. Given that PYRIN is activated by a range of modifications to RHOA and that PYRIN does not directly interact with RHOA [101], it was hypothesised that, rather than sensing RHOA modification directly, PYRIN likely detects a downstream effect. More recently, it has been shown that RHOA mediated activation of the Ser/Thr protein kinases PKN1 and PKN2 leads to phosphorylation of PYRIN and its interaction with 14-3-3 proteins to maintain it in an inactive state [37,103,104]. Thus, inactivation of RHOA by bacterial toxins blocks PYRIN phosphorylation and prevents the inhibitory interaction with 14-3-3 proteins, whether a phosphatase is required for dephosphorylation to facilitate this step is unknown.

The dephosphorylation of PYRIN in monocytes from healthy human donors is insufficient to drive full activation, whereas it is sufficient in monocytes from sufferers of Familial Mediterranean Fever, who have mutations in PYRIN [105,106]. This data suggests that whilst dephosphorylation is a priming step for activation a second signal is required for assembly

and activation of wild-type PYRIN. Functioning microtubule dynamics are required downstream of dephosphorylation for PYRIN induced pyroptosis, likely because retrograde transport is required for PYRIN oligomerisation at the centrosome [103,107], however the precise nature of this second signal has remained elusive. Recently, steroid catabolites have been shown to facilitate PYRIN activation of constitutively primed PYRIN and thus function as the second signal in human monocytes [108]. Interestingly, activation of PYRIN by these catabolites was independent of RHOA inactivation and specific to human PYRIN, as mouse PYRIN does not contain the B30.2 domain that is sensitive to the catabolites. Whether further, unknown, ligands also facilitate PYRIN activation downstream of dephosphorylation in the context of pathogen infection remains to be determined.

In vivo, *Mefv*$^{-/-}$ mice display increased susceptibility to *B. cenocepacia* [99]. PYRIN induced pyroptosis is also important for defence against *Yersinia*, as both *Mefv*$^{-/-}$ and *Il18*$^{-/-}$ mice are hypersusceptible to *Y. pestis* [25] and *Y. pseudotuberculosis* mutants unable to block PYRIN activation are attenuated *in vivo* [100,109].

The non-canonical inflammasome initiates pyroptosis in response to LPS from Gram-negative bacteria

LPS is a well-known, highly immunostimulatory molecule found in the outer membrane of all Gram-negative pathogens. It consists of a lipid A moiety, which is a disaccharide that is usually hexa- or hepta-acylated and sits within the outer membrane, and a variable extracellular O-antigen of many repeating sugar units. Whilst extracellularly LPS has long been known to be recognised by receptors such as TLR4, MD2 and CD14, for many years a sensor of intracellular LPS was unknown. Seminal work revealed that human caspase-4/5 and murine caspase-11 recognise cytosolic LPS by binding the lipid A moiety and directly cleave GSDMD to bring about pyroptosis, without the need for the adaptor protein ASC [11,13,110]. Whilst caspase-4 is essential for the non-canonical inflammasome in macrophages and epithelial cells, the role of caspase-5 is less clear. Following LPS exposure, monocytes cleave caspase-5 but not caspase-4, however knockdown of either diminishes their ability to secrete IL-1β following extracellular LPS exposure [111]. Unlike caspase-1, the caspases of the non-canonical inflammasome cannot cleave pro-IL-1β and therefore its processing and secretion rely on the secondary activation of the NLRP3 inflammasome resulting from K^+ efflux through the GSDMD pores [10].

In order to facilitate the recognition of LPS associated with the bacterial surface, incorporated into bacterial outer membrane vesicles (OMV), or transfected into cells using lipid-based reagents additional proteins are required. Priming cells, for example with IFN-γ or LPS, induces the expression of GBPs, of which there are 7 in humans and 11 in mice [112]. The formation of a coat of these GBPs on the bacterial surface is required for caspases to recognise the LPS and to drive pyroptosis [113–116]. In humans, hGBP1 is the gatekeeper and directly binds LPS to initiate coat formation [117–120]. This recruitment is followed by hGBPs 2, 3 and 4 which are required for the recruitment and activation of caspase-4. In the mouse system, the 5 GBPs on chromosome 3 (Gbp^{Chr3}), namely mGBPs 1, 2, 3, 5, and 7, are required for efficient caspase-11 activation, with $Gbp2^{-/-}$ macrophages showing a similar phenotype to those lacking all 5 of these GBPs, demonstrating that mGBP2 is critical. However, it remains unknown precisely which of the remaining 4 GBPs are also required for caspase-11 recruitment and activation. Further complexity is added as in the mouse system caspase-11 activation can still occur in the absence of the *Gbps* on Chr3, it is simply delayed [114,121], whereas in the human system caspase-4 activation absolutely requires GBPs [118].

How GBPs facilitate LPS sensing by the caspases remains unclear. Whilst it was initially thought the GBPs targeted the pathogen containing vacuole for lysis, thus facilitating access of the bacteria and therefore LPS to the cytosol [113], the localisation of GBPs directly on the bacterial surface suggests this is not the case. Indeed for bacteria including *S. flexneri*, *S.* Typhimurium and *F. novicida* cytosolic access has been shown to be independent of the GBPs and IFN-γ stimulated genes [92,117,122]. Moreover, kinetic analysis of membrane damage, as marked by Galectin recruitment, and GBP recruitment to *Salmonella* in THP-1 cells has shown that GBP recruitment occurs downstream of the membrane damage event and that Galectin-8 recruitment is not altered in $GBP1^{-/-}$ cells [120]. Indeed, binding and polymerisation of human GBP1 on the bacterial outer membrane is sufficient to destabilise the envelope to facilitate caspase-4 binding to lipid A as well as enhancing sensitivity to the antibiotic polymyxin B [123]. The structure of the GBP coatomer, recently published in a pre-print, revealed a 260 Å unfolding of human GBP1 during binding to the bacterial outer membrane, which is proposed to destabilise the bacterial membrane sufficiently to release LPS for caspase-4 sensing [124]. In mouse macrophages, GBP recruitment is coupled with bacteriolysis by IRGB10 [93]. This bacteriolysis liberates many PAMPs, including LPS for sensing by caspase-11, and IRGB10 knockout macrophages show decreased activation of the non-canonical inflammasome by pathogens, but not transfected LPS. In humans, there is no homologue of IRGB10 thus suggesting that the GBPs may act through a different mechanism here. Interestingly, the apolipoprotein APOL3 was recently identified as a novel

mediator of bacteriolysis in human macrophages, and this required additional IFN inducible factors, including GBP1, to disrupt the O-antigen and allow access of APOL3 to the inner membrane [125]. Whether knockout of APOL3 has an effect on non-canonical inflammasome activation remains to be tested. It is tempting to speculate that the human GBPs perturb the outer bacterial membrane to facilitate bacteriolysis by APOL3 to liberate LPS for caspase-4 sensing, however direct evidence of this is lacking.

More recently, further regulators of the non-canonical inflammasome have been identified. It has long been known that cells deficient in the autophagy pathway demonstrate increased sensitivity to LPS [126], this has been further expanded to demonstrate that the ATG8 family protein GABARAPL2 (also known as GATE-16) is required to control caspase-11 activation following gram-negative infection in mouse and human macrophages [127,128]. Furthermore, two groups recently identified a mouse-specific interferon-inducible GTPase, IRGM2, as a negative regulator of the non-canonical inflammasome with *Irgm2*$^{-/-}$ macrophages undergoing increased pyroptosis following LPS challenge [127,129]. GABARAPL2 and IRGM2 interact and are likely involved in the same pathway that restricts caspase-11 activation, however the mechanistic details of this pathway remain to be elucidated. Moreover, caspase-6 can cleave caspase-11 into its active form and *Casp6*-deficient cells have defects in caspase-11 activation in response to LPS [130]. Finally, GALECTIN-3, a cytosolic β-galactoside binding protein linked with responses to endomembrane damage [1,131], associates with LPS bound to caspase-4/11 and promotes their oligomerisation and activation and enhancing pyroptosis [132].

During infection with several Gram-negative pathogens, *Casp11*-deficiency increases susceptibility. *Casp11*$^{-/-}$ mice have increased mortality and higher bacterial burdens following challenge with pathogens such as *S.* Typhimurium [26], and *Brucella abortus* [133]. Interestingly, activation of caspase-11 in the absence of caspase-1 during *S.* Typhimurium infection *in vivo* leads to a dramatic increase in bacterial burdens in the liver and spleen due to decreased neutrophil-mediated clearance of bacteria released from pyroptotic macrophages and increased extracellular replication of *S.* Typhimurium [134]. On the other hand, activation of the non-canonical inflammasome is linked with sepsis, a severe inflammatory condition that results in multi-organ dysfunction and eventually death, during Gram-negative infection. Mice lacking caspase-11, or GSDMD, are protected from lethality following high-dose intraperitoneal injection with LPS as well as during caecal ligation and puncture [10,35]. Recently, it was shown that caspase-11 activation in monocytes and macrophages, but not neutrophils, is mostly responsible for LPS induced sepsis whereas

during *B. thailendensis* infection activation of caspase-11 in macrophages and neutrophils is protective but in dendritic cells or intestinal epithelial cells plays no role in host defence [135].

Subversion of inflammasome activation by bacterial pathogens

As successful intracellular pathogens, bacteria must subvert host defence mechanisms to preserve their replicative niche. In the context of pyroptosis, it is becoming increasingly appreciated that pathogens deploy a wide range of effectors that target this pathway at multiple stages to prevent both destruction of their host cell as well as to block the release of DAMPs and cytokines that would recruit further components of the immune system.

Several pathogens employ mechanisms to block the recognition of their PAMPs by the inflammasome sensors (**Figure 2**). *F. novicida* lipid A is tetra-acylated to reduce its ability to activate caspase-11, a mechanism similarly employed by *Helicobacter pylori* and *Y. pestis* [13,110]. This mechanism is not 100% effective as, in *Aim2*$^{-/-}$ BMDMs, caspase-11 knockdown slows down pyroptosis and reduces IL-1β secretion following *F. novicida* infection [136]. However, caspase-4 is still activated by this hypo-acylated LPS [137]. *S. flexneri* is able to ubiquitylate the GBPs following coating through the secreted E3 ligase IpaH9.8, leading to disassembly of the coat and thus rendering LPS recognition by caspase-4 ineffective [122,138]. *Y. pseudotuberculosis* fails to activate the NLRP3 and NLRC4 inflammasomes due to the virulence factor YopK, which interacts with the translocon of the T3SS to prevent its recognition [139]. Moreover, *S.* Typhimurium genetically lacking the SPI2 T3SS show enhanced SPI1 mediated activation of NLRC4 and NLRP3 in human macrophages, suggesting an active blockade of inflammasome activation by SPI2 or its secretion substrates [140].

Direct targeting of pyroptotic effector proteins such as the inflammatory caspases and GSDMD is employed by both intra- and extracellular pathogens to inhibit inflammasome activation (**Figure 2**). The *S. flexneri* effector OspC3 ADP-Riboxinates key arginine residues in caspase-11 and caspase-4 to prevent both their auto-processing and GSDMD binding in response to LPS [141–143]. Intriguingly, the OspC3 interaction with caspase-11 requires IFN-γ stimulation and is enhanced following cytosolic delivery of LPS, suggesting that OspC3 specifically inhibits activated caspase-11. The extracellular enteric pathogen EPEC utilises the effector NleF, which is injected into cells by the T3SS, to inhibit caspase-4 activation by, for example, OMVs [144]. Potentially one of the most efficient mechanisms to prevent pyroptosis is to target the terminal effector of the pathway, GSDMD, to block pyroptosis induced by separate inflammasomes. For example, the *S. flexneri* effector IpaH7.8 ubiquitinates human, but not murine, GSDMD to drive proteasomal degradation to block pore formation [145]. *Y. pestis and*

Y. pseudotuberculosis prevent activation of PYRIN following inactivation of RHOA through the secreted effector YopM, which directly activates Protein Kinase C family members, including PKN1/2 to maintain levels of PYRIN phosphorylation [25,100]. YopM can also inhibit pyroptosis by directly binding and inhibiting caspase-1 [146]. Further, YopM and YopJ doubly-deficient *Yersinia* elicit higher levels of pyroptosis than the YopM single mutant, demonstrating the YopJ also constrains PYRIN inflammasome activation although the mechanism remains to be determined [109,147].

As well as inhibiting inflammasome activation in order to facilitate pathogenesis, some pathogens use inflammasome activation to supress other host-protective responses. For example, *Pseudomonas aeruginosa* has adapted to subvert the host immune response by inducing pyroptosis in immune cells, especially alveolar macrophages, whilst remaining extracellular [50,148]. Through the action of their T3SS and flagellin, *P. aeruginosa* can activate the NLRC4 inflammasome leading to IL-18 secretion, which dampens a host-protective IL-17 mediated response [148].

However it is achieved, blockade of pyroptosis likely requires other effectors to block alternative cell death modalities to be effective. As is seen in many cases, removal of one cell death pathway leads to the alternative activation of another, for example loss of caspase-1 or GSDMD often results in apoptosis following a normally pyroptotic stimulus [149,150].

Future perspectives

There are several areas of research that show potential to provide exciting new insights into the mechanisms and consequences of pyroptosis during bacterial infection. Whilst new mechanistic insight into how buried PAMPs, such as lipid A and bacterial DNA, are liberated in both humans and mice has developed over recent years, several questions remain. For example, whilst we know that bacteriolysis occurs in the mouse and have identified some key players in this pathway, the importance of this in the human system is as yet underappreciated and novel bacteriolysis factors undoubtedly remain to be discovered. There are also several NLR proteins with currently unknown function, identifying their competency at inflammasome formation and identifying the triggers for their activation will be of interest. How inflammasome activation is regulated, both physiologically by the host as well as how it is manipulated by pathogens will be an important research topic in the coming years.

When considering the role of pyroptosis *in vivo* it remains to be determined why, in some contexts, pyroptosis is protective and others detrimental, for example NLRP6 activation during *C. rodentium* and *L. monocytogenes* infection, respectively [8,88]. Moreover, it remains to be

determined whether the physical death of infected cells or the release of PAMPs, DAMPs and cytokines is more important for organismal protection or demise. In some contexts, it appears to be largely driven by IL-1β and IL-18 [8,81], whereas in others the death of the cell rather than secretion of these cytokines is more critical [82]. Further, a highly unexplored area of research, mostly due to a lack of tools, is determining the effect of pyroptotic cells on their neighbours, something recently being explored *in vitro* [32].

Acknowledgements

We extend our apologies to all those whose important work could not be cited here due to space limitations.

Funding

This work was supported by an SNSF project grant (310030_192523) to P.B.

Figure legends

Figure 1: Mechanism and effect of inflammasome activation. Following sensing of activating signals such as PAMPs, disrupted cellular homeostasis or modification by bacterial effectors, a range of inflammasome sensors can become activated. For canonical inflammasomes, this drives the assembly of the ASC speck and activation of Caspase-1 to process Gasdermin-D and IL-1 family cytokines to initiate pyroptosis and enhance inflammatory responses. During activation of the non-canonical inflammasome, caspase-4 (-11), LPS binding to the caspase downstream of GBP recruitment allows activation of the caspase and Gasdermin-D processing and pyroptosis. Final plasma membrane rupture (PMR) and cellular lysis is mediated by NINJ-1.

Figure 2: Bacterial effectors subvert inflammasome responses. The activation of inflammasomes and pyroptosis is subverted at multiple stages by diverse pathogens. **a.** *Yersinia pestis* subverts activation of PYRIN downstream of RHOA manipulation by directly activating the kinases PKN1/2 to maintain PYRIN in its phosphorylated and inactive state. **b.** The steps following inflammasome activation that mediate pyroptosis can be subverted by *Y. pestis* YopM that binds and inactivates Caspase-1 or by *Shigella flexneri* IpaH7.8, which ubiquitinates Gasdermin-D for proteasomal degradation. **c.** To prevent liberation of LPS from the bacterial surface for caspase-4 (-11) sensing, *S. flexneri* ubiquitinates the GBPs through the secreted E3 ligase IpaH9.8 to target them for proteasomal degradation. **d.** Non-canonical inflammasome activation is disrupted by *Francisella novicida* as altered acylation of lipid A prevents its binding to capase-11. This inflammasome is also subverted by EPEC NleF and *S. flexneri* OspC3, both of which inhibit caspase-4 (-11).

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endogenous
danger signals

ASC

pathogens

pathogen-derived
molecular patterns

inactive
receptors

NLRP1/CARD8
NLRP3, NLRC4
Pyrin, AIM2

inflammasome
complex

Caspase-1

IL-1b
IL-18

cell death
(pyroptosis)

Gasdermin-D

NINJ-1

Gram-negative
bacteria

Lipopolysaccharide
(LPS)

GBP coatomer

1
2
3
4

Caspase-4 (-11)

plasma
membrane
rupture

a

YopT

bacterial toxins or
effectors (TecA, VopS,..)

RhoA
GTP

RhoA
GDP

Y. pestis
YopE

Yersinia pestis
YopM

PKN1/2

14-3-3
P

'inactive'

'active'

Pyrin

b

inflammasome
complex

Caspase-1

Yersinia pestis
YopM

Gasdermin-D

Shigella flexneri
IpaH7.8

Ub

Proteasome

c

Shigella flexneri
IpaH9.8

Ub

GBP
coatomer

Caspase-4

Proteasome

d

Gram-negative
bacteria

Lipopolysaccharide
(LPS)

Francisella

GBP coatomer

GBP coatomer

Caspase-4

S. flexneri
OspC3

EPEC
NleF