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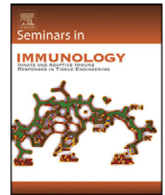

Review

NLR-mediated control of inflammasome assembly in the host response against bacterial pathogens

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ABSTRACT

The host response against diverse bacterial pathogens involves activation of specialized immune cells and elaboration of pro-inflammatory cytokines that help to coordinate appropriate host defense. Members of the interleukin-1 (IL-1) cytokine family, IL-1β and IL-18, are central players in this process. Extracellular release of the mature, active form of these cytokines requires their processing by the cysteine protease caspase-1, which therefore serves as a key regulator of the inflammatory response. In addition to its role in secretion of pro-inflammatory cytokines, caspase-1 is also required for a form of cell death, recently termed pyroptosis, that occurs in macrophages infected by certain bacterial pathogens. Caspase-1 itself is synthesized as a pro-enzyme, which must first be activated by autocatalytic cleavage. This activation requires recruitment of caspase-1 into multiprotein complexes known as inflammasomes. The Nod-like receptor (NLR) family of cytosolic proteins play an important role in detecting inflammatory stimuli and subsequently mediate inflammasome assembly. A common feature of NLR proteins that trigger inflammasome assembly in response to bacterial infection is that they appear to sense membrane perturbation or delivery of bacterial components into the cytosol through bacterial pore-forming toxins or bacterial secretion systems. This review will discuss the recent developments regarding caspase-1 activation in response to bacterial infection, cross-talk between caspase-1 and other pathways involved in regulating cell death, and recent findings that a number of bacterial pathogens possess mechanisms to inhibit caspase-1 activation.

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1. Introduction

Appropriate responses of multicellular eukaryotes against diverse microorganisms requires recruitment of inflammatory cells and release of pro-inflammatory and antimicrobial mediators. The Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are two major families of germ-line encoded receptors that play crucial roles in the host response to bacterial infection by detecting conserved microbial structural components such as lipopolysaccharide (LPS), peptidoglycan, or lipoteichoic acid, and initiating transcriptional programs that mediate activation of the host immune response. Throughout this review, we will use the newly established nomenclature in referring to members of the NLR family [1].

TLRs are membrane-bound receptors located on the plasma membrane and on vesicles of the endocytic pathway, while NLRs are present within the cytosol. Although TLRs and their downstream signaling pathways are essential for recognition of microbial components that were originally termed 'pathogen associated molecular patterns' (PAMPs), they are insufficient to distinguish between pathogenic and non-pathogenic bacteria because they recognize molecules present in both classes of bacteria. That role appears to be played at least partially by certain members of the NLR family, which detect the presence of microbial components within the host cell cytosol and respond by triggering caspase-1 activation. For example, the microbial components that have been identified as being detected by NLRs, such as muramyl di-peptide (MDP) [2,3] and flagellin [4,5], are not unique to pathogenic bacteria. However, the presence of these microbial components within the cytosol of the host cell appears to depend upon their delivery via specialized secretion systems or pore-forming toxins. The ability of NLRs to respond specifically to pathogenic microbes is thus linked to an activity – the formation of pores in cellular membranes – that appears to be preferentially associated with microbial pathogens.

NLRs possess a characteristic domain architecture, consisting of an N-terminal Pyrin domain (PYD) or caspase recruitment domain (CARD), a central nucleotide binding/oligomerization domain (NOD, also known as NACHT), and C-terminal leucine-rich repeats (LRRs) (Fig. 1). The CARD or PYD mediate homophilic protein-protein interactions with other CARD or PYD containing proteins.

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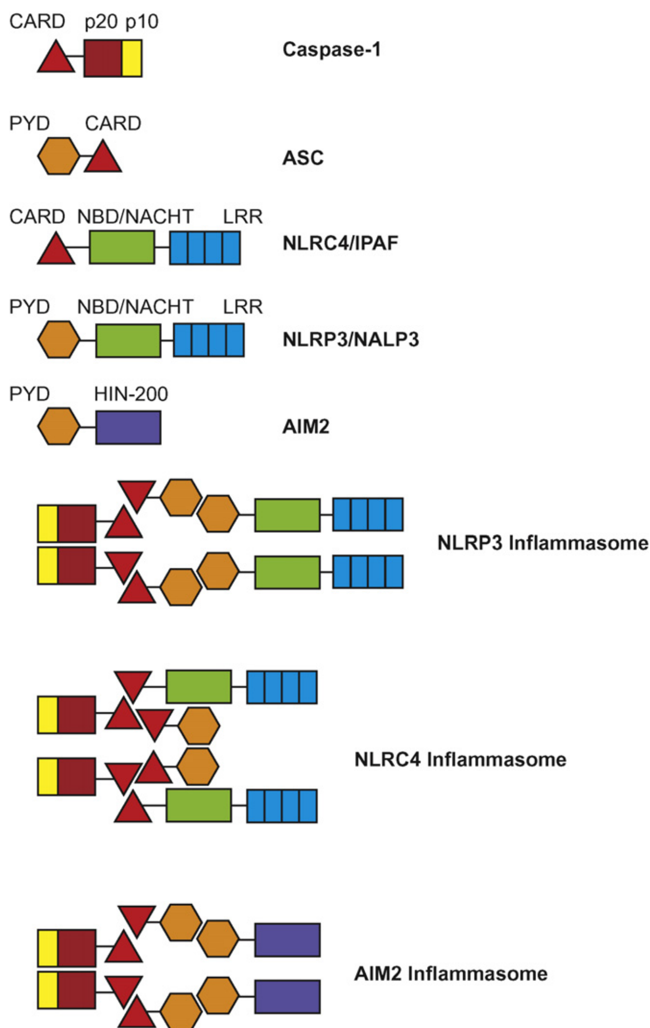

Fig. 1. Inflammasome components and inflammasomes. Inflammasomes are assembled through protein–protein interactions between proteins containing Caspase recruitment domains (CARD) or Pyrin domains (PYD). Members of the NLR family, such as NLRP3 and NLRC4 contain either N-terminal PYD or CARD (respectively) as well as a central nucleotide binding domain (NBD), also known as a NACHT domain. The leucine rich repeat domain (LRR) is thought to act as an auto-inhibitory domain responsible for sensing (either directly or indirectly) stimuli that trigger inflammasome assembly. AIM2, a member of a family of proteins that contain a PYD and a DNA binding domain known as HIN-200, is an interferon-inducible protein responsible for assembly of a recently described inflammasome that responds to cytosolic DNA.

NLRs bear striking similarity to the resistance, or R, proteins of plants, which also contain a variable N-terminus, central NOD domain, and C-terminal LRR [6]. Like mammalian NLRs, plant R proteins play a critical role in immunity of plants to infection with microbial pathogens. Unlike mammalian NLRs that have been characterized thus far however, plant R proteins detect the presence of virulence factors, either via a direct interaction, or through an intermediary protein that is modified by the virulence factor. Based on analogy with R proteins as well as mammalian Apaf-1, which also contains a similar domain architecture to NLRs, the LRRs are believed to maintain the NLRs in an auto-inhibited state, and to mediate recognition of a specific signal leading to a conformational change that promotes oligomerization of the NLR into a multiprotein complex, termed the inflammasome, within which caspase-1 is activated [7].

Caspase-1 is the founding member of a family of cysteine proteases that cleave proteins at specific sequences following aspartyl residues [8]. Originally termed Interleukin Converting Enzyme (ICE), caspase-1 was identified based on its ability to cleave the pro-form of IL-1β to mature, active IL-1β [9]. Since then, a great deal of research has focused on the mechanisms by which caspase-1 is activated. Discovery in the late 1990s of a large family of cytosolic proteins containing C-terminal LRRs, a central nucleotide binding domain, and N-terminal homotypic protein–protein interaction domains of the death domain superfamily led to the emerging idea that these proteins were regulators of cell death and pro-inflammatory signaling [10,11]. The apoptosis associated speck-like protein containing a CARD (ASC), which contains both a CARD and PYD, was also identified as playing a role in regulating caspase-1 activation, although it was initially unclear whether ASC was a positive or negative regulator of caspase-1 [12].

The fields of caspase-1 biology and bacterial pathogenesis converged with a series of studies demonstrating that infection of macrophages by different bacterial pathogens resulted in a caspase-1-dependent cell death [13,14]. Due to the release of the pro-inflammatory cytokines, IL-1β and IL-18, upon caspase-1 activation, this form of cell death was termed 'pyroptosis' to reflect its pro-inflammatory nature and distinguish it from classical apoptosis [15,16]. The caspase-1-dependent death of macrophages in response to bacterial infection was shown to depend on the presence of bacterial virulence determinants, notably bacterial secretion systems [13,14,17,18] or pore-forming toxins [19]. Generation of mice deficient in ASC or different NLRs led to the discovery that different NLRs were important for activation of caspase-1 in response to different signals [20]. Furthermore, ASC was defined as an adaptor necessary for bridging the CARD of caspase-1 and the PYD of the NLRP proteins, thus playing a positive role in caspase-1 activation [7,21].

Much of this literature has recently been reviewed elsewhere [22–25]. In this review, we discuss a number of outstanding questions that remain concerning the nature of the signals that are sensed by different inflammasomes, cross-talk between inflammasomes and other signaling pathways, and the recent observations that some bacterial pathogens can interfere with caspase-1 activation. We apologize to those whose work could not be cited due to space constraints.

2. Role of NLRs in detection of bacterial pathogens

The human genome encodes 23 NLR family members, while mice encode at least 34 due to the presence of multiple paralogs of certain NLRs [24]. The founding members of the NLR family, Nod1 and Nod2, activate NF-κB- and MAPK-dependent gene expression programs via a CARD-dependent association with the signaling kinase RIP2, also called RICK [26–28]. Indeed, in the context of infection with the pathogen *Helicobacter pylori*, Nod1 activates NF-κB signaling in response to peptidoglycan delivered through the *Helicobacter* Type IV secretion system [29]. The other members of the NLR family that have been described are associated with activation of caspase-1 and consequent release of mature IL-1β, rather than with control of gene expression. However, it was recently demonstrated that in addition to its role in transcriptional responses, Nod2 is necessary for the activation of caspase-1 in response to both MDP and anthrax lethal toxin [30]. In the case of both MDP and anthrax toxin, the Nod2-dependent activation of caspase-1 was independent of RIP2, but rather depended on an association between Nod2 and NLRP1b. NLRP1b has previously been shown to be responsible for caspase-1 activation in response to anthrax lethal toxin [31]. Thus, it appears that in at least some cases, multiple NLRs can be recruited to a single inflammasome, indicating that some inflammasomes contain oligomers of a single NLR, while others contain multiple different NLRs. Such a mechanism shares conceptual similarity with the TLR system, in that some TLRs, like TLR4, function as a homodimer, while TLR2 forms heterodimers with other TLRs to

trigger different signaling programs [32]. Although inflammasomes containing heterodimers or hetoroligomers of different NLRs have only been described for Nod2 and NLRP1b, it is intriguing to speculate that the ability of other NLRs to also heterodimerize could potentially enable a greater diversity of NLR-containing inflammasome complexes.

2.1. Detection of cytosolic flagellin

Unlike Nod2, the other NLRs that have been shown to respond to bacterial infection, Nlrp3 (formerly Nalp3, Cias1, or Cryopyrin) and Nlrc4 (formerly Ipaf), have only been linked to inflammasome assembly and caspase-1 activation, and are not known to contribute to transcriptional activation through RIP2 or NF-κB signaling. Initially identified based on its similarity to the protein Apaf1 [33], Nlrc4 was the first NLR shown to be required for caspase-1 activation in response to bacterial infection by the enteric pathogen *Salmonella*, and this activation was shown to depend upon the presence of a functional bacterial type III secretion system (T3SS) [20]. Subsequent work demonstrated that activation of caspase-1 through Nlrc4 required delivery of bacterial flagellin into the host cell cytoplasm, which was hypothesized to occur at some low level through the T3SS due to the evolutionary connection between the T3SS and flagellar biosynthesis machinery [4,5]. In addition to *Salmonella* flagellin, *Legionella pneumophila* and *Pseudomonas aeruginosa* flagellin also activate caspase-1 [34–36], although in the case of *Legionella* the protein, Naip5 (formerly Birc1e) is also required [36,37]. In the case of *Legionella*, it was hypothesized that delivery of flagellin occurred through a Type IV secretion system T4SS [36], which, although structurally and evolutionarily distinct from the T3SS, also delivers bacterial virulence proteins into the cytosol of infected cells. A reporter protein (such as adenylate cyclase) fused to flagellin would definitively establish whether flagellin is delivered through the Type IV secretion system. Such a reporter-based approach recently demonstrated that intracellular *Salmonella* deliver flagellin into the host cell cytosol by means of the T3SS [38]. Earlier studies have found some degree of promiscuity in secretion of particular T3SS substrates by different secretion systems [39], as well as the secretion of non-flagellar proteins by the flagellar secretion machinery [40]. The evolutionary relationship between the T3SS and the flagellar biosynthesis machinery and the physical constraints on the structure of flagellin may therefore prevent bacteria from evolving a mechanism to avoid secreting flagellin through the T3SS and thus, avoid innate immune surveillance mechanisms. However, *Salmonella* may evade recognition by downregulating the expression of flagellin during systemic infection [41]. Indeed, a *Salmonella* in which flagellin is constitutively expressed during infection are highly attenuated [42], although it is unclear whether this is directly due to increased activation of caspase-1 during infection or increased recognition of the bacteria through TLR5.

In the context of *Salmonella* or *Legionella* infection of macrophages, flagellin detection by Nlrc4 or Nlrc4 together with Naip5 (respectively) leads to caspase-1 activation and macrophage cell death [4,5,37] (Fig. 2). However, a number of studies suggest that this model may be incomplete. Notably, infection of macrophages with *Shigella flexneri* also leads to caspase-1 activation via a pathway that requires Nlrc4 [43]. *Shigella flexneri* does not express flagellin [44], raising the question of what signal is being sensed by Nlrc4 during *Shigella* infection. Furthermore, two different groups have come to precisely opposite conclusions regarding the role of flagellin in Nlrc4-dependent caspase-1 activation during infection with *Pseudomonas aeruginosa* [18,34]. Specifically, Miao et al. demonstrated that *Pseudomonas* strain PAO1 activates caspase-1 in a manner that requires host Nlrc4 and bacterial expression of flagellin and a TTSS [34]. In contrast, using either the naturally non-flagellated clinical isolate PA103, or a strain in which the *fliC* gene encoding flagellin was deleted, Sutterwalla et al. concluded that flagellin was not required for *Pseudomonas* to activate caspase-1 through Nlrc4, analogous to the flagellin independent induction of caspase-1 in *Shigella* infected cells [18]. The basis for this seeming discrepancy currently remains unresolved.

It is possible that strain-specific differences or differences in experimental procedures are responsible; however, it is difficult to imagine how such differences would account for these fundamentally different observations. Another possibility is that like Nlrp3, which is required to trigger caspase-1 activation in response to a diverse number of chemically and structurally unrelated stimuli (discussed below in further detail), Nlrc4 can respond to multiple stimuli, of which flagellin is but one. Recent work has demonstrated that retroviral transduction of a construct encoding the C-terminal 35 amino acids of *Salmonella*, *Pseudomonas*, or *Legionella* flagellin is sufficient to trigger Nlrc4-dependent caspase-1 activation in bone marrow derived macrophages [45]. This region of flagellin is highly conserved and appears to be sensed by Naip5, as retroviral transduction of this portion of flagellin fused to GFP was cytotoxic to wild-type, but not Naip5−/− macrophages. However, inflammasome activation by full length flagellin appears to be only partially dependent on Naip5 [45]. These findings suggest either direct recognition of this portion of flagellin by Naip5, which then triggers caspase-1 activation through Nlrc4, or the presence of an additional accessory protein upstream of Naip5 that transduces a flagellin-dependent signal that leads to assembly of a Naip5/Nlrc4 inflammasome. These findings also suggest that portions of flagellin outside of this 35 amino acid region mediate Nlrc4-dependent, Naip5-independent caspase-1 activation in the context of infection with *Salmonella* or *Pseudomonas*. Whether other Naip proteins, which are closely related to Naip5, might be involved in assembly of distinict Nlrc4 inflammasome remains to be experimentally tested.

This study does not eliminate the possibility that in the context of an actual bacterial infection multiple signals might be simultaneously sensed by the Nlrc4 inflammasome. It does demonstrate that the presence of flagellin alone within the cytosol, absent any other bacterial components, is sufficient for Nlrc4-dependent caspase-1 activation. The non-flagellar signals that are responsible for activating caspase-1 through Nlrc4 in the context of infections with *Shigella* and certain strains of *Pseudomonas* therefore remain to be determined. Nlrc4 has been suggested to detect pore formation by the *P. aeruginosa* T3SS [18], but there is no direct evidence for this, and instead, membrane perturbation or its downstream consequences appear to be preferentially detected by the Nlrp3 inflammasome.

2.2. Detection of membrane disruption during bacterial infection

Infection of macrophages with bacteria that elaborate pore-forming toxins, such as *Listeria monocytogenes* or *Staphylococcus aureus*, triggers caspase-1 activation through an inflammasome containing Nlrp3 and ASC [46]. The precise signal that is detected by Nlrp3 remains unclear. In addition to bacteria and their toxins, crystalline compounds of both biological and non-biological origin [22,47–49], as well as cytosolic delivery of bacterial or viral nucleic acids [50,51] appear to be sufficient to induce Nlrp3-dependent caspase-1 activation. The common terminal signal responsible for triggering assembly of the Nlrp3 inflammasome appears to be efflux of intracellular potassium, because Nlrp3-dependent activation of caspase-1 by all of these signals is prevented by the addition of high potassium to the extracellular medium [52]. In contrast, caspase-1 activation by Nlrc4 is not blocked by high potassium, thereby mechanistically separating the signaling pathways leading to assembly of these two inflammasomes.

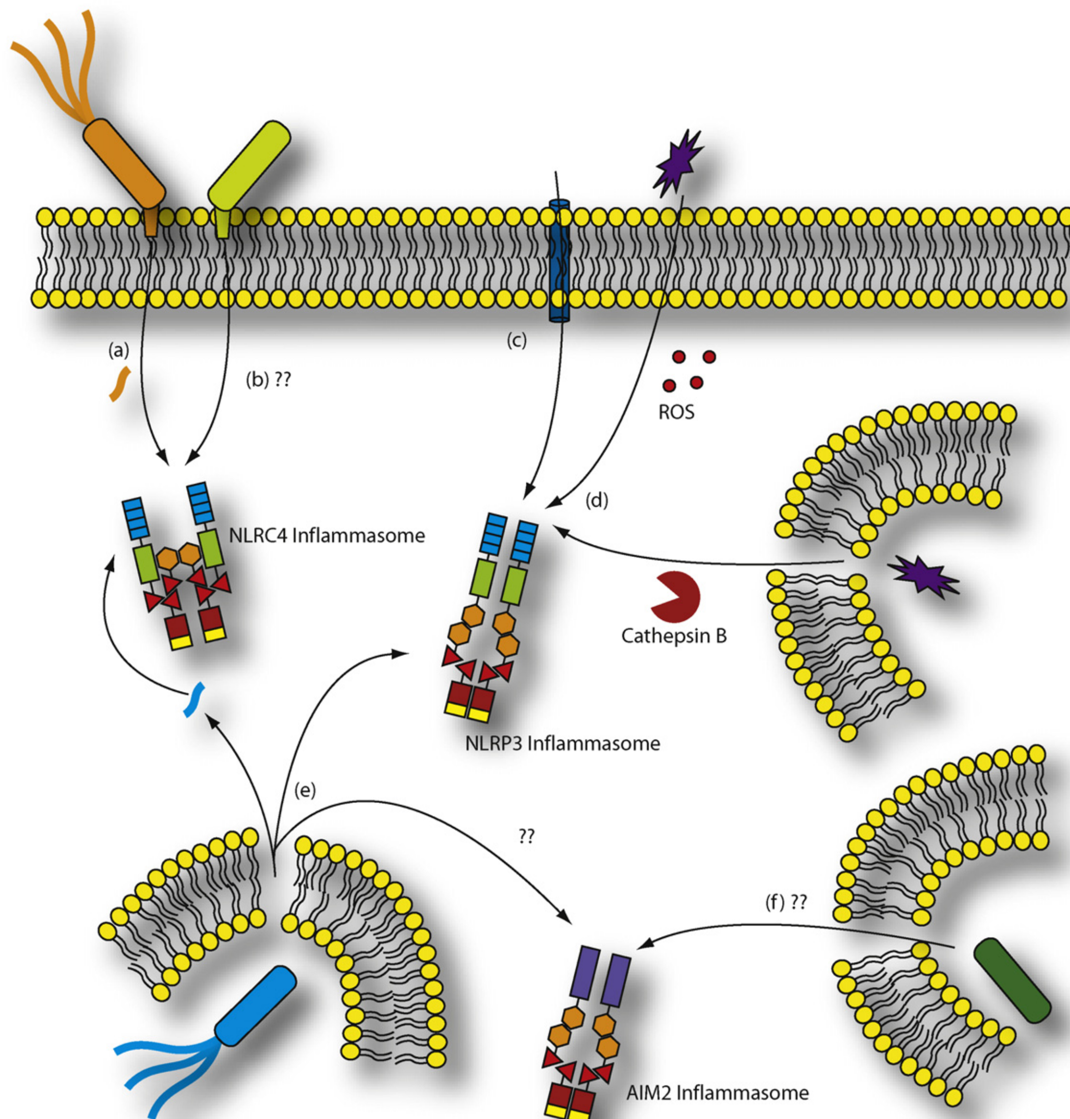

Fig. 2. Inflammasome inducing stimuli. Activation of caspase-1 through the NLRC4 inflammasome occurs in response to flagellin delivered through the secretion systems of *Salmonella* (orange bacteria) (a) or in response to a non-flagellar signal also delivered by the Type III secretion system of *Shigella* and some strains of *Pseudomonas* (green bacteria) (b). The NLRP3 inflammasome is responsive to a number of different stimuli involving the formation of different pores in the plasma membrane (c) as well as reactive oxygen species (ROS) generated in response to the presence of large crystals (purple star) (d). An alternative model for activation of the NLRP3 inflammasome in response to crystals involves lysosomal disruption, accompanied by the release of the lysosomal protease cathepsin B (d). *Listeria monocytogenes* that express the pore-forming toxin LLO (blue bacteria) can activate both the NLRC4 and NLRP3 inflammasomes, and at least one other inflammasome that also depends on ASC, possibly the recently described AIM2 inflammasome (e). *Francisella* that escape from the phagosome (dark green bacteria) also activate caspase-1 through an inflammasome that requires ASC but is distinct from NLRC3 or NLRP4, again potentially the interferon-inducible AIM2 inflammasome (f). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In addition, LPS followed by ATP treatment triggers Nlrp3-dependent caspase-1 activation by a mechanism that requires $P2X_7$ receptor stimulation which causes massive K^+ efflux. $P2X_7$ receptor activation leads to the assembly of a large hemichannel composed of the protein pannexin-1 in the plasma membrane that enables the release of small cytoplasmic solutes into the extracellular medium [53]. Thus, it has been proposed that pannexin-1 is responsible for the large change in intracellular K^+ concentration and subsequent caspase-1 activation. However, although inhibition of pannexin-1 blocks IL-1β maturation and release, it has no effect on ATP-stimulated K^+ efflux [53]. Furthermore, K^+ efflux alone induced by ATP is not sufficient to drive Nlrp3 inflammasome activation, suggesting that the ion-carrying activity of pannexin-1 activation is unnecessary for inflammasome activation. Alternatively, bacterial components such as LPS or peptidoglycan, have been proposed to be delivered into the cytosol via pannexin-1 [54,55]. Triggering of the Nlrp3 inflammasome in response to bacterial components delivered through pores formed in cellular membranes would therefore be analogous to intracytosolic flagellin leading to assembly of the Nlrc4 inflammasome (Fig. 2).

One set of experiments consistent with the role of membrane disruption leading to Nlrp3-mediated caspase-1 activation showed that disruption of endocytic vesicular membranes triggers caspase-1 through release of lysosomal proteases, particularly cathepsin B [56]. Notably, Nlrp3-dependent activation of caspase-1 could be achieved through the hypotonic lysis of endocytic vesicles within intact cells even in the absence of exogenous stimuli [56]. This argues that disruption of lysosomal membranes, either itself, or

due to concomitant release of lysosomal contents, is a key trigger of Nlrp3-dependent caspase-1 activation. However, like the Nlrc4 inflammasome, some debate currently exists as to the mechanism by which these stimuli activate caspase-1 through Nlrp3. An alternative model argues that large particles, such as asbestos fibers, silica, or monosodium urate (MSU) crystals, are too large to be efficiently phagocytosed, leading to a process termed "frustrated phagocytosis" [48,57]. The inability of macrophages to phagocytose these crystals is coupled with sustained recruitment of lysosomes to the site of attempted phagocytosis, leading to release of large amounts of reactive oxygen species (ROS), which has been proposed to play a role in caspase-1 activation (Fig. 2). Support for this model is provided by the observation that pharmacological inhibition of the NADPH oxidase complex with ROS scavengers (e.g. N-acetyl-cysteine) inhibit caspase-1 activation in response to monosodium urate crystals. In addition, siRNA-mediated downregulation of the NADPH oxidase subunit $p22^{phox}$ highly diminished IL-1β secretion [48].

However, the role of ROS as a signal is unclear, as Nlrp3-dependent activation of caspase-1 triggered by silica, MSU crystals, ATP or poly(dA:dT) appears normal in macrophages from mice deficient for NADPH oxidase subunit $gp91^{phox}$ [56]. Furthermore, ROS production in macrophages deficient in the enzyme superoxide dismutase (SOD1) inhibited caspase-1 activation and secretion of IL-1β, due to oxidation and glutathionylation of two redox-sensitive cysteine residues of caspase-1 [58]. Furthermore, SOD1-deficient mice were protected from LPS-induced endotoxic shock and exhibited much lower levels of caspase-1-dependent serum cytokines. This data would imply that ROS production is a negative regulator of caspase-1 activation. Consistent with both models for the role of ROS in regulating caspase-1, incubation of macrophages with N-acetyl cysteine or DPI (an inhibitor of NADPH oxidase) inhibits nigericin or LPS/ATP-induced processing of IL-1β, while incubation of macrophages with SOD promotes IL-1β processing [59]. It is possible that the two membrane-bound subunits of NADPH oxidase, $gp91^{phox}$ and $gp22^{phox}$, participate in distinct complexes with different roles in ROS production upstream of inflammasome activation. Thus, the role of ROS generation in activation of caspase-1 through the Nlrp3 inflammasome is unclear and requires additional studies for clarification.

Precisely how crystalline stimuli lead to caspase-1 activation is therefore not definitively established. However, consistent with a role for phagosomal disruption in inflammasome assembly, activation of caspase-1 in the context of certain intracellular bacterial infections, such as *Listeria monocytogenes* and *Francisella tularensis*, requires lysis of the phagosomal membrane accompanied by escape of the bacteria into the cytosol [46,60,61]. Nevertheless, because intracytosolic *Listeria* and *Francisella* manipulate other key aspects of host cell biology during the course of their replication in the cytosol, it is possible that other signals also play a role in caspase-1 activation in response to infection with these bacterial pathogens.

Interestingly, infection of macrophages with the intracellular bacterial pathogen *Francisella tularensis* also triggers caspase-1 activation in a manner that requires bacterial escape into the cytosol as well as ASC, but is independent of Nlrp3 [46]. One possible explanation for this is that multiple different NLR-containing infammasomes are activated in response to *F. tularensis* infection, and that other inflammasomes activate caspase-1 in response to *Francisella* that escape the phagosome even in the absence of Nlrp3. In contrast to infection with other pathogenic bacteria, activation of caspase-1 in response to *Francisella* requires signaling through the type I interferon pathway [61], suggesting that signals and inflammasomes other than those previously described are involved in the response to *Francisella*. Consistent with the idea of different inflammasomes responding to infection with individual bacterial species, *Listeria*-infected macrophages have been suggested to simultaneously assemble different inflammasomes involving Nlrp3 or Nlrc4, as well as another yet-to-be-identified inflammasome that contains ASC and an uncharacterized NLR [62]. The nature of the NLR protein that activates caspase-1 in response to *Francisella* is currently unclear, but has been suggested to potentially involve a class of interferon-inducible pyrin domain containing proteins [61]. The recent identification of AIM2, a member of this family, as the protein responsible for activation of caspase-1 in response to cytosolic DNA [63–65] suggests that it may also be involved in caspase-1 activation following *Francisella* or *Listeria* infection.

The precise nature of the signal responsible for assembly of the Nlrp3 inflammasome remains mysterious. Given the large variety of structurally diverse stimuli that trigger caspase-1 activation through Nlrp3, it is unlikely that any one of them is directly recognized by Nlrp3. Although the NLR proteins share domain organization features with Apaf1, which directly binds cytochrome c released from mitochondria, the steps leading from membrane disruption to potassium efflux to Nlrp3 inflammasome assembly are poorly defined. Indeed, the other NLRs have also not been shown to directly bind to the molecules or proteins that are responsible for assembly of their inflammasomes in vivo. In vitro reconstitution of the NLRP1 inflammasome has led to a proposed two-step model of inflammasome assembly in response to MDP [66]. In this model, binding of MDP to the LRR domain allows oligomerization of NLRP1 in the presence of ATP. However, the existence of a stable MDP–NLRP1 complex in vivo, or direct binding of any of the NLRs by the molecules that trigger assembly of their respective inflammasomes, awaits further experimental verification.

3. Cross-talk between inflammasomes and other cellular pathways

3.1. Inflammasome activation and apoptosis

Recent work has revealed the existence of cross-talk between inflammasome-mediated activation of caspase-1 and a number of other cellular signaling pathways that also play a central role in the response to pathogens. Two studies have demonstrated that NF-κB-dependent gene products are responsible for regulating caspase-1 activation in response to microbial products. A study by Bruey et al. demonstrated that the proteins Bcl-2 and Bcl-X_L, both of which are negative regulators of apoptosis at the level of mitochondrial cytochrome c release, are also negative regulators of NLRP1-mediated caspase-1 activation in response to purified MDP [67]. This study demonstrated using THP1-derived macrophages, as well as biochemical approaches with extracts from transfected cells, that Bcl-2 and Bcl-X_L can both directly bind NLRP1, and that this binding interfered with the association of NLRP1 with ASC, thereby inhibiting assembly of the NLRP1 inflammasome. Interestingly, Bcl-2 and Bcl-X_L were the only anti-apoptotic proteins of the Bcl-2 family to bind any of the NLR proteins, and NLRP1 was the only NLR with which they could associate. NLRP1 is distinct from other NLRP proteins in that it contains two additional domains, FIIND and a CARD following the LRR domain which is the C-terminal domain in all other NLRs. However, these domains were not responsible for the binding of NLRP1 to Bcl-X_L, as it was demonstrated that the LRR domain was necessary but insufficient for this interaction. This work demonstrated the existence of regulation of caspase-1 activation by members of the Bcl-2 family of anti-apoptotic proteins. A separate study by Greten et al., also showed negative regulation of caspase-1 by another anti-apoptotic protein, plasminogen activator inhibitor 2, or PAI-2 [68].

Similar to Bcl-2 and Bcl-X_L, PAI-2 expression is induced by NF-κB and protects macrophages from apoptosis that is triggered by inhibiting gene transcription in the context of LPS treatment [69].

Transcription of NF-κB-dependent genes requires degradation of the inhibitor of NF-κB, IκB, which is initiated by its phosphorylation by the upstream kinase IKKβ. In macrophages selectively deficient in IKKβ expression, Greten et al. observed that levels of IL-1β transcript were markedly reduced, but levels of secreted mature IL-1β were significantly higher in response to LPS treatment, correlating with increased activation of caspase-1 [68]. Precisely how caspase-1 inhibition by PAI-2 occurs is currently unclear. PAI-2 is a serine protease inhibitor, and is therefore unlikely to inhibit caspase-1 directly. However, it may inhibit a proteolysis-sensitive step upstream of inflammasome assembly. Caspase-1 activation is currently believed to initiate a pro-inflammatory form of cell death, pyroptosis, which proceeds in a mechanistically different fashion from apoptosis and leads to functionally different consequences [16]. These studies demonstrated the role of anti-apoptotic gene products in the regulation of caspase-1 activation, suggesting cross-talk between caspase-1 driven pyroptosis and other cell-death activation pathways.

3.2. Inflammasome activation and autophagy

In addition to regulation of caspase-1 activation by anti-apoptotic proteins, two recent studies have demonstrated that inflammasome activation and autophagy appear to negatively regulate one another [43,70]. Autophagy is an evolutionarily conserved process involving the sequestering of cytoplasmic contents, including excess or damaged organelles, into a double-membrane compartment, termed the autophagosome, which eventually fuses with lysosomes, allowing degradation and recycling of cellular contents [71]. Some degree of autophagy occurs under steady state conditions, but is upregulated in response to certain stimuli. These stimuli include (but are not limited to), amino acid starvation, certain kinds of bacterial infection, and inflammatory stimuli such as the cytokine IFN-γ or LPS. In wild-type macrophages, LPS alone is insufficient to activate caspase-1, as caspase-1 activation is thought to require two signals, a priming signal such as LPS, and a second activating signal, such as ATP or pore formation and subsequent cytosolic delivery of bacterial components during bacterial infection [72]. However, in macrophages lacking Atg16L1, an essential component of autophagosome formation, LPS treatment alone was sufficient to induce robust activation of caspase-1 and release of mature IL-1β [70]. Furthermore, treatment of wild-type macrophages with 3-methyladenine (3MA), a chemical inhibitor of autophagy, enhanced production of IL-1β and cell death in response to LPS treatment [70]. This indicates that under normal circumstances, basal or LPS-induced autophagy prevents activation of caspase-1 in response to LPS. Similar to *Nod2*, in which polymorphisms have been linked to Crohn's disease and ulcerative colitis [73,74], human *Atg16L1* has been identified as a candidate susceptibility gene for Crohn's disease [75,76].

The data therefore suggest that mutations in Atg16L1 that interfere with its function may lead to inappropriate inflammasome activation in response to commensal bacteria and aberrant production of IL-1β, thereby leading to increased intestinal inflammation. Indeed, Atg16L1-deficient chimeric mice were extremely susceptible to DSS-induced colitis, which was ameliorated by neutralizing antibodies against IL-1β and IL-18 [70]. Additionally, caspase-1 activation in Atg16L1-deficient cells that occurred in response to LPS treatment required generation of reactive oxygen species (ROS) as well as potassium efflux, suggesting that this caspase-1 activation is mediated by the Nlrp3 inflammasome. It is possible that macrophages from $Atg16L1^{-/-}Nlrp3^{-/-}$ mice might therefore have reduced caspase-1 activation and IL-1β production. The mechanism by which inhibition of autophagy might result in activation of the Nlrp3 inflammasome in response to LPS remains to be determined, but is independent of signaling through the $P2X_7$ receptor, which triggers caspase-1 activation through Nlrp3 following stimulation with LPS and ATP [46,77]. Interestingly, the activation of caspase-1 in Atg16L1-deficient cells required the adaptor protein TRIF, as LPS failed to activate caspase-1 in $Atg16L^{-/-}Trif^{-/-}$ cells [70]. This indicates that autophagy inhibits caspase-1 activation downstream of TRIF signaling, either by competing for limiting shared common pathway components, or, more likely, by packaging important caspase-1 activating components into autophagosomes.

The interplay between the autophagic pathway and NLRC3-mediated caspase-1 activation has also been implicated during the course of *Shigella* infection. Autophagy was previously suggested to be a host defense response in epithelial cells against *Shigella* infection, as it was triggered in response to *Shigella* undergoing actin-based motility within the cytoplasm of infected cells and served to limit bacterial replication [78]. Furthermore, a *Shigella* type III secreted protein (IcsB) interfered with autophagy by inhibiting cellular recognition of the VirG (also called IcsA) protein which is responsible for directing *Shigella* actin-based motility. Recent studies with macrophages demonstrated that caspase-1 deficient macrophages exhibited a much higher degree of autophagosome formation than wild-type macrophages in response to *Shigella*, and that inhibition of autophagy by 3MA led to increased macrophage death following *Shigella* infection [43]. This finding is consistent with the possibility that activation of cell death and autophagy during bacterial infection or in response to detection of bacterial PAMPS may inversely regulate one another. However, the increased death of *Shigella*-infected cells following 3MA treatment occurred in the absence of caspase-1, suggesting that inhibition of autophagy may have effects on caspase-1 independent cell death pathways as well.

A role for the NLR protein Naip5 as a regulator of the balance between caspase-1 and autophagy during infection with *Legionella pneumophila* has also been suggested [79]. This suggestion is based on data demonstrating that *L. pneumophila* can be observed within autophagosomes, and that Naip5 function correlates with more extensive autophagosome maturation [80] and increased caspase-1 mediated cell death following *Legionella* infection [35–37]. However, since deficiency in the autophagy pathway in the free-living amoeba *Dicteostylium discoideum* has no effect on *L. pneumophila* replication [81], direct evidence for a role of autophagy in control of *Legionella* replication or *Legionella*-induced death of mammalian macrophages awaits further experimentation. Collectively, these studies linking autophagy to the regulation of caspase-1 activation suggest that the initiation of autophagy may indicate successful control of bacterial replication, thereby triggering a signaling pathway that prevents caspase-1 activation. Conversely, bacterial inhibition of autophagy or the inability of autophagy to contain bacterial infection could potentially act as another type of signal that triggers caspase-1 activation.

4. Blocking of caspase-1 activation by bacterial pathogens

Since caspase-1 activation and release of its processed products IL-1β and IL-18 plays such a central role in host defense against bacterial pathogens, it seems likely that microbes would have evolved mechanisms to evade this innate immune pathway through the production of virulence factors that interfere with caspase-1 activation. Indeed, three recent studies have demonstrated the existence of such virulence factors in bacterial pathogens unrelated by either genetics or lifestyle. Notably, *Mycobacterium tuberculosis*, *Francisella tularensis*, and *Pseudomonas aeruginosa* virulence factors were demonstrated to inhibit caspase-1 activation in infected primary macrophages [18,82,83]. Additionally, earlier investigations with *Yersinia enterocolitica* indicated that pore formation by the bacterial TTSS triggered caspase-1 activation and IL-1β release in cultured cell lines, and that this was inhibited by bacterial TTSS

effector proteins, possibly the Rho-GTPase activating protein YopE [84]. Interestingly, *Yersinia pestis* was recently shown to alter the balance between caspase-1-dependent, pyroptotic, cell death, and caspase-3-dependent, apoptotic, cell death, depending on the activation state of the infected macrophages [85].

Investigations into the basis for why mycobacterial infections are not typically associated with high levels of IL-1β revealed that *Mycobacterium tuberculosis* expresses a zinc metalloprotease, termed Zmp1, which prevents caspase-1 activation and IL-1β release from infected macrophages [82]. Interestingly, in addition to blocking caspase-1 mediated release of mature IL-1β, Zmp1 also appeared to inhibit lysosomal maturation of mycobacteria-containing phagosomes; absence of Zmp1 resulted in increased lysosomal trafficking of mycobacteria-containing phagosomes, which was abrogated in caspase-1-deficient macrophages [82]. This suggests that in addition to its well-appreciated role in the secretion of pro-inflammatory cytokines, caspase-1 may also play some role in endosome–lysosome trafficking. Whether the Nlrc4, Nlrp3, or an as-yet-unidentified inflammasome is responsible for caspase-1 activation in primary macrophages in response to *M. tuberculosis* that lack Zmp1 has not yet been determined, although knockdown of either Nlrc4, ASC, or caspase-1 in RAW264.7 macrophage-like cells enhanced intracellular survival of *Mycobacterium* [82]. Collectively, these data suggest that Zmp1 might somehow inhibit the Nlrc4 inflammasome. Interestingly, knockdown of IL-1β caused the same enhancement in mycobacterial survival as knockdown of the inflammasome components themselves, suggesting that paracrine signaling through the IL-1 receptor might enhance or in some way contribute to lysosomal trafficking of mycobacterial phagosomes.

Evidence for the existence of other bacterial virulence factors that inhibit caspase-1 activation was also obtained from a genome-wide negative selection screen in *Francisella tularensis* to identify bacterial genes necessary for virulence in a mouse model of tularemia [83]. Surprisingly, two of the genes identified as being negatively selected in this screen were responsible for inhibiting *Francisella*-induced macrophage cell death, as mutation of either of these genes resulted in very rapid IL-1β release from infected macrophages and host cell death, compared with macrophages infected with wild-type bacteria. This rapid macrophage death was dependent on both ASC and caspase-1, suggesting that these genes were specifically involved in inhibiting rapid caspase-1 activation in response to *Francisella* infection. This is particularly interesting in light of observations that caspase-1 is still activated, albeit with delayed kinetics, in response to infection with wild-type *Francisella* [61]. This indicates that caspase-1 activation per se does not affect *Francisella* virulence, but that somehow rapid activation of caspase-1 limits replication of *Francisella* at systemic sites. One possible explanation might be that since the delayed caspase-1 activation occurs in response to *Francisella* escape from the phagosome, the delay in caspase-1 activation allows *Francisella* to replicate within the phagosome or to activate appropriate gene expression programs to enable replication within the cytosol. However, neither of these mutants exhibited a replication defect following in vitro infection of bone marrow derived macrophages [83]. This suggests that the delay in caspase-1 activation may promote either the spread of bacteria to systemic sites from the initial site of infection, or reinfection of macrophages in vivo, rather than promoting bacterial replication within individual infected cells.

In addition to the discovery that the intracellular pathogens *Mycobacterium tuberculosis* and *Francisella tularensis* can interfere with caspase-1 activation, macrophages infected by the extracellular pathogen *Pseudomonas aeruginosa* activate caspase-1, secrete IL-1β, and undergo cell death in response to bacteria lacking all type III secreted effectors, but fail to do so in response to bacteria that express the secreted effector protein ExoU [18]. The ExoU protein possesses phospholipase A_2 activity, and this activity appears to be important for its ability to inhibit caspase-1 activation, as cells pretreated with a phospholipase A_2 inhibitor were able to activate caspase-1, even in response to bacteria expressing ExoU. This conclusion was supported by additional experiments using an ExoU point mutant which was deficient in PLA_2 activity. However, in contrast to the function of Zmp1 of Mycobacteria, the consequence of ExoU inhibition of caspase-1 was activation of a caspase-1 independent cell death pathway. Because *Pseudomonas* is an extracellular, rather than intracellular pathogen, it may be that the function of ExoU is to trigger a caspase-1 independent death and prevent IL-1β/IL-18 release by blocking caspase-1 activation. Indeed, clinical isolates of *Pseudomonas aeuruginosa* vary with regard to the presence of the *ExoU* gene, and *ExoU*-positive isolates display a higher degree of virulence in clinical infections [86]. Alternatively, caspase-1 independent death may be a host defense response to the detection of caspase-1 inhibition in the context of bacterial infection.

4.1. Conclusions and future perspectives

A substantial amount of research over the last several years by many different laboratories has led to a dramatic increase in our understanding of the mechanisms underlying caspase-1 activation in response to infection with bacterial pathogens. Central to the process of caspase-1 activation are the NLR proteins, which are currently believed to detect particular stimuli by means of their C-terminal LRR domain, and mediate assembly of caspase-1-containing inflammasome complexes via their N-terminal CARD or PYD domains. While this basic framework is now well established, along with a key role in caspase-1 activation for the CARD-PYD containing adaptor protein ASC, a number of outstanding questions remain that will drive the next stages of research in this important area. Notably, whether NLR proteins directly sense the molecules responsible for activation of caspase-1, as has been suggested for flagellin and Nlrc4, or whether caspase-1 activating stimuli interact with a different sensor that triggers a signaling pathway leading to inflammasome assembly, as appears to be the most likely scenario for Nlrp3, remains to be formally demonstrated. In the case of flagellin and Nlrc4, it has also been proposed that rather than acting as a sensor, Nlrc4 acts as a scaffold for the assembly of multiple different kinds of flagellin-responsive inflammasomes [45]. Further investigations will no doubt shed further light on this issue. Recent studies have also demonstrated that caspase-1 activation has additional roles other than triggering pyroptosis and release of active IL-1β and IL-18. In epithelial cells treated with the pore-forming toxin aerolysin, caspase-1 appears to be necessary for activating a membrane repair program through the cleavage and release of the membrane-bound transcription factor SREBP [87]. This suggests that there may be cell-type specificity for the functions of caspase-1, the basis and functional consequences of which remain to be determined.

Additionally, caspase-1 appears to be responsible for the non-conventional secretion of a large number of proteins, other than the IL-1 family cytokines [88]. The vast majority of these proteins do not themselves appear to be substrates for cleavage by caspase-1, but nonetheless depend on caspase-1 activity for their secretion. One possibility is that these proteins are packaged into an exocytic compartment whose fusion with the plasma membrane and release of its contents depends on caspase-1. It seems that this might be the most efficient way to accomplish the simultaneous bulk secretion of a large number of proteins, but at least one caspase-1-dependent protein, IL-1α, appears to be bound directly by caspase-1 even though it is not a substrate, raising the possibility that caspase-1 binding is a prerequisite for non-conventional secretion. The mechanism of caspase-1 mediated non-conventional secretion also remains an area for further exploration.

Finally the recent observations that caspase-1 activation can be inhibited by virulence factors of bacterial pathogens suggests that like other central host defense pathways, such as MAPK and NF-κB signaling, which are well-established targets of virulence factors of diverse pathogens, targeting of caspase-1 is an emerging common theme in the interaction between bacterial pathogens and eukaryotic hosts. This raises the possibility that other bacterial pathogens not yet known to inhibit caspase-1 will be found to possess virulence factors capable of doing so. Future studies will therefore need to be designed to identify these factors and to determine how they contribute to the virulence of their respective bacterial pathogens. Such studies are likely to provide new insights into mechanisms of how pathogens disrupt key aspects of innate immune function and host defense.

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