Diverse mechanisms for inflammasome sensing of cytosolic bacteria and bacterial virulence

Yue Zhao and Feng Shao

Addresses

National Institute of Biological Sciences, Beijing, 102206, China;

Corresponding author:

Feng Shao, Ph.D.

National Institute of Biological Sciences, Beijing

#7 Science Park Rd

Zhongguancun Life Science Park

Beijing, China, 102206

Telephone: +86-10-80728593

Fax: +86-10-80728046

E-mail: shaofeng@nibs.ac.cn

Abstract

The inflammasomes are emerging cytosolic defenses against bacterial infections. The inflammasomes converge on inflammatory caspases activation that triggers pyroptosis, and interleukin-1 β /18 maturation in the case of caspase-1 activation. The inflammasomes not only detect major bacterial molecules but also sense bacterial virulence activity. Among the canonical caspase-1-activating inflammasomes, the NAIP subfamily of NLR proteins serves as the receptors for bacterial flagellin and type III secretion apparatus; Pyrin indirectly senses Rho modification/inactivation by various bacterial agents; NLRP1 in mice/rats detects the protease activity of anthrax lethal toxin by serving as its substrate. Caspase-11 and caspase-4/5 directly recognize bacterial LPS and then become activated. Inflammasome sensing of cytosolic bacteria employs much more diversified biochemical mechanisms, compared with Toll-like receptors-mediated recognition on the membrane.

Introduction

The inflammasome is critical for cytosolic defense against infection [1]. The inflammasome activates caspase-1 by inducing its autoproteolytic processing. Active caspase-1 triggers macrophage pyroptosis and interleukin (IL)-1 β /18 maturation for clearing infection. For inflammasome assembly, a pattern recognition receptor (PRR), very often a nucleotide-binding domain (NBD), leucine-rich repeat (NLR) protein, is postulated to recognize a pathogen-associated molecular pattern (PAMP). The process often involves the PYRIN domain (PYD)-caspase activation and recruitment domain (CARD) adaptor ASC that links the NLR to caspase-1. Caspase-1 belongs to the inflammatory caspase group that also includes human caspase-4/5 and murine caspase-11, all containing an N-terminal CARD. Recent discovery of caspase-11 functioning in detecting Gram-negative bacteria has led to classification of canonical and noncanonical inflammasomes for activating caspase-1 and caspase-11, respectively. Active caspase-1 and caspase-11 both trigger pyroptosis by cleaving the newly identified Gasdermin D (GSDMD) protein [2-3].

Key to understanding inflammasome-mediated defense is to identify the PRRs for major bacterial signals and elucidate their mechanism of action. In this review, we discuss how various bacterial products and toxins are sensed by the canonical NAIP/NLRC4, Pyrin and NLRP1 inflammasomes as well as the “noncanonical inflammasome”. The NLRP3 and AIM2 inflammasomes (not dedicated to detecting bacteria) are not covered due to space limitation. We emphasize the diverse biochemical strategies employed by the inflammasomes to sense various bacterial signals.

Direct recognition of bacterial structural components

Recognition of flagellin and type III secretion apparatus by the NAIPs

Many Gram-negative bacteria harbor a type III secretion system (T3SS) that

injects virulence effectors into host cells. The T3SS evolved from bacterial flagella and structurally resembles the flagellar export system. Assembly of a functional T3SS critically requires a rod and a needle component. The rod protein polymerizes into a ring structure in the bacterial periplasm but can reach host cytosol by erroneous type III secretion or other nonspecific means; the highly conserved needle protein forms the translocation channel appended to the bacterial surface. While the flagella are prevalent in both pathogenic and nonpathogenic bacteria, the T3SS mainly exists in pathogenic bacteria. Flagellin, the monomeric subunit of flagella, is a major target of host innate immunity. The membrane-bound Toll-like receptor (TLR5) recognizes extracellular flagellin to induce cytokine transcription. Genetic studies of *Legionella pneumophila* evasion of macrophage immunity suggest a requirement of flagellin for caspase-1-mediated pyroptosis [4-5]. Flagellin entering into host cytosol from the bacteria can trigger caspase-1 activation, which also requires NLRC4 [6-7], an NLR protein known to mediate *Salmonella typhimurium*-induced caspase-1 activation [8]. Miao *et al.* further observed that NLRC4 could also sense the rod component of the T3SS in *S. typhimurium* [9].

How are cytosolic flagellin and the T3SS rod protein detected by the inflammasome? Two studies independently identified the NAIP subfamily of NLRs as the responsible receptors [10-11]. The mouse encodes seven homologous NAIPs (NAIP1-7) while humans only have one (hNAIP). In mice, flagellin is recognized by NAIP5 and its closest paralog NAIP6, which stimulates a physical association of NAIP5/6 with NLRC4; the two NLRs then co-oligomerize to form a large complex for caspase-1 activation (Figure 1). The T3SS rod protein is detected similarly by the NAIP2 receptor. Human monocytes do not respond to cytosolic flagellin and the rod protein, but are sensitive to the T3SS needle protein [11-12]. hNAIP directly binds to

the needle protein to induce caspase-1 activation. This inflammasome pathway operates similarly in mice with NAIP1 being the receptor [12-13]. Recognition of T3SS rod/needle proteins by the NAIPs activates caspase-1 through NLRC4, similar to the flagellin-NAIP5/6-NLRC4 inflammasome (Figure 1).

The needle and rod proteins are structurally similar, featuring a hydrophobic helix hairpin. An analogous structural element is present in flagellin and determines NLRC4 inflammasome activation. Ligand recognition by the NAIP is mediated by sequence regions associated with the NBD rather than the C-terminal leucine-rich repeat (LRR) domain [14]. Crystal structure of NLRC4 Δ CARD shows an autoinhibitory conformation, similar to Apaf-1 in apoptosis [15]; ligand binding likely induces conformational changes of the NAIP and NLRC4 that are then assembled into an inflammasome complex through NBD-mediated oligomerization [11,14,16]. A role of infection-induced NLRC4 phosphorylation has been suggested in this activation process [17]. The NAIP-NLRC4 inflammasome has a dominant role in macrophage sensing of various bacterial infections including *S. typhimurium* and *Shigella flexneri*. A recent study in mice also suggests an epithelium-intrinsic NAIP-NLRC4 inflammasome that limits *S. typhimurium* intraepithelial proliferation [18].

LPS recognition by inflammatory caspase-11/4/5

LPS (lipopolysaccharide) is the major component of Gram-negative bacterial cell wall. For a long time, understanding of innate recognition of LPS was limited to its membrane receptor TLR4/MD2-stimulated cytokine transcription. Recent studies of caspase-11 function suggest a role in sensing Gram-negative bacteria of both pathogenic and non-pathogenic nature [19-23]. Cytosolic LPS is indeed the responsible factor for triggering caspase-11-mediated pyroptosis in mouse macrophages [24-25]. Cytosolic delivery of LPS alone is sufficient to activate

caspase-11, which is independent of TLR4. When primed with TLR3 agonist, *Tlr4*^{-/-} and wild-type mice are equally susceptible to LPS-induced sepsis, establishing a critical role of caspase-11 in endotoxic shock. This intracellular LPS sensing requires bacterial escape from the vacuole [23] and is promoted by interferon-inducible GTPase [26-27].

Knowing the existence of intracellular LPS sensing is paradigm-shifting in innate immunity. However, it was unclear whether the pathway operates in human. A recent study shows that human U937 and THP1 monocytes undergo massive pyroptosis following cytosolic delivery of LPS and this effect is mediated by caspase-4 [28]. Caspase-4 and caspase-11 can functionally complement each other in LPS sensing. Whereas caspase-11 is transcriptionally upregulated by interferon or TLR signaling [19,24-25,29], caspase-4 is constitutively and highly expressed in various cell types including keratinocytes and epithelial cells, rendering these cells highly sensitive to cytosolic LPS. A crucial role for caspase-4 sensing of LPS in polarized epithelial cells has also been suggested recently [30]. These observations urge re-thinking the relevance of mouse endotoxic shock model to Gram-negative bacteria-induced sepsis in humans.

Knowledge established from apoptosis and caspase-1 researches led to a natural belief that a CARD-containing protein acts as an LPS sensor upstream of caspase-11. Shi *et al.* found that recombinant caspase-4/11 purified from insect cells are monomers while those from *E. coli* are ~ 600-kDa multimers; the former can undergo oligomerization upon incubation with Gram-negative lysates or LPS [28]. Biochemical analyses revealed a specific and high-affinity binding between caspase-4/11 and LPS (or its active component lipid A). The binding induces caspase-4/11 oligomerization and importantly activation of the caspases. The CARD

is required and sufficient for LPS binding, which also oligomerizes upon LPS binding. LPS binding-deficient point mutants of caspase-11/4 are inert in LPS-induced oligomerization/activation and cell pyroptosis. Under-acylated lipid A and LPS variants can bind to caspase-4/11, but do not induce high-order oligomerization and cell pyroptosis [24-25,28]. This echoes LPS recognition by human TLR4/MD2 complex. Biochemically, caspase-5 can also bind to LPS and trigger pyroptosis. Thus, caspase-4/5/11 are innate receptors for cytosolic LPS (Figure 1). The combination of a pattern-recognition sensor (the CARD) and an executioner (the protease domain) in a single polypeptide allows for efficient responses to LPS, guarding the cytosol from Gram-negative invaders.

Detection of bacterial virulence activity

Sensing bacterial modifications of Rho GTPase by Pyrin

Bacterial pathogens often target Rho GTPases to modulate host actin cytoskeleton for virulence purposes. Studies have identified numerous Rho-targeting bacterial toxins and effectors that covalently modify Rho GTPases or modulate their nucleotide cycling [31]. Among them, toxin A/B from *Clostridium difficile* (TcdA/B) inactivate Rho by monoglucosylation of a threonine (Thr-37 in RhoA) [32], which is crucial for *C. difficile* pathogenesis [33]. Xu *et al.* recently discovered that the glucosyltransferase activity of TcdB could induce caspase-1 activation in mouse and human macrophages [34]. This activation is absent in *Asc*^{-/-} cells, suggesting a requirement of a PYD-containing sensor. Reconstitution of TcdB-induced ASC speck formation in 293T cells led to the identification of Pyrin. Genetic ablation of Pyrin could completely block TcdB-induced inflammasome activation, uncovering the long sought physiological function of Pyrin in innate immunity. Consistently, Pyrin is expressed in innate immune cells and its overexpression can induce ASC-dependent

caspase-1 activation [35-36]. Gain-of-function mutation in Pyrin-encoding gene *MEFV* causes familial Mediterranean fever (FMF), a common autoinflammatory disease [37].

The T3SS effector VopS from *Vibrio parahaemolyticus* and *Histophilus somni* IbpA also modify Thr-37 (in RhoA) or the nearby Tyr-34 by adenylation. The *C. botulinum* C3 toxin ADP-ribosylates Asn-41 in RhoA with a high selectivity for the Rho subfamily. These toxins all modify the GTP-binding switch I region, leading to Rho inactivation. When delivered into macrophages, VopS, IbpA and C3 toxin all can induce caspase-1 activation mediated by the Pyrin/ASC inflammasome [34]. Moreover, *Burkholderia cenocepacia* can also inactivate Rho GTPases [38-39], resulting from a putative type VI secretion system (T6SS) effector-mediated deamidation of Asn-41 (in RhoA) [34]. Consistently, *B. cenocepacia* activates the Pyrin inflammasome with severe inflammation in the lung of infected mice. Thus, the Pyrin inflammasome detects diverse modifications-induced Rho inactivation and can potentially cope with a wide spectrum of bacterial infections (Figure 1).

Pyrin does not interact directly with Rho or the modified GTPase. It is most possible that dysfunctioning of the actin cytoskeleton caused by Rho modification triggers Pyrin activation [34]. This model is supported by a recent follow-up study [40]. Mice homozygous for a hypomorphic allele of *Wdr1* (encoding an actin-depolymerizing cofactor) exhibit spontaneous autoinflammatory disease and thrombocytopenia. The autoinflammation mainly results from Pyrin inflammasome activation [40]. Thus, Pyrin likely senses pathological perturbation of the actin polymerization dynamics. It is worth noting that the actin polymerization-inhibiting agents, cytochalasin D and the actin crosslinking domain of *Vibrio cholerae* RTX toxin, can not induce Pyrin activation in mouse primary bone macrophages. This

suggests that Pyrin is not sensitive to all types of actin cytoskeleton disruption and instead detects a specific signaling event of non-physiological nature.

Sensing the metalloprotease activity of anthrax lethal toxin by NLRP1B

Lethal toxin (LT), a key virulence determinant of *Bacillus anthracis*, contains the lethal factor (LF) subunit and protective antigen (for receptor binding and endocytic delivery of the holotoxin). LF is a metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKKs) and blocks MAPK-mediated defenses [41]. LT triggers caspase-1 activation and pyroptosis in mouse macrophages, requiring its protease activity [42-43]. LT-induced killing only occurs in macrophages of certain genetic background [41], and this allows for positional cloning of mouse *Nlrp1b* [44]. The polymorphism in rat *Nlrp1* also determines rat macrophage sensitivity to LT intoxicification [45].

NLRP1B is the mouse homolog of NLRP1 in human and rat. The *Nlrp1b* locus in mice has two paralogues *Nlrp1a* and a pseudogene *Nlrp1c*. It is now well accepted that NLRP1B and rat NLRP1 serve as the inflammasome sensor for LT protease activity (Figure 1). The NLRP1 subfamily all contains a function to find domain (FIIND) and a CARD following the NBD domain. Different from human NLRP1, the N-termini of mouse and rat NLRP1 lack a PYD or CARD. Sequence differences in the N-terminal 100 residues in LT-sensitive and -resistant rat NLRP1 correlate with phenotypic differences [45], leading to identification of a putative LT cleavage site in LT-sensitive NLRP1 [46]. Indeed LT could specifically cleave the sensitive rat NLRP1; the cleavage is required for caspase-1 activation reconstituted in nonmacrophage cells. LT also cleaves the N terminus of mouse NLRP1B that lacks a canonical cleavage site [47]. By engineering a TEV protease cleavage site into NLRP1B, Chavarria-Smith and Vance demonstrated that proteolytic cleavage in the N terminus of NLRP1B is

sufficient to initiate inflammasome responses [48].

Different from Pyrin sensing of toxin virulence activity, NLRP1B itself is a substrate of LT protease activity (Figure 1). LT cleavage of NLRP1B likely releases the N-terminal autoinhibitory state of NLRP1B. However, this model can not explain several known observations. LT cleaves NLRP1B from both toxin-sensitive and -resistant mice [47], inconsistent with *Nlrp1b* being the genetic determinant for mouse sensitivity to LT. Secondly, LT killing of macrophages is sensitive to proteasome and N-end rule inhibitors [49-50], but these protein degradation inhibitors do not block LT cleavage of NLRP1B [46,48]. Despite the incomplete mechanism, LT does represent another example of inflammasome sensing of bacterial virulence. Given that NLRP1B activation does not require a fixed cleavage site, NLRP1B may also function as an inflammasome sensor for other microbial protease activity [48].

Conclusions and perspectives

LPS and flagellin are general bacterial PAMPs that are also present in nonpathogenic strains. Inflammasome sensing of LPS and flagellin helps to ensure an effective defense upon cytosol contamination by a bacterium. The T3SS and Rho-modifying toxins are unique to pathogens, and their detection allows the innate immune response to distinguish virulent bacteria from avirulent ones. This is likely significant for immune homeostasis in microflora containing mostly commensals. Pyrin and NLRP1 are unique PRRs that sense bacterial virulence activity. The mode of Pyrin action echoes the guard model in plant innate immunity, in which the disease resistance NB-LRR protein often perceives effector inactivation of its host target [51]. Pyrin appears to guard Rho GTPase or its downstream actin cytoskeleton from pathogen destruction. Different from the NB-LRRs, Pyrin presumably can sense many effectors with Rho-modifying activity, and such feature allows mammals to use a few

PRRs to deal with a large number of pathogens.

In innate immunity, TLRs adopt a common mechanism to recognize different PAMPs. In contrast, inflammasome sensing of cytosolic bacteria involves diversified biochemical strategies. Sensing of flagellin and the T3SS requires two NLRs with NAIP being a receptor and NLRC4 being a signaling adaptor; Rho-modifying toxins are detected by a non-NLR protein Pyrin; LPS is recognized by inflammatory caspases harboring both the sensor and executioner function. Other new or unexpected mechanisms for sensing cytosolic bacteria will likely appear in future studies. Despite multiple and diverse inflammasomes, a certain inflammasome often dominates over others in detecting a particular bacterium. For example, caspase-11 detection of LPS is sufficient to protect mice from lethal challenge with *B. pseudomallei* [23]. Meanwhile, the physiological and pathogenic diversity among bacteria provide a valuable toolbox for discovering new sensing mechanisms. Common to inflammasome-mediated defenses is the activation of inflammatory caspases, but how inflammatory caspases induce pyroptosis remains completely unknown.

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Figure Legend

Figure 1. Inflammasomes-mediated innate immune detection of cytosolic bacteria and bacterial virulence activity.

The canonical inflammasomes activate caspase-1 to induce pyroptosis and IL-1 β /IL-18 maturation, which relies on a central scaffold protein (AIM2, NAIPs, Pyrin or NLRP1B) to sense the bacteria. AIM2 is the receptor for dsDNA including that from bacteria while the NAIPs directly recognize bacterial flagellin and components of the toxin-injecting type III secretion apparatus. Pyrin indirectly senses bacterial virulence activities that can covalently modify and inactivate host Rho GTPases; NLRP1B detects the protease activity of anthrax lethal toxin by serving as its substrate. Upon detecting the cognate bacterial signals, these scaffold proteins undergo oligomerization, which often involves adaptor proteins like ASC and/or NLRC4, to assemble the canonical inflammasome complex. For cytosolic LPS, it is directly recognized by caspase-11 in mice and caspase-4/5 in human, resulting in oligomerization and catalytic activation of the caspases. Active caspase-4/5/11 also triggers pyroptosis as well as activation of the canonical NLRP3/ASC inflammasome. These inflammasome pathways employ diverse mechanisms for sensing various bacterial products or virulence activities, playing a critical role in cytosolic defenses against a wide spectrum of bacterial infections.

Figure