

REVIEW

Sensing bacterial infections by NAIP receptors in NLRC4 inflammasome activation

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ABSTRACT

The inflammasome is an emerging new pathway in innate immune defense against microbial infection or endogenous danger signals. The inflammasome stimulates activation of inflammatory caspases, mainly caspase-1. Caspase-1 activation is responsible for processing and secretion of IL-1 β and IL-18 as well as for inducing macrophage pyroptotic death. Assembly of the large cytoplasmic inflammasome complex is thought to be mediated by members of NOD-like receptor (NLR) family. While functions of most of the NLR proteins remain to be defined, several NLR proteins including NLRC4 have been shown to assemble distinct inflammasome complexes. These inflammasome pathways, particularly the NLRC4 inflammasome, play a critical role in sensing and restricting diverse types of bacterial infections. Here we review recent advances in defining the exact bacterial ligands and the ligand-binding receptors involved in NLRC4 inflammasome activation. Implications of the discovery of the NAIP family of inflammasome receptors for bacterial flagellin and type III secretion apparatus on future inflammasome and bacterial infection studies are also discussed.

KEYWORDS inflammasome, NOD-like receptors, NLRC4, caspase-1, NAIP, type III secretion system, flagellin, *Salmonella*, *Legionella*, enteropathogenic *E. coli*, *Burkholderia*

INTRODUCTION OF INFLAMMASOME IN INNATE IMMUNITY

The innate immune system in mammals defends against

microbial infection. To discriminate “microbial foreigners” from “self,” the innate immune system has evolved an array of germline-encoded pattern recognition receptors (PRRs), which exhibits a broad specificity to a wide spectrum of pathogen-associated molecular patterns (PAMPs) (Takeuchi and Akira, 2010). Engagement of PAMPs by the cognate PRRs mounts an inflammatory response, exemplified by expression and secretion of a plethora of inflammatory cytokines, which functions to restrict microbial infection and promote subsequent adaptive immune response. The membrane-bound Toll-like receptors (TLRs) are established to directly recognize specific microbial products and thereby stimulate the proinflammatory NF- κ B and interferon signaling, leading to transcriptional induction of many cytokines. The cytosolic NOD-like receptors (NLRs) (Chen et al., 2009) are another family of PRRs that are also thought to function to sense microbial products, serving as an intracellular innate immune surveillance mechanism. NLR proteins generally contain an N-terminal caspase recruitment domain (CARD) or a pyrin domain, a central nucleotide-binding and oligomerization domain (NOD) and a C-terminal leucine-rich repeat (LRR) domain. This structural organization is similar to Apaf-1 in apoptosis as well as plant disease resistance (*R*) gene product, the latter of which mediates innate immune defense in plants. In mammals, the NLR family contains 23 members in human and 34 members in mice (Ting et al., 2006; Davis et al., 2011).

NLR proteins can mediate the assembly of the so-called inflammasome complex mainly in macrophage or dendritic cells. The inflammasome acts as a signaling platform for inducing caspase-1 autoprocessing and activation, which further leads to maturation and secretion of IL-1 β and IL-18 (Schroder and Tschoop, 2010). Caspase-1 activation often triggers a special type of macrophage inflammatory death termed pyroptosis (Fink and Cookson, 2006; Brodsky and

Medzhitov, 2011). Both interleukin production and macrophage pyroptosis serve as effector mechanisms in limiting bacterial replication and defending against infection (Miao et al., 2010a). The CARD or PYD domains in NLR proteins signal downstream caspase-1 activation through direct CARD–CARD interaction with caspase-1 or indirectly through the inflammasome adaptor protein ASC. Similarly to Apaf-1 in apoptosome, NLR protein, upon sensing of the PAMP signal or certain endogenous danger signal, oligomerizes to form the large inflammasome complex, which requires nucleotide binding to its NOD domain (Duncan et al., 2007; Faustin et al., 2007). Four NLR proteins including NLRP3 (Anand et al., 2011), NLRP6 (Chen et al., 2011; Elinav et al., 2011), NLRC4 and mouse Nalp1b (Boyden and Dietrich, 2006), together with a non-NLR protein AIM2 (Hornung and Latz, 2010), have been demonstrated to function in the inflammasome pathway and play important but distinct roles in detecting and counteracting bacterial infection.

NLRC4 bears an N-terminal CARD domain that activates caspase-1 and downstream inflammasome signaling in both ASC-dependent and -independent manners (Broz et al., 2010b). Earlier genetic studies have established that NLRC is responsible for sensing cytosolic presence of bacterial flagellin in macrophage cells (Franchi et al., 2006; Miao et al., 2006). Recent studies on the function and mechanism of NLRC4 inflammasome activation have identified components of bacterial type III secretion apparatus as additional bacterial ligands for NLRC4 inflammasome and also the NAIP-family NLR proteins as the corresponding inflammasome receptors, which are the focus of the present review.

BACTERIAL TYPE III SECRETION SYSTEM AND BACTERIAL INFECTION

Bacterial pathogens and their eukaryotic hosts have been engaged in a constant battle during evolution. Bacterial pathogens usually harbor specialized secretion systems to inject virulence effector proteins into host cells to manipulate or disrupt various host processes, particularly the innate immune defense pathway. Many Gram-negative bacterial pathogens, such as *Yersinia* spp. (plague and gastrointestinal disorders), *Salmonella* spp. (typhoid or non-typhoid fever), *Shigella* spp. (bacillary dysentery), food-poisoning pathogenic *Escherichia coli*, *Burkholderia* spp. (glanders in human and animals), *Pseudomonas* spp. (that can infect humans, animals and plants), and the rare human pathogen *Chromobacterium violaceum* (that potentially can cause liver abscesses and sepsis), all harbor a so-called type III secretion system (Galán and Wolf-Watz, 2006; Cornelis, 2010). The type III secretion system is an essential virulence mechanism for these pathogens as it usually delivers multiple effectors with extremely potent and harmful activities into host cells (Cui and Shao, 2011). The type III secretion system, composed of more than 20 different protein subunits, is a sophis-

ticated translocation apparatus characterized by a multi-ring base and an inner rod (Marlovits et al., 2006), located on the bacterial membranes and periplasm, as well as a protruding needle that forms a conduit for effector secretion (Deane et al., 2006). Many of the type III secretion apparatus subunits share sequence and structural similarity to components of bacterial flagellar export system that translocates monomeric flagellin out of the bacteria for flagellar assembly. Therefore, it is generally believed that two systems are evolutionarily related (Erhardt et al., 2010). Although type III secretion-containing bacterial pathogens, except for *Shigella flexneri* that is nonflagellar but does harbor a flagellin-encoding gene, usually come with the flagellar appendage for motility, there are also flagellar bacteria that do not harbor the type III secretion system. For example, *Legionella pneumophila*, the causative agent of Legionnaires' pneumonia and a model intracellular pathogen, features a type IV secretion system that is required for *L. pneumophila* replication within host alveolar macrophage cells (Isberg et al., 2009; Ge and Shao, 2011).

FLAGELLIN RECOGNITION BY NAIP5(6) RECEPTORS

Earlier studies showed that several *Salmonella* spp. are cytotoxic to macrophage or macrophage-like cells (Chen et al., 1996). *Salmonella* infection triggers caspase-1 activation, and blocking caspase-1 activation by a caspase-1-specific inhibitor or genetic ablation inhibits *Salmonella* infection-induced macrophage death (Hersh et al., 1999), suggesting inflammasome activation upon macrophage sensing of *Salmonella* infection. *Salmonella*-induced inflammasome activation entails the NLR protein NLRC4 as bone marrow macrophages derived from *Nlrc4* knockout mice show no caspase-1 activation and can survive *Salmonella* infection (Mariathasan et al., 2004). On the bacterial side, the gene encoding the monomeric flagellin, the building blocks of bacterial flagella, is responsible for NLRC4-dependent caspase-1 activation (Franchi et al., 2006; Miao et al., 2006; Broz et al., 2010a). Mutant *S. typhimurium* strain lacking flagellin is deficient in activation of caspase-1 and IL-1 β secretion; cytoplasmic delivery of purified flagellin into macrophages by cationic liposome or pore-forming toxins can trigger NLRC4 inflammasome activation (Franchi et al., 2006; Miao et al., 2006; Molofsky et al., 2006). Consistent with the wide presence of the flagella among bacterial species, NLRC4 inflammasome-mediated innate immune detection has been observed with many other types of bacterial pathogens including *Pseudomonas aeruginosa* (Franchi et al., 2007; Suterwala et al., 2007; Galle et al., 2008; Miao et al., 2008), *L. pneumophila* (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Case et al., 2009), and the Gram-positive *Listeria monocytogenes* (Warren et al., 2008; Tsuchiya et al., 2010; Wu et al., 2010). However, a defined biochemical assay us-

ing the amino-terminal domain of anthrax lethal factor-mediated intracellular delivery revealed that recombinant flagellin derived from several bacterial species, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *S. flexneri*, *C. violaceum* and *Burkholderia thailandensis*, is biochemically inactive in stimulating NLRC4 inflammasome activation (Zhao et al., 2011). Consistently, genetic ablation of flagellin-encoding genes from EPEC, *C. violaceum* and *B. thailandensis* has little effects on infection-induced caspase-1 activation. Thus, although flagellin detection appears to be a conserved mechanism for the host to recognize invading pathogenic bacteria, certain bacterial species have escaped from this NLRC inflammasome-mediated innate immune detection likely due to modification of their flagellin sequence.

A flagellar mutant of *L. pneumophila* that expresses flagellin but does not assemble the flagellum still induces flagellin-dependent macrophage cell death (Ren et al., 2006), suggesting that inflammasome sensing of flagellin does not require an intact flagellum structure. It has been shown that the C-terminal D0 region of flagellin is responsible for activating the inflammasome pathway (Lightfield et al., 2008). In fact, the C-terminal 35 amino acids of *L. pneumophila* flagellin are required and sufficient for stimulating NLRC4 inflammasome activation. Mutation of three leucine residues that disrupts a hydrophobic patch within the 35 amino acid region also abolishes inflammasome-mediated detection of flagellin from *L. pneumophila* (Lightfield et al., 2008; Zhao et al., 2011) and *P. aeruginosa* (Franchi et al., 2007). The inflammasome recognition region in flagellin is distinct from that by the plasma membrane-bound TLR family member TLR5 that is established to be the flagellin receptor for activating the NF- κ B signaling. TLR5 appears to recognize the D1 region of flagellin and an I411A mutation in *L. pneumophila* flagellin that disrupts TLR5 binding is fully functional in activating NLRC4 inflammasome (Lightfield et al., 2008). Macrophages derived from TLR5 knockout mice are still responsive in flagellin stimulation of the inflammasome pathway (Franchi et al., 2006; Miao et al., 2006). This further suggests that TLR5 is not the flagellin receptor for inflammasome activation.

What is the cytosolic receptor for flagellin in inflammasome activation? NLRC4 was the most attractive candidate when the genetic relation between bacterial flagellin and NLRC4 was first established. However, direct binding of flagellin to NLRC4 has never been recorded in the literature. Two recent studies propose that NAIP5 (probably also NAIP6), a member of the BIR domain-containing NLR protein family, serves as the innate immune receptor for flagellin in inflammasome activation in mice (Kofoed and Vance, 2011; Zhao et al., 2011). NAIP5 was originally identified as the genetic determinant for controlling *L. pneumophila* replication in murine macrophages (Diez et al., 2003; Wright et al., 2003; Derré and Isberg, 2004; Fortier et al., 2007). A natural *Naip5* mutant

allele renders macrophages from the A/J mouse permissive to *L. pneumophila* intracellular replication. Further studies showed that flagellin sensing by wild-type NAIP5 restricts *L. pneumophila* infection (Zamboni et al., 2006; Fortier et al., 2007; Lamkanfi et al., 2007; Fortier et al., 2009) and that macrophages derived from NAIP5 knockout mice is defective in flagellin stimulation of caspase-1 activation during *L. pneumophila* infection (Lightfield et al., 2008). The biochemical assay established by Zhao et al. further revealed a universal role for NAIP5 in NLRC4 inflammasome activation in response to different bacterial flagellins (Zhao et al., 2011). In both yeast two-hybrid and co-immunoprecipitation assays, flagellin shows a robust direct interaction with NAIP5 and also the closely related NAIP6 (Zhao et al., 2011). Furthermore, the ability of flagellins from ten different bacterial pathogens to bind to NAIP5 correlates well with their differential inflammasome-stimulating activity (Zhao et al., 2011). This further strengthens the idea that flagellin is directly recognized by NAIP5 in triggering NLRC4 inflammasome activation. In fact, flagellin binding promotes a physical association between NAIP5(6) and NLRC4 (Zhao et al., 2011), resulting in formation of an oligomeric NLRC4 inflammasome complex and full reconstitution of flagellin stimulation of IL-1 β production in non-macrophage cells (Kofoed and Vance, 2011; Zhao et al., 2011). Thus, NAIP5(6) serves as the inflammasome receptor for bacterial flagellin and NLRC4 indeed functions as an adaptor that relays the NAIP5 signaling to downstream caspase-1 activation and macrophage inflammation (Fig. 1).

Flagellin can be synthesized *de novo* upon sensing of host-produced lysophospholipids (Subramanian and Qadri, 2006). Translocation of flagellin monomers into macrophage cytosol for inflammasome stimulation is not through the flagellar secretion system (Miao et al., 2006), and instead is mediated by bacterial type III or type IV secretion system. Therefore, flagellin stimulation of the inflammasome pathway is usually absent or significantly attenuated for bacterial mutants harboring a genetically disrupted type III or type IV secretion system. For example, deletion of *sipB* that encodes a key component in the type III secretion translocator pore from *S. typhimurium* diminishes infection-induced caspase-1 activation and macrophage inflammatory death (Hersh et al., 1999). Similarly, the *L. pneumophila dotA* mutant deficient in type IV-mediated effector translocation is also unable to stimulate inflammasome activation. Using the β -lactamase reporter secretion assay, it has been demonstrated that *S. typhimurium* flagellin can be injected into host cells through *Salmonella* Pathogenicity Island 1 (SPI-1) type III secretion system (Sun et al., 2007). Moreover, it is also a possibility that lysis of intracellular bacteria can lead to cytosolic presence of bacteria flagellin, which might explain activation of NLRC4 inflammasome by flagellin from *L. monocytogenes* that harbors neither type III nor type IV secretion system (Warren et al., 2008; Tsuchiya et al., 2010; Wu et al., 2010).

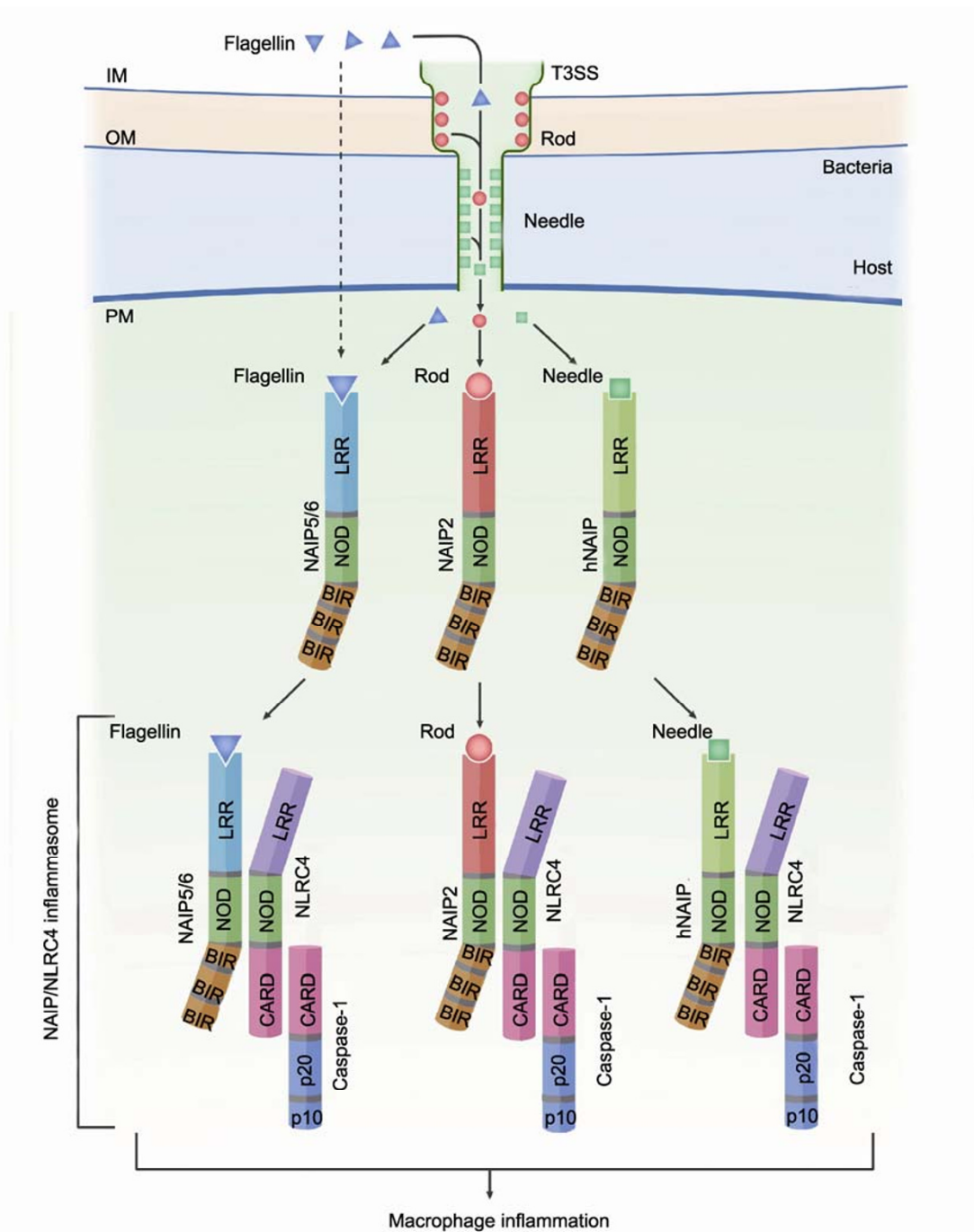


Figure 1. Model of NAIP receptors for recognizing bacterial products in stimulating NLRC4 inflammasome activation. The NAIP family of NLR proteins contains three BIR domains at the N termini and leucine-rich repeat (LRR) domain at the C termini. Murine NAIP5 (possibly also the closely related NAIP6) directly recognizes monomeric bacterial flagellin (blue triangle) that accidentally gets into macrophage cytosol through the type III secretion system (T3SS) (solid line) or other T3SS-independent means (dashed line). Another murine NAIP-family member NAIP2 recognizes the rod component (red circle) of the T3SS while the sole NAIP protein in human (hNAIP) serves as the inflammasome receptor for the T3SS needle subunit (green square). Binding of NAIP receptors by corresponding bacterial ligands induces a physical interaction between NAIP and NLRC4, leading to formation of an oligomeric NLRC4 inflammasome complex. This type III secretion system serves as a platform for inducing downstream caspase-1 activation through the CARD–CARD interaction between NLRC4 and caspase-1. Caspase-1 activation initiates macrophage inflammatory responses including processing and maturation of IL-1 β /IL-18 and macrophage pyroptotic cell death. PM: eukaryotic cell plasma membrane; OM: bacterial outer membrane; IM: bacterial inner membrane.

RECOGNITION OF BACTERIAL TYPE III SECRETION SYSTEM BY MURINE NAIP2 AND HUMAN NAIP

Given the evolutionary and functional connection between the type III secretion system and the flagellar system, it has been proposed that inflammasome sensing of a small portion of newly synthesized flagellin monomers, which are accidentally translocated into host cytosol through the type III secretion system, serves as an indirect means for the host to detect an active virulent effector secretion system (Miao and Warren, 2010). However, some bacterial pathogens such as EPEC, *C. violaceum* and *B. thailandensis* have evaded from NAIP5(6)-mediated innate immune recognition (Zhao et al., 2011), and bacterium like *Yersinia enterocolitica* shuts down its flagellin expression at the infection temperature of 37°C. Therefore, evidences have been provided that the NLRC4 inflammasome can also respond to the rod subunit of the type III secretion system, as has been observed with PrgJ in *Salmonella* SPI-1 in mouse macrophage infection (Miao et al., 2010b). Zhao et al. further noted that the NLRC4 inflammasome in human monocytic U937 cells specifically responds to the needle subunit of the type III secretion system, such as PrgI in *S. typhimurium* and CprI in *C. violaceum* (Zhao et al., 2011). Cytoplasmic delivery of purified recombinant type III secretion rod or needle subunit by using the N-terminal domain of anthrax lethal factor can stimulate NLRC4-dependent caspase-1 activation and macrophage inflammation, indicating an inflammasome-stimulating activity of these secretion system components by themselves (Zhao et al., 2011). Interestingly, similarly to that observed with flagellin, the rod and needle proteins from different bacterial species also show differential inflammasome-stimulating activity. Thus, detection of both flagellin and the type III secretion apparatus components by the NLRC4 inflammasome can maximize the capacity of this arm of innate immunity to ensure an effective response to the similar type of bacterial activities.

Analogous to flagellin recognition by NAIP5(6) receptor, the rod protein is recognized by another NAIP family member, NAIP2 in mice (Kofoed and Vance, 2011; Zhao et al., 2011). Mouse genome encodes seven homologous NAIP proteins, and NAIP2 is most distantly related to NAIP5 in primary sequence. RNA interference knockdown of NAIP2 in mouse bone marrow macrophages attenuates the rod protein-triggered inflammasome activation during EPEC and *S. typhimurium* infection. NAIP2 can directly bind to the rod protein such as PrgJ of *S. typhimurium* and BsaK of *B. thailandensis* (Zhao et al., 2011), and this binding also stimulates a complex formation between NAIP2 and NLRC4 (Fig. 1), rendering functional reconstitution of an oligomeric NLRC4 inflammasome complex in nonmacrophage cells (Kofoed and Vance., 2011; Zhao et al., 2011). Human only harbors one

NAIP family member and human monocytic U937 cells are defective in sensing bacterial flagellin and the type III secretion rod subunit (Zhao et al., 2011). Genetic studies of *C. violaceum* infection of U937 cells revealed that the type III secretion needle subunit CprI is responsible for infection-triggered NLRC4 inflammasome activation. Biochemically, both CprI and PrgI of *S. typhimurium* are proficient in direct interaction with human NAIP, suggesting that human NAIP functions in a similar way as NAIP5(6)/2 in mice and serves as the receptor for the type III secretion needle subunit (Fig. 1) (Zhao et al., 2011). Furthermore, human NAIP, but not murine NAIP5(6) and NAIP2, supports reconstitution of an active NLRC4 inflammasome that can specifically respond to intracellular delivery of recombinant CprI or PrgI. Other homologous needle subunits from EHEC, *B. thailandensis*, *P. aeruginosa*, *S. flexneri* and *S. typhimurium* can also stimulate NLRC4 inflammasome activation in human cells (Zhao et al., 2011). This suggests that direct recognition of the type III secretion needle subunits by human NAIP represents another general innate immune response to bacterial infection.

Previous studies on the mechanism of type III secretion suggest that both the rod and needle subunits are secreted prior to initiation of effector secretion. The rod protein could also be accidentally exported out of the bacteria. This provides an explanation for how these secretion apparatus components can be exposed to recognition by the NAIP receptors in macrophage cytosol. This is certainly the case for some bacterial pathogens. For example, mutation of the essential ATPase component in *S. typhimurium* SPI-1 blocks *S. typhimurium*-induced inflammasome activation in human U937 cells that results from activity of the needle subunit PrgI. However, the situation appears to be different for *C. violaceum* infection as the needle protein CprI-triggered caspase-1 activation in U937 cells still occurs in the absence of the two type III secretion ATPase subunits (Zhao et al., 2011). This indicates that entry of the needle subunit into macrophage cytosol does not absolutely require a functional type III secretion system. Consistently, ectopic expression of *S. typhimurium* SPI-1 rod protein PrgJ in *L. monocytogenes* can potently stimulate NAIP2-dependent NLRC4 inflammasome activation (Kofoed and Vance., 2011). Therefore, the rod and needle proteins can get into macrophage cytosol in both type III secretion-dependent and -independent manners (for instance, bacterial lysis), and the relative contribution of each channel depends on the particular pathogen and likely also the infection condition.

CONCLUSION AND PERSPECTIVE

Thus, in addition to sensing cytoplasmic presence of bacterial flagellin by the NAIP5 receptor, the innate immune system in mammals employ other NAIP proteins such as NAIP2 in mice and NAIP in human to directly recognize components of

bacterial type III secretion system. Engagement of NAIP receptors by corresponding bacterial ligands promotes their physical interaction with NLRC4 and formation of an oligomeric NLRC4 inflammasome complex, resulting in activation of caspase-1 and macrophage innate immunity (Fig. 1). Similarly to that of flagellin, the inflammasome-stimulating activities of type III secretion rod and needle proteins also reside in their C-terminal helical hairpin regions that are enriched in hydrophobic residues. Therefore, other homologous NAIP family members in mice might recognize bacterial products of similar biochemical features. The arrival of seven NAIPs in the mouse system, probably resulting from gene duplication during the evolution, likely reflects the need of a broad-spectrum innate immune surveillance for the mouse to cope with diverse bacterial infections it encounters. Moreover, it is also interesting to note here that the NLR-like protein Apaf-1 recognizes mitochondrion-derived cytochrome c to form the oligomeric apoptosome complex for caspase-9 activation, particularly given the evolutionary connection between mitochondria and bacteria.

Two important questions remain regarding the mechanism and other functional aspects of the NAIP-NLRC4 inflammasome axis in anti-bacterial innate immunity. Firstly, what is the biochemical basis for specific recognition of flagellin and the type III secretion rod and needle proteins by different NAIP receptors? Answer to this question requires future structural studies of NAIP receptors in complex with the particular bacterial ligand. Secondly, are there additional functions of the NLRC4 inflammasome in innate immune detection of other bacterial infection? For example, genetic deletion of *zmp1* from *Mycobacterium tuberculosis*, which encodes a putative metalloprotease, results in elevated inflammasome activation that is mediated by NLRC4 and ASC (Master et al., 2008). This suggests that the NLRC4 inflammasome likely detects an unknown signal from *M. tuberculosis*. It is worthwhile to further probe whether this activation also involves a NAIP family receptor and what is the biochemical mechanism underlying *Zmp1* inhibition of NLRC4 inflammasome activation.

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ABBREVIATIONS

CARD, caspase recruitment domain; EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; LRR, leucine-rich repeat; NLRs, NOD-like receptors; NOD, oligomerization domain; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; TLRs, Toll-like receptors.

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