The inflammasome in pathogen recognition and inflammation

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Abstract: The nucleotide-binding oligomerization domain-like receptor (NLR) family of proteins is involved in the regulation of innate immune responses and cell death pathways. Some NLR family members promote the activation of proinflammatory caspases within multiprotein complexes, called inflammasomes. Recent studies analyzing mice deficient in various components of the inflammasome have provided insight into the role of these molecules in host defense against pathogens and autoinflammatory disorders. Here, we review these studies and propose that membrane disruption leads to activation of the inflammasome. J. Leukoc. Biol. 82: 000–000; 2007.

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INTRODUCTION

The mammalian nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family of intracellular proteins is characterized by a unique nucleotide-binding domain—found in neuronal apoptosis inhibitory protein (NAIP), Class II trans-activator (CIITA), incompatibility locus protein from Podospora anserina (HET-E), and telomerase-associated protein (TP-1; NACHT)—which is located at the center of the molecule between an N-terminal, protein-binding domain caspase-recruitment domain (CARD), pyrin domain (PYD), or baculovirus inhibitor of apoptosis repeat (BIR) and a C-terminal leucine-rich-repeat (LRR) domain [1–4] (Fig. 1). The NLR family members Nod3, Nod4, and Nod5 contain an as-of-yet undefined domain at their N terminus. The structure of NLR proteins resembles that of Apaf-1 of vertebrates and insects, Ced-4 of C. elegans, and the nucleotide-binding site (NBS)-LRR-type, disease-resistance proteins (R proteins) of plants, all of which contain another type of nucleotide-binding domain, NB-ARC or apoptotic ATPase, which is also centrally located between an N-terminal protein-binding domain (CARD, TIR, or CC domain) and a C-terminal WD40 or LRR domain [5]. Our understanding of the mechanism of NLR molecules is based on evolutionary similarities with other innate immune receptors, which contain a LRR motif (i.e., TLRs and plant NBS-LRR-type R proteins); NLR molecules are postulated to function as intracellular pathogen recognition molecules. The NACHT domain regulates activity of the nucleotide in a way that is dependent on ligand binding to the LRR motif, and the N-terminal protein-binding domain then transmits a signal to specific downstream effectors.

The plant NBS-LRR-type R proteins have evolved divergently to protect the host from various pathogens, and some NBS-LRR-type R proteins have been demonstrated to bind pathogen-derived molecules [6, 7]. However, it remains unclear whether the NBS-LRR-type R proteins are direct pathogen recognition receptors or sensors of cellular distress. For example, a NBS-LRR protein of Arabidopsis, RPS5, recognizes a fragment of the host protein PBS1, which is generated by the protease activity of the Pseudomonas syringae protein AvrPphB following its injection into host cells by the bacteria [8]. This finding implies that this NBS-LRR protein recognizes a consequence of the activity of pathogens rather than the presence of the pathogen itself.

THE INFLAMMASOME: A MOLECULAR PLATFORM TO ACTIVATE CASPASE-1

Although caspases are known to play an essential role in apoptotic cell death, a member of the caspase family, caspase-1, regulates the secretion of the proinflammatory cytokines IL-1β[UNMAPED CHARACTER &x0200;] and IL-18 by cleaving the corresponding, immature proforms of these cytokines, pro-IL-1β and pro-IL-18. IL-33, a cytokine involved in generation of Th2 responses, is also processed by caspase-1 [9]. Besides caspase-1, four more caspases, which are phylogenetically related to caspase-1, are also implicated in the regulation of inflammation and therefore, called inflammatory caspases. These are caspase-1, caspase-4, and caspase-5 in humans and caspase-1, caspase-11, and caspase-12 in the mouse [10]. Although human and mouse caspase-1 are orthologs, human caspase-4 and caspase-5 are likely to have arisen from gene duplication of caspase-11 [10]. In contrast to murine caspase-12, human caspase-12 appears to be inactive [11].

Based on analogy to the induced-proximity model for the activation of the apoptotic caspases 8 and 9 by CD95/Fas-death-inducing signaling complex (DISC) and the Apaf-1-apoptosome, respectively, a putative, molecular platform to activate the inflammatory caspases has been termed the “inflammasome” [12–14]. Tschopp and colleagues [12, 13] proposed...
an inflammasome model based on biochemical analysis of three NLR proteins, NALP1, NALP2, and NALP3, and NALP3 (also known as cryopyrin, CIAS-1, Pypaf-1, or CLR1.1), which has a PYD at its N terminus, binds to two kinds of adaptor proteins, ASC and a CARD-containing protein (CARDINAL); both of these adaptors bind to caspase-1 through CARD-CARD interactions so that a complex composed of a single NALP3, ASC, and CARDINAL molecule can attract two caspase-1 molecules to this caspase-1 activation platform, the NALP3 inflammasome. There is no CARDINAL homologue in the mouse, and hence, murine NALP3 is thought to recruit only a single caspase-1 molecule (Fig. 2). NALP1 and NALP2 also have been shown to form similar caspase-1-activating platforms. It remains unclear whether the NALP3 inflammasome is stably present in the cytoplasm or if it is assembled by upstream signals as in the apoptosome or DISC.

NALP3 AND DANGER SIGNALS

Macrophages secrete IL-1β in response to serial stimulation with LPS followed by ATP. In this experimental system, LPS or ATP alone are not sufficient to induce IL-1β secretion, and LPS is required to induce pro-IL-1β and to prime cells for caspase-1 activation in response to ATP [20]. The stimulation of cells by high concentrations of ATP is thought to mimic the rapid release of ATP from activated platelets, neurons, antigen-stimulated T cells, and injured cells. The effect of ATP is mediated by P2X7, which causes a rapid K+ efflux from the cytosol upon activation [21, 22]. As potassium ionophores, membrane-permeabilizing agents, pore-forming agents, and K+ depletion from cell culture media are also known to induce IL-1β secretion, the efflux of cytosolic potassium is thought to be an essential trigger of caspase-1 activation [23–27]. Recent studies have identified pannexin-1, a membrane protein that
NALP3 detects endogenous danger signals. Following cell death may be an additional way in which the cellular milieu by necrotic cells. Recognition of uric acid is a major component released into the extracellular space. Inflammatory joint disease. In addition to its role in gout, uric acid is a major component released into the extracellular space. NALP3-dependent manner. Deposition of MSU and CPPD crystals activate caspase-1 in a NALP3-dependent manner. Studies using macrophages from NALP3-deficient mice have shown that NALP3 plays a central role in caspase-1 activation in response to TLR stimulation combined with potassium efflux (Fig. 2). NALP3-deficient macrophages stimulated with TLR agonists plus ATP, nigericin, or maitotoxin failed to secrete IL-1β and IL-18 [30–32]. This defect in IL-1β and IL-18 secretion was found to occur at the level of caspase-1 activation, as stimulation of NALP3-deficient macrophages with LPS plus ATP did not result in the autocatalytic cleavage of procaspase-1 into its p20 and p10 subunits. In vivo, mice challenged with LPS showed increased resistance to endotoxic shock in the absence of ASC or NALP3 [30, 31]. ASC-deficient mice, however, tolerated higher doses of LPS compared with NALP3-deficient mice, suggesting that ASC, being a common downstream adaptor for multiple NLR molecules, may play additional roles in endotoxic shock, which do not overlap with those of NALP3.

In a separate study, Martinon and colleagues [32] demonstrated that monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) crystals activate caspase-1 in a NALP3-dependent manner. Deposition of MSU and CPPD crystals in joints is responsible for the inflammatory conditions gout and pseudogout, respectively. It is unclear if MSU and CPPD activation of the NALP3 inflammasome is dependent on K+ effluxes, similar to other NALP3 inflammasome-activating agents such as ATP and nigericin. This study identifies NALP3 as playing a potentially important role in inflammatory joint disease. In addition to its role in gout, uric acid is a major component released into the extracellular milieu by necrotic cells. Recognition of uric acid following cell death may be an additional way in which NALP3 detects endogenous danger signals.

**BACTERIAL ACTIVATION OF THE NALP3 INFLAMMASOME**

Secrecion of IL-1β and IL-18 by macrophages in response to infection with *S. aureus* and *L. monocytogenes* is dependent on ASC and NALP3 [31] (Fig. 2). Mutant *L. monocytogenes* deficient in LLO were unable to induce caspase-1 activation in wild-type macrophages, suggesting that LLO-mediated escape of *L. monocytogenes* from the phagosome into the cytosol was crucial for activation of the NALP3 inflammasome, presumably by allowing the detection of pathogen-associated molecular patterns (PAMPs) now released into the cytosol. We however found that LLO-containing supernatants, in the absence of bacteria, added to macrophages extracellularly, result in the NALP3-dependent activation of caspase-1 (Y. Ogura and R. A. Flavell, unpublished observations). These data suggest that the pore-forming capacity of LLO on the plasma membrane of the macrophage results in a potassium efflux, which in turn leads to the activation of the NALP3 inflammasome. Unlike LLO-deficient *L. monocytogenes*, *S. aureus* singly deficient in α-, β-, or γ-hemolysin were still capable of activating caspase-1 [31]. This may be a result of redundant membrane disruptive toxins still present when only a single hemolysin is absent.

The pore-forming toxin aerolysin from *Aeromonas hydrophila* is also able to activate caspase-1 by mediating a potassium efflux [33]. Using small interfering RNA to silence expression of NALP3, ASC, or IPAF in HeLa cells, the NALP3 and IPAF inflammasomes were implicated in mediating caspase-1 activation in response to aerolysin [33]. It will be informative to determine the roles of NALP3 and IPAF in aerolysin-mediated caspase-1 activation using primary cells from NALP3- and IPAF-deficient mice.

Kanneganti et al. [34, 35] have suggested that NALP3 and ASC are essential for caspase-1 activation in response, specifically to bacterial RNA, the imidazoquinoline compounds R837 and R848, and viral dsRNA. Their studies found that these PAMPs were capable of activating caspase-1 in the *Listeria monocytogenes* and *Staphylococcus aureus*, microbial toxins (maitotoxin, aerolysin, and nigericin), and danger signals (ATP and uric acid) have all been implicated in the activation of the NALP3 inflammasome. A number of these stimuli mediate a potassium efflux; however, it is still unclear if NALP3 inflammasome activation by uric acid and *S. aureus* is dependent on potassium. These stimuli likely lead to a conformational change in NALP3 by an unknown mechanism, which allows NALP3 to oligomerize. Following oligomerization, NALP3 recruits ASC through a homophilic PYD-PYD interaction. ASC, in turn, recruits procaspase-1 via homophilic CARD-CARD interactions, which lead to activation of caspase-1, and active caspase-1 can then process pro-IL-1β and pro-IL-18 and induce macrophage cell death. LLO, Listeriolysin-O; P2X7, an ionotropic ATP receptor.
absence of ATP, which is in contrast to the findings of others. In addition, this study failed to observe ATP-mediated caspase-1 activation in response to other TLR ligands such as LPS, lipoteichoic acid, and Pam3CSK4. The reason for the discrepancy between this study [34] and the others [30–32] remains unclear.

**BACTERIAL ACTIVATION OF THE IPAF INFLAMMASOME**

Caspase-1 is important for host defense to a number of pathogens. Infection of macrophages with *Salmonella*, *Shigella*, *Legionella*, and *Pseudomonas* all leads to caspase-1 activation, release of IL-1β, and rapid cell death. The caspase-1-mediated cell death caused by infection with *Salmonella typhimurium* was found to be independent of NALP3 [30, 31]. *S. typhimurium*-infected macrophages did, however, require IPAF to activate caspase-1 [36] (Fig. 3). Recent studies in our lab suggest that *P. aeruginosa*-induced activation of caspase-1 is also dependent on IPAF (F. S. Sutterwala and R. A. Flavell, unpublished observations). The role for ASC in IPAF-mediated caspase-1 activation is unclear. Although IPAF can interact directly with procaspase-1, ASC-deficient macrophages have delayed kinetics of cell death following infection with *S. typhimurium* compared with wild-type. This suggests ASC may serve a role stabilizing the interaction between IPAF and procaspase-1.

Despite the dramatic in vitro effects of IPAF deficiency on *S. typhimurium*-induced macrophage death, IPAF-deficient mice infected orally with *S. typhimurium* did not display enhanced susceptibility to infection [37]. However, caspase-1-deficient mice infected with *S. typhimurium* were more susceptible to infection, as demonstrated by a shorter time until death and higher bacterial burdens in spleen and mesenteric lymph nodes. The in vivo difference seen between caspase-1-deficient mice and IPAF-deficient mice may be a result of additional, undefined pathways, which result in caspase-1 activation, or a synergistic effect among IPAF, NALP3, and ASC deficiency.

*L. pneumophila* can also mediate macrophage cell death through a caspase-1-dependent manner [38] (Fig. 3). The activation of caspase-1 by *L. pneumophila* is dependent on IPAF but not NALP3 [38, 39]. *L. pneumophila* is unique in that another NLR member, Naip5 (Birc1e), is also involved in susceptibility to infection with *L. pneumophila* [40, 41]. These findings link Naip5 to the caspase-1 pathway, which is supported by the observation that Naip5 and IPAF can interact physically [38].

Recent studies have begun to provide insight into how the IPAF inflammasome is activated. *S. typhimurium* [42, 43] and *L. pneumophila* [44, 45] strains deficient in flagellin were found to be defective in their ability to activate caspase-1. Purified flagellin delivered to the macrophage cytosol by transfection was also capable of activating caspase-1 [42, 43]. These findings led to the hypothesis that cytosolic flagellin contributes to the activation of caspase-1. However, the direct activation of IPAF by cytosolic flagellin remains controversial for several reasons. First, the *P. aeruginosa* mutant PAKΔflc, which is deficient in flagellin, is still capable of activating caspase-1 in an IPAF-dependent manner (F. S. Sutterwala and R. A. Flavell, unpublished observations). Second, the nonflagellated bacterium *Shigella flexneri* has also been suggested to activate caspase-1 [42, 43]. These findings led to the hypothesis that cytosolic flagellin contributes to the activation of caspase-1. However, the direct activation of IPAF by cytosolic flagellin remains controversial for several reasons. First, the *P. aeruginosa* mutant PAKΔflc, which is deficient in flagellin, is still capable of activating caspase-1 in an IPAF-dependent manner [46]. Finally, Miao et al. [43] showed that at a high multiplicity of infection, flagellin-deficient *S. typhimurium* strains were still capable of inducing macrophage secretion of IL-1β.

A common feature that *S. typhimurium*, *S. flexneri*, and *P. aeruginosa* share is their requirement for an intact, Type III secretion system (TTSS) to activate caspase-1 [47, 48]. *L. pneumophila*-induced caspase-1 is dependent on the Dot-Icm Type IV secretion system, which is unrelated structurally but similar functionally to the TTSS [38]. As flagellin alone is not sufficient to activate the IPAF inflammasome, it is possible that rather than detecting a specific pathogen-derived molecule,
IPAF detects membrane damage induced by Type III or Type IV secretion systems. Flagellin may serve to enhance Type III or Type IV secretion system-dependent caspase-1 activation through aiding bacterial adhesion to the host cell. Alternatively, flagellin may interact with Naip5, which in turn associates with IPAF.

MEMBRANE DISRUPTION AS A POSSIBLE TRIGGER FOR INFLAMMASOME ACTIVATION

Mariathasan and colleagues [49] have recently shown that F. tularensis is also capable of mediating macrophage cell death in a manner that is dependent on ASC and caspase-1, and caspase-1- and ASC-deficient mice infected in vivo with F. tularensis showed increased susceptibility to infection compared with wild-type mice. NALP3 and IPAF were not found to be responsible for mediating caspase-1 activation in response to infection with F. tularensis, suggesting another NLR family member plays a role in the detection of F. tularensis. It is interesting that it was found that F. tularensis mutants, which could not escape from the phagosome, did not induce caspase-1 activity [49]. This suggests that disruption of the phagosome membrane may be yet another signal to activate a specific NLR pathway.

Caspase-1 has also been found to play a role in membrane repair. Gureel et al. [33] found that aerolysin-induced membrane disruption leads to a K⁺ efflux, which is responsible for activation of NALP3 and IPAF inflammasomes [33]. Caspase-1, which is activated in this manner, induces the activation of sterol regulatory element-binding proteins, which promote cell survival through membrane repair [33]. It is unclear why activation of caspase-1 in response to pathogens such as Salmonella, Shigella, and Pseudomonas leads to cell death, whereas caspase-1 activation by aerolysin toxin leads to increased cell survival. Specific activation of caspase-1 via NALP3 or IPAF inflammasomes may be responsible for guiding this process.

Although rapid progress has been made in identifying how the inflammasome is activated, many crucial questions remain. It is unclear if NLRs serve as direct receptors of PAMPs or if they sense the consequences of infection or inflammatory stimuli indirectly. We propose that NLR molecules recognize and respond to membrane disruption itself as opposed to the concept that membrane disruption serves as a portal for entry of PAMPs into the cytosol. The NALP3 inflammasome appears to be activated following a potassium efflux mediated by membrane disruption of the plasma membrane by pore-forming toxins or through the engagement of the P2X₇ receptor by ATP. The IPAF inflammasome, conversely, is activated by membrane disruption mediated by Type III or Type IV secretion systems. A third pathway resulting in phagosome membrane disruption, such as that caused by the pathogen F. tularensis, may lead to an as-of-yet undefined NLR pathway. Hence, it appears that where the pathogen resides and what intracellular compartments the pathogen uses are crucial to which NLR pathway is activated. Further studies to elucidate the downstream events leading to activation of the inflammasome will be required to help elucidate how the NLRs function. Multiple NLR molecules remain, which still have unidentified functions. Identifying activation signals as well as effector functions for these NLRs will help in determining the role of these molecules in host defense against pathogens and in autoinflammatory disorders.

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