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NLRC4/IPAF: a CARD carrying member of the NLR family

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Abstract

The NOD-like receptor (NLR) family of proteins is involved in the regulation of innate immune responses and cell death pathways. Recent findings show that the NLR family member NLRC4 (also known as IPAF) has important roles in innate immune responses to Gram-negative bacteria. Macrophages infected with *Legionella pneumophila*, *Salmonella typhimurium*, *Shigella flexneri*, or *Pseudomonas aeruginosa* activate caspase-1 in an NLRC4-dependent manner leading to macrophage cell death and the release of proinflammatory cytokines. This review will discuss these findings as well as the role of bacterial type III and type IV secretion systems and flagellin in NLRC4-mediated caspase-1 activation.

Keywords

Innate Immunity; NOD-like receptors; NLRC4; Caspase-1; Inflammasome; Macrophage; Flagellin; Type 3 Secretion System; type 4 Secretion System

Introduction

The innate immune system possesses numerous germline-encoded pattern recognition receptors that are capable of recognizing highly conserved pathogen-associated molecular patterns (PAMPs). These receptors include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like RNA helicases (RLHs) and C-type lectin receptors (CLRs). In addition to PAMPs, NLRs can sense endogenous danger signals (danger-associated molecular patterns; DAMPs) released by cellular damage or stress in response to either invading pathogens or sterile inflammatory responses.

The importance of the NLRs in innate immunity can be demonstrated by the finding that mutations within a number of the NLR genes have been associated with autoimmune and autoinflammatory disorders in humans (Table 1). Mutations in NOD2 have been associated with Crohn's disease and Blau syndrome [14, 15]. Mutations in NLRP3 (also known as NALP3, CIAS1 and cryopyrin) are responsible for the autoinflammatory syndromes, Muckle-Wells

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syndrome, familial cold autoinflammatory syndrome, and neonatal-onset multisystem inflammatory disease [14,15]. Most recently sequence variants in the *NLRP1* (also known as NALP1 and DEFCAP) gene have been linked to autoimmune and autoinflammatory diseases associated with vitiligo [7].

The NLR family

The human NLR family of intracellular proteins contains 22 members including 14 NLRP members, 5 members of the NLRC subfamily, NAIP, NLRX, and CIITA [16]. Currently, a number of different names have been assigned to the members of the NLR family. Recently a standardized nomenclature has been proposed which we will use throughout this review [17]. NLRs have a unique nucleotide-binding domain called NACHT, which is located at the center of the molecule between an N-terminal protein-binding domain (CARD (caspase-recruitment domain), PYD (pyrin domain) or BIR (Baculovirus IAP repeat)) and a C-terminal LRR (Leucine-rich repeat) domain.

A number of the NLR molecules have been shown to interact with and modulate the function of the cysteine protease caspase-1. Based on elegant biochemical analysis of three NLR proteins, NLRP1, NLRP2 and NLRP3, a model was proposed in which caspase-1 was activated within a multiprotein complex termed the inflammasome [18]. The structure and components of these inflammasomes have recently been reviewed [16]. In addition to NLRP1, NLRP2, and NLRP3, a number of other NLR molecules including, NLRC4, NLRP6, NLRP7, NLRP10 and NLRP12 have also been reported to modulate caspase-1 activity [16]. However, so far only NLRP1, NLRP3 and NLRC4 have been shown to have clear physiologic roles.

The NLRC4 inflammasome

NLRC4 (also known as IPAF, Card12 and CLAN) is expressed in myeloid cells where it has been shown to regulate caspase-1 activation and IL-1 β processing. NLRC4 contains an N-terminal CARD, a central NACHT domain and C-terminal LRRs. Deletion of the LRR domain of NLRC4 results in a constitutively active form of the molecule suggesting that NLRC4 activation occurs in a similar manner to other NLRs that also gain activity upon deletion of their LRR domain [19]. NLRC4, similar to other NLRs such as NLRP3, NOD1 and NOD2, also interacts with the chaperone protein heat-shock protein 90 (HSP90) and the ubiquitin ligase-associated protein SGT1 [20,21]. HSP90 and SGT1 have been shown to be required for NLRP3, NOD1 and NOD2 activation and are predicted to also be required for NLRC4 [20, 21].

Despite the many similarities between NLRC4 and other NLR proteins there are also striking differences. Activation of caspase-1 through an NLRC4 dependent pathway is closely associated with the subsequent death of the cell. However, stimuli that activate caspase-1 through NLRP3 predominantly result in the processing and secretion of IL-1 β and IL-18 without necessarily resulting in cell death. Hence, NLRP3 and NLRC4 may play an important role in directing the final fate of caspase-1 activity either towards processing of proinflammatory cytokines or initiation of cell death pathways and may therefore add a layer of specificity to the innate immune response against specific subgroups of pathogens.

Many studies have demonstrated the importance of a potassium efflux in the activation of the NLRP3 inflammasome [22–24]. Activation of caspase-1 through NLRC4 however does not require potassium, further demonstrating the differences between these two pathways [25].

Bacterial activation of the NLRC4 inflammasome

NLRC4-mediated activation of caspase-1 has been shown to be important in host defense for a number of pathogens. Infection of macrophages with *Salmonella*, *Shigella*, *Legionella*, and *Pseudomonas* all lead to caspase-1 activation, release of IL-1 β and rapid cell death. The first study to demonstrate NLRC4's role in host defense examined the pathogen *S. typhimurium* [26]. NLRC4-deficient macrophages infected with *S. typhimurium* had a marked defect in their ability to activate caspase-1 and secrete IL-1 β and IL-18. In addition, *S. typhimurium*-induced macrophage death was also retarded in NLRC4-deficient macrophages [26]. However, despite the dramatic *in vitro* effects of NLRC4-deficiency on *S. typhimurium*-induced macrophage caspase-1 activation, NLRC4-deficient mice infected orally with *S. typhimurium* did not display enhanced susceptibility to infection [27]. Caspase-1-deficient mice infected with *S. typhimurium* were however more susceptible to infection with *S. typhimurium* [27]. The *in vivo* difference seen between caspase-1-deficient mice and NLRC4-deficient mice may be due to additional undefined pathways that lead to *Salmonella*-induced caspase-1 activation that are not mediated through NLRC4.

Legionella pneumophila is also capable of mediating macrophage cell death through a caspase-1-dependent manner. The activation of caspase-1 by *L. pneumophila* has been demonstrated to be dependent on NLRC4 [28]. *L. pneumophila* is unique in that another NLR member, Naip5 (Birc1e), is also involved in susceptibility to infection with *L. pneumophila*. Naip5 and NLRC4 have been shown to physically interact linking Naip5 to the caspase-1 pathway [28], however the role of Naip5 in caspase-1 activation remains unclear as discussed below. NLRC4 has also been shown to play an important role in *L. pneumophila* mediated phagosome maturation. *L. pneumophila*-containing phagosomes avoid fusion with the lysosome in NLRC4- and caspase-1-deficient macrophages hence allowing bacterial replication [29]. These findings demonstrate a novel function for the NLRC4 inflammasome in response to intracellular bacteria.

S. flexneri has also been shown to activate caspase-1 in an NLRC4 dependent manner [30]. This intriguing study also shows that shigella-induced autophagy was increased in the absence of NLRC4 or caspase-1 and that autophagy may protect macrophages from caspase-1-mediated pyroptosis [30]. Finally, three recent studies have demonstrated a clear role for NLRC4 in host defense against *P. aeruginosa* [31–33]. NLRC4-deficient macrophages were markedly diminished in their ability to activate caspase-1 and secrete IL-1 β in response to *P. aeruginosa*. Additionally, in both a pulmonary and a peritoneal *in vivo* model of *P. aeruginosa* infection NLRC4-deficient mice were more susceptible to infection [31,32]. Interestingly, a subset of *P. aeruginosa* strains that express the effector molecule ExoU were able to inhibit caspase-1 activation through ExoU phospholipase activity [32]. The *P. aeruginosa* effector molecule ExoS has also been shown to inhibit IL-1 β maturation in a manner dependent on its ADP ribosyltransferase activity [34]. The fact that *P. aeruginosa* appears to have evolved mechanisms to inhibit NLRC4-mediated caspase-1 activation further suggests that this pathway is important for host defense against Gram-negative bacterial infections.

ASC and Naip5 in NLRC4 mediated caspase-1 activation

As NLRC4 can interact directly with pro-caspase-1 through a CARD-CARD interaction, the role of the adaptor molecule ASC in NLRC4-mediated caspase-1 activation is unclear. In a number of infectious models including *Salmonella*, *Shigella* and *Pseudomonas*, ASC-deficient macrophages demonstrated defective caspase-1 activation and IL-1 β secretion; however, these infected ASC-deficient macrophages still underwent cell death in response to infection with similar kinetics compared to WT macrophages [26,30,32]. These observations suggest that

ASC is in fact crucial for NLRC4 mediated caspase-1 activation and IL-1 β secretion but does not play a role in NLRC4-mediated cell death. As there is no detectable caspase-1 activation in ASC-deficient macrophages infected with Salmonella, Shigella or Pseudomonas, it remains to be determined the mechanism by which ASC-deficient macrophages are undergoing cell death.

Macrophages from A/J mice have a mutant *Naip5* allele and are permissive for the intracellular growth of *L. pneumophila*. Given that caspase-1- and IPAF-deficient macrophages were also permissive for replication of *L. pneumophila* and that IPAF and Naip5 could physically interact, it was postulated that Naip5 played a role in the IPAF inflammasome pathway [28,35]. A number of studies have found that the restriction of Legionella growth is far more complex and requires IPAF-dependent activation of caspase-1 as well as Naip5 signaling [36,37]. Miao and colleagues also examined the role of Naip5 in caspase-1 activation and found that A/J (Naip5-deficient) macrophages were able to secrete IL-1 β in response to infection with *S. typhimurium*, *P. aeruginosa*, and *Listeria monocytogenes* suggesting that Naip5 is dispensable for caspase-1 activation [33].

Does cytosolic flagellin trigger NLRC4-mediated caspase-1 activation?

Recent studies have begun to elucidate how the NLRC4-inflammasome is activated. One component that is critical for pathogens, such as Salmonella, Legionella, Shigella and Pseudomonas, to activate caspase-1 is a functional bacterial type III (T3SS) or type IV (T4SS) secretion system [28,32,38,39]. The T3SS is a complex macromolecular structure that spans both bacterial membranes and includes a long needle-like structure through which effector molecules pass. Access of the effector molecules into the cytoplasm of the host cell requires disruption of the plasma membrane of the host cell by a proteinaceous pore called the translocon. *L. pneumophila* possesses a Dot-Icm T4SS that is structurally unrelated but functionally similar to the T3SS. Bacteria defective in T3SS or T4SS activity were also found to be unable to activate macrophage caspase-1. *S. typhimurium* and *L. pneumophila* strains deficient in flagellin are also defective in their ability to activate caspase-1 following macrophage infection [28,35,40–42]. In two independent studies, by the groups of Aderem and Núñez, delivery of purified flagellin into the macrophage cytosol by transfection was capable of activating caspase-1 in an NLRC4-dependent manner [41,42].

These findings led to the hypothesis that flagellin monomers gain entry into the cytosol of infected cells through T3SS or T4SS and lead to the activation of caspase-1 [41,42]. Recently Sun et al. demonstrated that the T3SS can in fact serve as a conduit for flagellin to pass into the cytosol of the infected cell (Figure 1) [43]. However, the activation of NLRC4 by cytosolic flagellin is not the complete story. The non-flagellated bacterium *S. flexneri* is capable of activating caspase-1 in an NLRC4-dependent manner [18]. We have also demonstrated that the *P. aeruginosa* mutant PAK Δ *fliC*, which is deficient in flagellin, is still capable of activating caspase-1 also in an NLRC4-dependent manner [32]. The groups of Aderem and Núñez did not observe caspase-1 activation using flagellin-deficient *P. aeruginosa* strains [31,33], but this may reflect differences in multiplicities of infection and infection times between these studies. Miao et al. showed that at a high multiplicity of infection flagellin-deficient *S. typhimurium* strains were still capable of inducing macrophage secretion of IL-1 β [41]. These data suggest that the NLRC4 inflammasome can also be activated independently of flagellin (Figure 1). Although flagellin may augment NLRC4-mediated caspase-1 activation, the direct ligand for NLRC4, as for most of the NLR family members, remains to be determined. Identification of the ligand for NLRC4 will help resolve many of the above questions and will enhance our understanding of the specificity built into the innate immune system.

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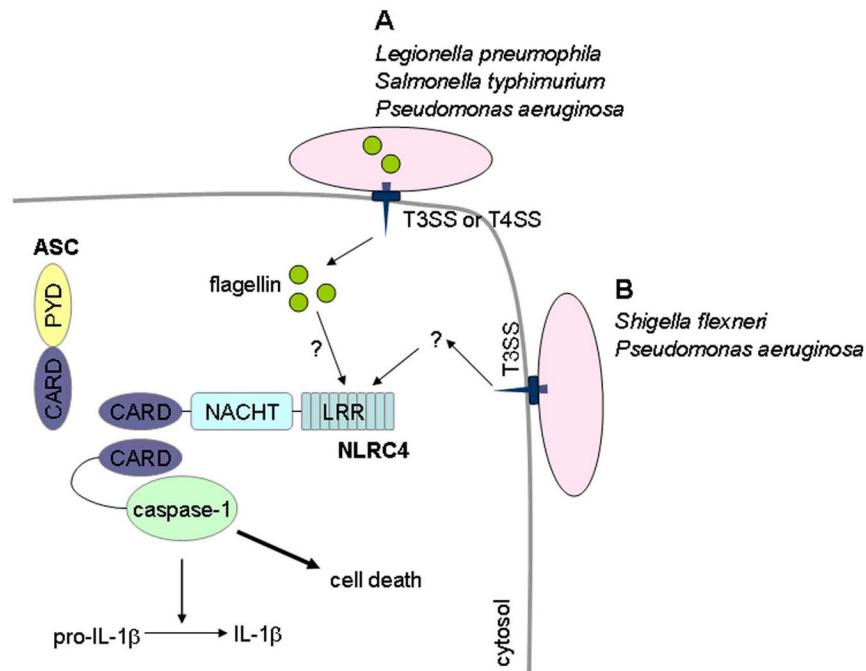


Figure 1. Activation of the NLRC4 inflammasome by Gram negative bacteria. Activation of caspase-1 following infection of macrophages with *S. typhimurium*, *P. aeruginosa*, *S. flexneri* or *L. pneumophila* requires a functional type III or type IV secretion system. Infection causes NLRC4 to undergo a conformational change by an unknown mechanism, which allows NLRC4 to oligomerize. Following oligomerization, NLRC4 recruits pro-caspase-1 via homophilic CARD-CARD interactions, which leads to activation of caspase-1. ASC is required for NLRC4-mediated caspase-1 activation although its exact role remains unclear. (A) Bacterial-derived cytosolic flagellin augments caspase-1 activation following infection with *L. pneumophila*, *S. typhimurium*, and *P. aeruginosa*. (B) Activation of caspase-1 following infection of macrophages with *S. flexneri* and *P. aeruginosa* can occur independently of flagellin.

Table 1

Overview of human NLR associated diseases

	Synonyms	Associated Disease	Reference
NOD2	NLRC2; CARD15; CLR16.3	Crohn's disease; Blau Syndrome; Early-onset sarcoidosis	[1–6]
NLRP1	NALP1; DEFCAP; NAC; CARD7; CLR17.1	Vitiligo-related autoimmune disorders	[7]
NLRP3	NALP3; CIAS1; PYPAF1; Cryopyrin; CLR1.1	Muckle Wells Syndrome; Familial Cold Autoinflammatory Syndrome; CINCA	[8–9]
NLRP7	NALP7; PYPAF3; NOD12; PAN7; CLR19.4	Hydatiform mole	[10]
NLRP12	NALP12; PYPAF7; Monarch1; PAN6; CLR19.3	Hereditary periodic fevers	[11]
NAIP	Birc1; CLR5.1; NLRB1	Spinal muscular atrophy	[12]
CIITA	MHCIITA; NLRA	Bare lymphocyte syndrome	[13]