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Nlrc4/Ipaf/CLAN/CARD12: more than a flagellin sensor

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Abstract

Nlrc4 is a member of the Nod-like receptors (NLRs), a family of cytosolic receptors involved in sensing bacterial molecules. NLRs are a group of proteins containing spans of leucine-rich repeats that senses bacterial factors within the eukaryotic cytosol. The recognition of bacterial factors provokes the formation of the inflammasome complex which includes specific NLRs. The inflammasome is responsible for caspase-1 activation which leads to the cleavage and maturation of inflammatory cytokines such as IL-1β and IL-18. Nlrc4 was considered to be a devoted flagellin sensor in eukaryotic cells. However, studies using a variety of pathogens such as *Salmonella*, *Legionella*, *Shigella* and *Pseudomonas* at high bacterial burdens revealed that Nlrc4 can mediate caspase-1 activation independent of bacterial flagellin. On the other hand, new reports showed that Nlrc4 can restrict bacterial infection independently of caspase-1. Therefore, Nlrc4 maybe involved in sensing more than one bacterial molecule and may participate in several immune complexes.

1. Introduction

The recognition of conserved microbial structures known as PAMP (pathogen associated molecular patterns) is accomplished by membrane bound Toll-like receptors (TLRs) and cytoplasmic NOD (nucleotide oligomerization domain) like receptors (NLRs) (Lamkanfi *et al.*, 2007a). There are 23 NLR in human genome, while mouse genome contains about 34 NLR-encoding genes (Amer, 2009). NLRs include NALPs (NACHT-, LRR-, and pyrin domain-containing proteins), Nlrc4 (Ipaf, CLAN or CARD12), and NAIPs (neuronal apoptosis inhibitory proteins) (Ting *et al.*, 2008). Upon sensing of PAMPs, the NLRs interact with members of the inflammasome complex. Consequently, inflammasomes are named after the specific NLR involved such as the NLRP1, NLRP3, NLRC4, AIM2 and the pyrin inflammasomes (Lamkanfi *et al.*, 2007a).

Assembly of the inflammasome complex leads to the cleavage and activation of procaspase-1 (Figure 1). Once activated, caspase-1 promotes the proteolytic maturation and activation of IL-1β, IL-18 and caspase-7 and the deactivation of IL-33 (Lamkanfi *et al.*, 2007a). Active caspase-1 also mediates pyroptotic cell death (Bergsbaken *et al.*, 2009).

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Nlrc4 was identified in 2001 as a novel member of the NLR family (Damiano *et al.*, 2001; Poyet *et al.*, 2001). Studies showed that Nlrc4 is the sensor for bacterial flagellin during infection with *L. pneumophila*, *Salmonella*, and *Pseudomonas*. However, new reports are emerging showing that Nlrc4 mediates caspase-1 activation independently of bacterial flagellin in mice. Studies in human cells show that Nlrc4 mediates restriction of bacterial infections independently of flagellin or caspase-1 (Vinzing *et al.*, 2008). These new data suggest that Nlrc4 is involved in sensing PAMPs other than flagellin and contributing to complexes other than the typical caspase-1 inflammasome. The new data suggest the existence of a flagellin-independent Nlrc4 inflammasome confirming that NLRs may not be dedicated bacterial sensors after all. Here we will discuss the new findings supporting the role of Nlrc4 in sensing flagellated and non-flagellated pathogens.

2. Structure

Nlrc4 is encoded by a genomic locus on human chromosome 2 p21-p22 (Damiano *et al.*, 2001). The transcript of this locus is 3.3 kb that forms an open reading frame for 1024 amino acid residue with a predicted molecular mass of 113 kDa (Geddes *et al.*, 2001). Like all NLR, Nlrc4 consists of three domains N Terminal CARD domain (residues 1-88), this domain is the effector domain that can interact with CARD of ASC as well as CARD of caspase-1 (Figure 2). A central NACHT domain (residues 163-457), which shows distinct motifs including ATP/GTPase, specific P-loop, and magnesium binding motif. This domain functions as nucleotide binding and oligomerization domain (NBD). C-terminal Leucine Rich Repeats (LRR) (residues 656-1024) contains at least 13 LRR motifs (Figure 2). The LRR domain detects upstream signal resulting in the activation of Nlrc4 (Poyet *et al.*, 2001). Deletion of this domain from Nlrc4 results in a constitutively active protein. Although CARD truncated NLRC4 is inactive, CARD motif alone show slight activity. So both CARD and NBD are crucial for Nlrc4 activity.

3. Biological functions

3.1 Nlrc4 and Shigella

Shigella flexneri (*Shigella*) infection induces caspase-1 activation, IL-1β processing and cell death in macrophages. These responses require a functional bacterial type III secretion system and the host NLR Nlrc4 (Schroeder *et al.*, 2007; Suzuki *et al.*, 2007). Interestingly, caspase-1 activation by *Shigella* is independent of flagellin but requires a bacterial protein secreted by the type III secretion system called IpaB. Caspase-1 can also be activated by lipopolysaccharide moiety released from the bacteria (Suzuki *et al.*, 2007). Notably, in the absence of Nlrc4, autophagy is detected in *Shigella* infected macrophages. It is possible that autophagy is induced independently of Nlrc4 but only apparent when pyropotosis is abolished in macrophages lacking Nlrc4. Alternatively, it is possible that Nlrc4 suppresses autophagy by an unkown mechanism.

3.2 Nlrc4 and Salmonella typhimurium

Infection of macrophages with *Salmonella typhimurium* (*Salmonella*) results in the activation of caspase-1 in an Nlrc4 dependent manner (Franchi *et al.*, 2006). Active caspase-1 cleaves pro-IL1β and IL18, and induces cell death and caspase-7 activation (Lamkanfi *et al.*, 2008; Damiano *et al.*, 2004). Overexpression of Nlrc4 in human cell lines restricted *Salmonella* during moderate levels of infection. On the other hand, overexpression of Nlrc4 predisposes macrophages to cell death upon exposure to large burdens of *Salmonella* (Damiano *et al.*, 2001). These findings suggest that the role of Nlrc4 differs according to the bacterial burden. The activation of caspase-1 requires functional *Salmonella* Pathogenicity Island 1 (SPI1) encoding type III secretion system (T3SS). There are several proteins secreted by this system, however, none of them is implicated in caspase-1 activation (Ehrbar *et al.*, 2002; Lostroh and

Lee, 2001). On the other hand, purified *Salmonella* flagellin delivered into the cell by lyposomes or by pore forming proteins induced Nlrc4 inflammasome recruitment and subsequent activation of caspase-1 (Sun *et al.*, 2007; Amer *et al.*, 2006). So how could T3SS deliver flagellin into host cytosol? It is hypothesized that flagellin monomers leak from bacterial cytoplasm into host cytosol through T3SS with the transfer of other virulence factors (Sun *et al.*, 2007).

3.3 Nlrc4 and Legionella pneumophila

Macrophages derived from most mouse strains activate caspase-1 in response to *Legionella pneumophila* (*L. pneumophila*) infection. Caspase-1 activation is accompanied with restriction of intracellular *L. pneumophila* growth whereas macrophages derived from Nlrc4 or caspase-1 knockout mice allow *L. pneumophila* growth (Amer, 2009). At physiological (low) levels of bacterial burden, caspase-1 activation is dependent on bacterial flagellin and on host Nlrc4. The activation of caspase-1 by *L. pneumophila* also requires a functional bacterial Dot/Icm type IV secretion system (Amer, 2009; Amer *et al.*, 2006). Similar to *Salmonella*, caspase-7 is activated down stream of caspase-1 during *L. pneumophila* infection (Lamkanfi *et al.*, 2008). However, Naip5 (Birc1e) is also essential for restriction of *L. pneumophila* (Amer, 2009). Naip5 is one of the NLRs containing 3 BIR motifs as signaling domain (Lamkanfi *et al.*, 2007a). A/J derived macrophages which express mutant Naip5 cannot restrict *L. pneumophila* infection, although they do activate caspase-1 in response to infection (Akhter *et al.*, 2009; Lamkanfi *et al.*, 2007b). However, the activation of caspase-7 in A/J derived macrophages (expressing mutant Naip5) is lacking, suggesting that Naip5 is involved in caspase-7 activation downstream of the Nlrc4 inflammasome (Akhter *et al.*, 2009; Amer, 2009). Therefore, inflammasome complexes may harbor more than one NLR once activated. On the other hand, at high bacterial burdens, *L. pneumophila* mutants lacking flagellin still activated caspase-1, but independently of Nlrc4 (Case *et al.*, 2009). Taken together, it seems that NLRC4 mediate caspase-1 activation during physiological levels of infection but when the eukaryotic cell encounters high bacterial burdens, Nlrc4 is dispensable for capsase-1 activation.

In primary human-derived macrophages, caspase-1 is not activated in response to *L. pneumophila*. Yet, the ablation of human Nlrc4 by gene silencing lead to enhanced growth of flagellated bacteria (Vinzing *et al.*, 2008). These data suggest that Nlrc4 restricts *L. pneumophila* infection in human-derived macrophages independently of caspase-1 (Akhter *et al.*, 2009) .

3.4 Nlrc4 and Pseudomonas aerugenosa

Pseudomonas aerugenosa (P. aeruginosa) is a pathogenic bacterium which causes marked induction of inflammatory cytokines such as IL-1β and IL-18 in host tissues. Alveolar macrophages infected with *P. aerugenosa* activate caspase-1 in an Nlrc4 dependent mechanism (Sutterwala *et al.*, 2007). Type III secretion system is required for virulence of *P. aeruginosa* and is critical for caspase-1 activation through Nlrc4 (Sutterwala *et al.*, 2007). None of the four known virulence factors (Exo Y, ExoS, Exo T, ExoU) secreted by this system is implicated in activation of caspase-1. However, during low bacterial burden, flagellin is involved in caspase-1 activation during *P. aeruginosa* infection. Similar to *L. pneumophila*, at high levels of bacterial infection, *P. aeruginosa* mutants lacking flagellin are still capable of activating caspase1. Yet, unlike *L. pneumophila* the activation of caspase-1 by *P. aeruginosa* flagellin mutants is still dependent on Nlrc4 (Sutterwala *et al.*, 2007). These data confirm the possibility that Nlrc4 can sense bacterial molecules other than flagellin.

4. Medical disorders

The dis-regulation of the innate immune system appears crucial for the pathogenesis of many autoimmune and inflammatory diseases (Lamkanfi *et al.*, 2007a). Variation in Nlrc4 plays a role in diseases such as Kawasaki disease and atopic dermatitis. Kawasaki disease is characterized by a marked activation of the immune system with elevations of serum proinflammatory cytokines and chemokines at acute phase (Ikeda *et al.*, 2009). The major complications of this disease are vasculitis, damage of the coronary artery, myocardial infarction, and sudden death (Ikeda *et al.*, 2009). The recent study by Ikeda *et al* showed that Nlrc4 is one of five genes up-regulated in acute phase of Kawasaki disease and that the innate immune response plays a major role in the pathogenesis and pathophysiology of Kawasaki disease. On the other hand, atopic dermatitis is a chronic skin disease characterized by excessive immune reactions to ubiquitous antigens (Macaluso *et al.*, 2007). Gene-gene interaction studies present evidence for an interaction between the promoter SNPs in the Nlrc4 and NALP1 genes (Macaluso *et al.*, 2007). Taken together, these findings reveal that Nlrc4 is not only involved in detecting flagellin but also in sensing danger signals and if dis-regulated, it will lead to diseases.

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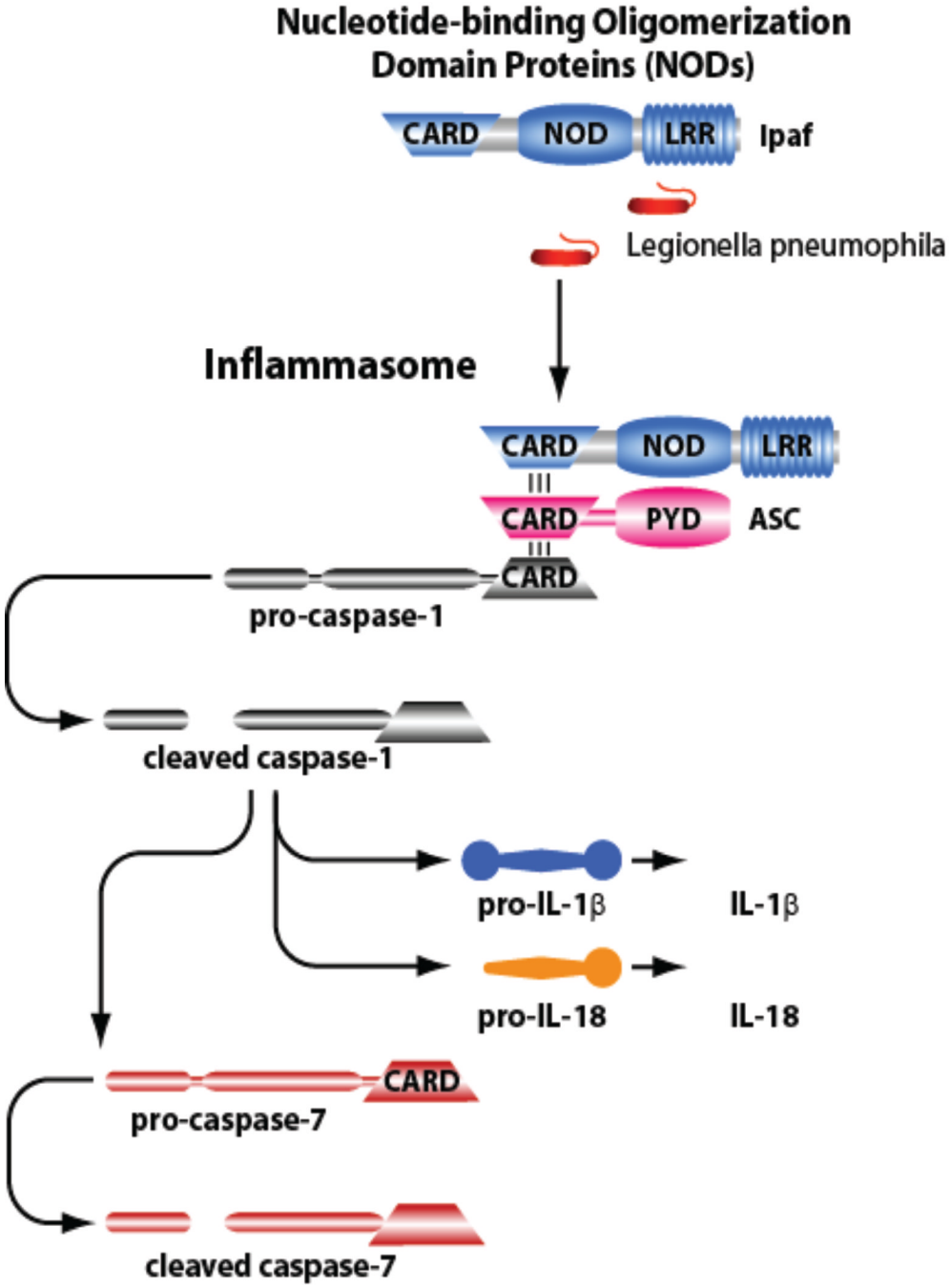

Figure 1.
Schematic representation of the structure of Nlrc4. The positions of the first and last residues for the different domains of Nlrc4 are indicated.

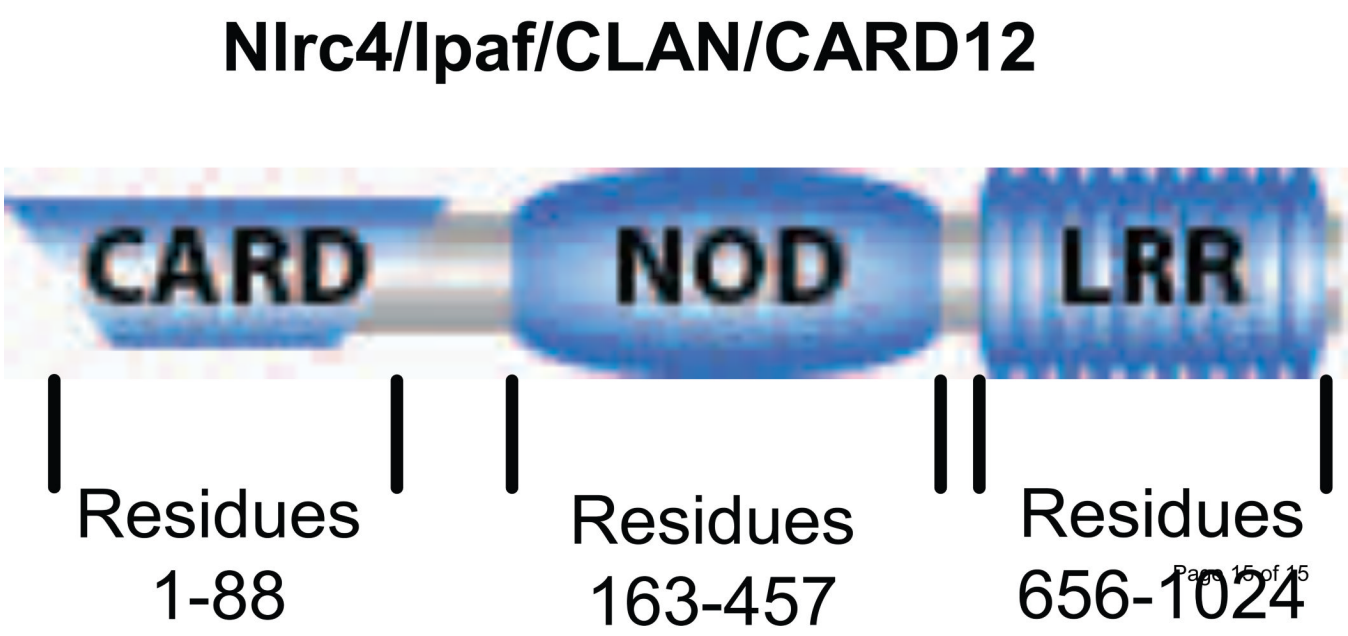

Figure 2.
The Nlrc4 inflammasome is composed of Ipaf, ASC, and caspase-1. Nlrc4 accepts ASC then caspase-1 via their CARD domains. Then, caspase-1 is activated and activates IL-1β, IL-18 and caspase-7.