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The NAIP/NLRC4 Inflammasomes

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Abstract

Inflammasomes comprise a family of cytosolic multi-protein complexes that sense infection, or other threats, and initiate inflammation via the recruitment and activation of the Caspase-1 protease. Although the precise molecular mechanism by which most inflammasomes are activated remains a subject of considerable debate, the NAIP/NLRC4 sub-family of inflammasomes is increasingly well understood. A crystal structure of NLRC4 was recently reported, and a domain in NAIPs that recognizes bacterial ligands was identified. In addition, gain-of-function mutations in NLRC4 have been shown to cause an auto-inflammatory syndrome in humans. Lastly, the NAIP/NLRC4 inflammasome has been shown to provide a novel form of cell intrinsic defense against *Salmonella* infection, involving expulsion of infected cells from the intestinal epithelium.

Introduction

Nucleotide binding domain and leucine rich repeat containing proteins (NLRs) are a functionally diverse protein family that includes approximately 20 members in both mouse and humans. The NLR family of apoptosis inhibitory proteins (NAIPs) are one particularly well-characterized NLR sub-family. In the mouse, NAIPs are encoded within a small cluster of genes that vary significantly in number and nucleotide sequence among different inbred strains. Classic genetic studies published by the Dietrich and Gros groups in 2003 were the first to demonstrate the critical role of NAIPs in host defense against bacterial infection [1,2]. The Dietrich and Gros groups showed that one particular NAIP protein, encoded by the *Naip5* gene of the C57BL/6 mouse strain, functions to severely restrict intracellular replication of *Legionella pneumophila*, a bacterial pathogen that causes a severe pneumonia called Legionnaires' Disease. At the time, the mechanism by which NAIP5 restricts *L. pneumophila* replication was entirely unclear, as was the relevance of NAIPs for defense against other bacterial pathogens or human innate immunity. In the ensuing decade, however, work from many labs has revealed the critical role of NAIPs in innate immunity in

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both mice and humans. There is now clear evidence that NAIPs function as cytosolic innate immune receptors for specific bacterial protein ligands. Upon detection of these ligands, NAIPs co-assemble with a downstream protein called NLRC4 to form a multiprotein complex called an inflammasome. Inflammasomes, in turn, function as platforms that activate inflammatory caspase proteases. In this review, I highlight several recent advances in our understanding of the NAIP/NLRC4 inflammasomes, with a particular emphasis on how NAIP/NLRC4 can provide beneficial functions in host defense, and conversely, how inappropriate NAIP/NLRC4 activation can lead to inflammatory disease.

NAIP and NLRC4 proteins

NAIP proteins function as specific cytosolic receptors for a variety of bacterial protein ligands. Mouse NAIP5 recognizes flagellin [3-7], the primary protein constituent of the bacterial flagellum. Mouse NAIP1 and NAIP2 detect needle and inner rod proteins, respectively, which are key structural components of a bacterial secretion system called the type III secretion system (T3SS) [3,7-10] (Figure 1). Importantly, the single human NAIP protein that has been characterized appears only to recognize the T3SS needle protein and does not appear to respond to flagellin [10]. T3SSs are evolutionarily and structurally related to flagella, and are used by many gram negative bacterial pathogens to inject ‘effector’ proteins into the host cell cytosol. T3SS effector proteins vary significantly among bacteria but are typically enzymes that manipulate host cell biology to benefit the pathogen. Given the variability of T3SS effectors, innate immune detection of the more highly conserved needle or inner rod components may represent a strategy by which the NAIPs can respond to a wide range of bacterial pathogens. However, since needle, rod and flagellin proteins comprise structures on the bacterial surface, they are not likely to be abundantly secreted. Thus, NAIPs must be highly sensitive to the presumably low levels of their ligands that are apparently inadvertently released into the host cell cytosol.

Upon detection of their cognate ligands, NAIPs co-oligomerize with a downstream adaptor protein called NLRC4. Confusingly, many papers continue to refer to NLRC4 as a “sensor”, despite a lack of evidence that NLRC4 binds or senses microbial ligands at all. Instead, NLRC4 is responsible for the recruitment and activation of the Caspase-1 (CASP1) protease, downstream of NAIP activation [11,12]. CASP1 orchestrates innate anti-bacterial responses by inducing a rapid lytic cell death, called pyroptosis, that is believed to expel bacteria from their protected intracellular niche [13,14]. In addition, CASP1 also mediates the processing and release of the pro-inflammatory interleukins-1β and -18. A major innate function of IL-1β is to stimulate the recruitment of neutrophils that engulf and kill extracellular bacteria. Thus, two major effector functions of CASP1 — pyroptosis and neutrophil recruitment — appear to function synergistically to eliminate bacteria [14].

NAIPs and NLRC4 are homologous to each other (~20% amino acid identity) and are members of the functionally diverse NLR superfamily, so-named because they contain a Nucleotide-binding domain (NBD) and a Leucine-rich Repeat (LRR) domain. (Despite continued confusion, NLR is not an abbreviation for Nod-like receptor [15]; indeed many NLRs are likely not receptors at all [16]). Many NLRs, including NAIPs, NLRC4, NLRP3 and NLRP1, assemble into multi-protein complexes called ‘inflammasomes’ that recruit and

activate CASP1 [16,17]. Importantly, however, not all NLRs form inflammasomes; thus, the possibility of caspase-independent functions for NAIP/NLRC4 should always be considered. In addition to the NBD and LRR domains, all NLRs contain three helical domains associated with the NBD: Helical Domain 1 (HD1), Winged Helix Domain (WHD), and Helical Domain 2 (HD2) (Figure 2). Together, the NBD-HD1-WHD-HD2 module is sometimes referred to as a NOD or NACHT domain. NAIPs are distinguished from other NLRs by the presence of three (misleadingly named) Baculovirus Inhibitor-of-apoptosis Repeats (BIRs) at the N-terminus of the protein. The function of the BIRs in NAIPs remains unknown, but they are not believed to play any role in inhibition of apoptosis [18]. NLRC4 lacks BIRs, but contains an N-terminal Caspase Activation and Recruitment Domain (CARD) that can directly recruit and activate CASP1, or can indirectly recruit CASP1 via a CARD-containing adaptor protein called ASC.

In addition to the presence of the BIR domains in NAIPs, the NAIP/NLRC4 inflammasomes exhibit several unique characteristics that distinguish them from other NLR inflammasomes. First, the NAIP/NLRC4 inflammasomes are the only NLR inflammasomes shown to bind and be activated by specific protein ligands. Thus NAIP/NLRC4 inflammasomes provide a unique experimental system for dissecting ligand-dependent NLR activation (see below). Second, NAIP/NLRC4 inflammasomes are unusual in involving the cooperative activity of two different NLRs: a NAIP protein that functions to sense ligands, and NLRC4 that recruits and activates CASP1. Most inflammasomes are believed to include only a single NLR, though other NLR-NLR interactions have been proposed [19]. Lastly, whereas most inflammasomes require an adaptor protein called ASC in order to recruit and activate CASP1, the presence of a CARD domain in NLRC4 allows NAIP/NLRC4 inflammasomes (and the NLRP1 inflammasome) to recruit and activate CASP1 directly, independent of ASC [20].

Unique roles of the NAIP/NLRC4 inflammasomes in epithelial and other cells

Most studies to date have focused on the function of NAIP/NLRC4 inflammasomes in hematopoietic cells, especially macrophages and dendritic cells. In these cells, as described above, inflammasomes are believed to initiate host defense primarily by triggering pyroptosis and inflammatory cytokine processing. However, a new study [21] has now provided convincing evidence for an epithelial-intrinsic function for the NAIP/NLRC4 inflammasomes. This study describes a novel mouse line in which the entire *Naip* locus is flanked by loxP sites and thus can be deleted either constitutively or conditionally in specific tissues (e.g., specifically in intestinal epithelial cells by use of a Villin-Cre transgene). The intestinal epithelial cells of the novel $Naip^{\Delta/\Delta}$ mice, as well as the previously described $Nlrc4^{-/-}$ mice, exhibit elevated *Salmonella* burdens at early stages of infection (i.e. 12-36h post infection). The mechanism of epithelial-intrinsic resistance does not appear to involve inflammasome-dependent cytokines, but instead appears to involve selective inflammasome-dependent expulsion of infected epithelial cells into the intestinal lumen. The authors were careful not to call this unusual process ‘pyroptosis’ as their analysis suggests that it occurs without the overt and rapid cellular lysis that is a defining characteristic of pyroptosis in

macrophages. A similar mechanism may explain the apparent non-hematopoietic function of NLRC4 in resistance to *Citrobacter rodentium* infection [22].

The idea that NAIP/NLRC4 inflammasomes may exhibit unique effector functions in distinct cell types is supported by additional recent data. One report demonstrated that resident peritoneal macrophages (but not bone marrow-derived macrophages) produce significant amounts of pro-inflammatory signaling lipids (an 'eicosanoid storm') upon NAIP/NLRC4 inflammasome activation [23]. Another very recent report found, surprisingly, that neutrophils do not undergo pyroptosis upon NAIP/NLRC4 activation [24]. An important conceptual advance emerging from these studies therefore appears to be that inflammasomes can be deployed for unique effector functions in distinct cell types. This insight may be important when considering the *in vivo* functions of other inflammasomes as well.

Human gain-of-function mutations in NLRC4

To date, our knowledge of the NAIP/NLRC4 inflammasome is based primarily on *in vitro* and *in vivo* studies in the mouse. These studies have provided an essential foundation for understanding human NAIP/NLRC4, but whether the NAIP/NLRC4 inflammasome is associated with human disease has been an open question. This has now changed with three recent reports [25-27] that characterize a newly described auto-inflammatory disease in several human patients harboring heterozygous gain-of-function mutations in NLRC4. Three distinct disease-associated mutations were described, each resulting in single amino acid substitutions (V341A, T337S, and H443P) in the NLRC4 NBD-associated HD1 or WHD domains. The mutations appear to disrupt the ability of NLRC4 to maintain itself in an auto-inhibited state, resulting in an NLRC4 protein with an increased propensity for spontaneous activation of CASP1. The patients harboring these mutations presented with a novel auto-inflammatory syndrome characterized by fever, diarrhea, splenomegaly, duodenal inflammation, anemia, hypertriglyceridemia, and elevated levels of inflammatory markers such as ferritin, C-reactive protein, and interleukin-18. The syndrome resulted in the death of a 23 day-old infant, but not his 43 year-old father, indicating that these heterozygous gain-of-function mutations in NLRC4 can produce variable clinical outcomes that in some cases can be tolerated into adulthood. The syndrome was termed SCAN4 (syndrome of enterocolitis and auto-inflammation associated with mutation in NLRC4) [27]) or NLRC4-MAS (NLRC4 macrophage activation syndrome) [25].

The presence of gastrointestinal symptoms is the most notable feature of the newly described syndrome that distinguishes it from the previously characterized auto-inflammatory periodic fever syndromes associated with mutations in the related inflammasome component NLRP3 [28]. However, due to the limited experimental manipulations that are possible on rare human patients, it remains unclear precisely how spontaneous NLRC4 activation leads to these specific gastrointestinal symptoms or disease in general. In particular, although spontaneous NLRC4 activation was observed in macrophages derived from the patients, it is unclear whether NLRC4 activation in macrophages, or perhaps other cell types *in vivo*, is the primary driver of disease symptoms. In light of the reports discussed above that indicate that the NAIP/NLRC4 inflammasome is

highly expressed and functional in non-hematopoietic intestinal epithelial cells [21,22,26], it is tempting to speculate that some of the disease symptoms in the human patients are due to NLRC4 activation in these cells. It is also unclear whether disease in SCAN4/NLRC4-MAS is due primarily to the spontaneous production of inflammasome-dependent cytokines, such as IL-1β or IL-18, or whether pyroptotic cell death may also drive inflammation in these patients. One patient was treated with IL-1 receptor antagonist (anakinra) and this was found to relieve many clinical symptoms, implying that IL-1 is an important mediator of disease.

Specificity and assembly of the NAIP/NLRC4 inflammasomes

The serious auto-inflammatory phenotype that results from spontaneous NLRC4 activation implies that the NAIP/NLRC4 inflammasomes must normally be appropriately and strictly regulated. Thus, an important area of investigation is to understand how NLRs are maintained in an auto-inhibited state and are selectively activated only in response to appropriate cognate stimuli. Unfortunately, there are few biochemical studies of inflammasome activation. NLR proteins are relatively large (>100kDa) multi-domain proteins that tend to aggregate and have been difficult to purify. Thus, the recently reported 3.2Å crystal structure of NLRC4 in the closed (inactive and auto-inhibited) state represents an impressive technical achievement [29] (Figure 2). Removal of the CARD domain proved critical to permit crystallization, but nevertheless, the NLRC4 structure provides valuable insight into the mechanisms of NLR auto-inhibition. In general, the auto-inhibited structure of NLRC4 resembles that of the previously determined auto-inhibited structures of Apaf-1 [30,31], an NBD-containing protein that regulates apoptosis. Auto-inhibition of NLRC4 appears to involve inter-domain contacts that stabilize a compact tertiary fold. For example, the NBD of the auto-inhibited NLRC4 was found to be bound to ADP, and contacts between ADP and the WHD appear to help maintain NLRC4 in a closed compact state. Further inter-domain contacts between the NBD and the HD2 and LRR domains appear to further stabilize a closed and inactive conformation. What remains unclear from the NLRC4 structure is how these auto-inhibitory inter-domain interactions are ultimately relieved to allow for NLRC4 activation. Although the authors speculate that ligand binding may disrupt the inter-domain contacts, as discussed above, there is as yet no evidence that NLRC4 binds a ligand. Instead ligand detection and binding appears to be a function encoded by NAIPs. How active NAIPs then bind and induce NLRC4 oligomerization remains obscure.

An unexpected feature of the NLRC4 structure is that the auto-inhibited form of the protein that was crystallized was found to contain serine 533 in the phosphorylated state [29]. This is interesting because it appears at odds with a previous proposal that phosphorylation on Serine 533 is required for *activation* of NLRC4 [32]. One resolution to this conundrum is to suggest that the form of NLRC4 that was crystallized is actually partially transitioning to an activated state [33]. Another resolution would be to suggest that perhaps phosphorylation of NLRC4 is not critical for its activation. Indeed a recent report could find no role for a proposed NLRC4 kinase, PKCδ, in NLRC4 activation [34]. Shao and colleagues similarly found no role for serine 533 phosphoylation in NAIP/NLRC4 activation in a reconstituted assay system [10].

In contrast to NLRC4, no crystal structure has been reported for any of the NAIP proteins. Thus the mechanism by which different NAIP proteins are able to detect specific bacterial ligands remains poorly understood. Based on analogy to the ligand-binding function of the LRRs in Toll-like receptors, it has been widely presumed that the LRR domains of NLRs would be responsible for ligand recognition. Therefore it was unexpected when an analysis of the specificity of chimeric NAIP proteins mapped ligand recognition to the NAIP helical domains and an unannotated region between the helical domains and the LRRs [35]. Interestingly, this region of the NAIPs also appears to be under positive (diversifying) selection, commensurate with its role in recognition of rapidly evolving microbial ligands [35]. Whether other NLRs use the helical domains for ligand recognition remains to be ascertained.

Conclusion

The recent work discussed above highlights the precarious function of the NAIP/NLRC4 inflammasome in the innate immune system. On one hand, NAIP/NLRC4 activation provides a robust early detection system that initiates rapid responses to eliminate bacterial intruders. On the other hand, the rare human patients harboring gain-of-function NLRC4 mutations illustrate the potentially severe auto-inflammatory disease that can result from inappropriate NLRC4 activation. The NAIP/NLRC4 inflammasomes therefore play a high-stakes game in which inappropriate activation can produce autoimmune disease, but conversely, a lack of activation can increase susceptibility to infection. A future challenge is to understand the regulatory mechanisms that allow NAIP/NLRC4 to ‘win’ its high-stakes game and serve reliably in host defense.

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Highlights

- The NAIP/NLRC4 inflammasomes initiate inflammation in response to bacterial ligands
- A crystal structure of NLRC4 provides structural insights into its regulation
- NAIPs use their alpha helical domains to detect specific bacterial ligands
- Gain-of-function mutations in NLRC4 cause a rare but serious auto-inflammatory disease
- NAIP/NLRC4 functions in epithelial cells to provide immune defense against *Salmonella*

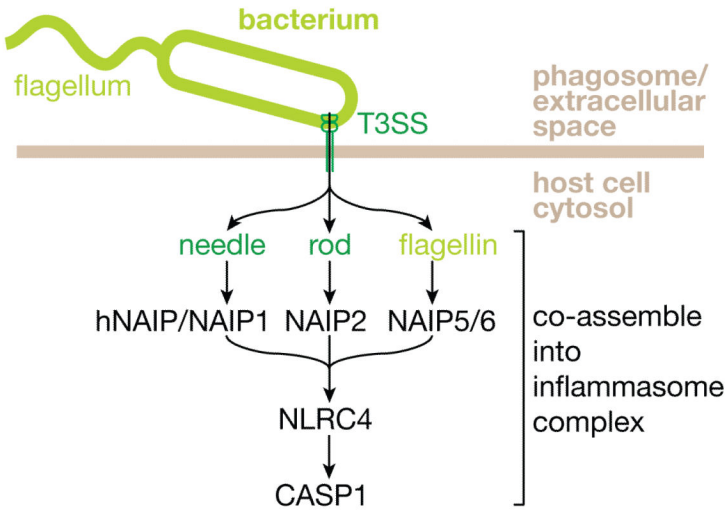

Figure 1. Specific detection of bacterial ligands by NAIPs

The needle or rod components of bacterial type III secretion systems (T3SS) are detected in the host cell cytosol by mouse NAIP1 or mouse NAIP2, respectively. Flagellin, the main structural protein comprising the flagellum, is detected by mouse NAIP5 or NAIP6. Human NAIP (hNAIP) detects the T3SS needle only and does not detect flagellin. Once NAIPs are activated in the presence of their cognate ligands, they co-assemble with NLRC4 to form an inflammasome that recruits and activates Caspase-1 (CASP1). The precise mechanism by which the needle and rod proteins access the host cell cytosol remains to be determined and may not involve direct secretion via the T3SS as shown.

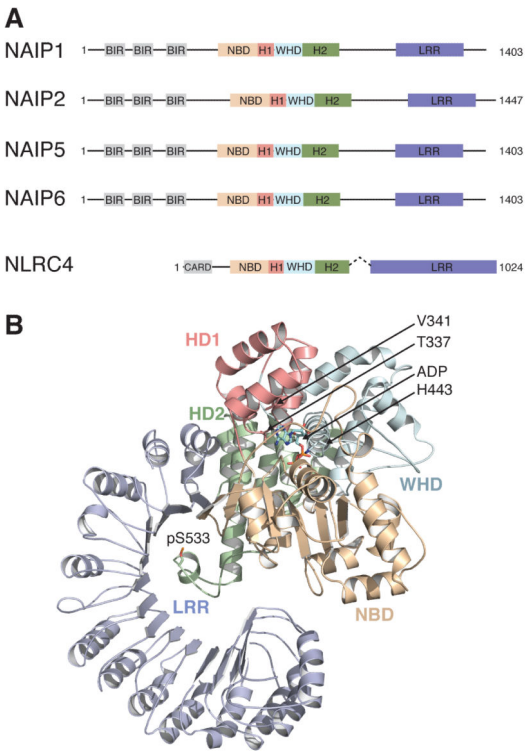

Figure 2. The NAIP and NLRC4 Proteins

A. Domain structures of mouse NAIPs and NLRC4. BIR, Baculovirus inhibitor-of-apoptosis repeat; NBD, nucleotide binding domain; H1, helical domain 1; WHD, winged helix domain; H2, helical domain 2; LRR, leucine rich repeat domain; CARD, caspase activation and recruitment domain. Numbers indicate the number of amino acids in the full length proteins.

B. Crystal structure of mouse NLRC4 in the auto-inhibited conformation. Structural coordinates are from the protein data bank (PDB accession number 4KXF) as reported by [29]. The NLRC4 CARD domain was truncated prior to crystallization and is therefore not shown. The positions of amino acids (Valine 341, Threonine 337, and Histidine 443) mutated in the recently reported human auto-inflammatory disease [25-27] are indicated, as is the phosphorylated serine 533.