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Orchestrating inflammasomes

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Recognition of microbes by the host is a critical step in initiating immune responses that culminate in the elimination or containment of invading microorganisms. The initial sensing of microbes is largely mediated by host pattern-recognition receptors that detect the presence of conserved microbial components or endogenous molecules generated in the setting of cellular injury (1). A major signaling pathway activated in response to microbial infection is the inflammasome, a multi-protein complex that activates the protease caspase-1. Once activated, caspase-1 proteolytically cleaves pro-IL1 β and pro-IL-18 into their biologically active forms. Upon release from phagocytes, IL-1 β and IL-18 act on neighboring cells to orchestrate a variety of immune responses that are important for pathogen clearance (2). To date, several inflammasomes have been identified including those triggered by the activation of NLRC4, NLRP1, and NLRP3, three members of the Nod-like receptor family (NLRs) of intracellular receptors. Of these inflammasomes, NLRC4 is activated upon infection by several Gram-negative bacteria, including *Legionella pneumophila*, *Salmonella enterica* serovar Typhimurium (*Salmonella*) and *Shigella flexneri* (2). The activation of the NLRC4 inflammasome requires the presence of an intact type III or IV secretion system, a molecular syringe-like apparatus that mediates the translocation of bacterial virulence factors as well as small amounts of flagellin or PrgJ-like rod proteins into the host cytosol which induce NLRC4 activation (3–5). Although the presence of flagellin or rod proteins is sufficient to activate the NLRC4 inflammasome, the precise molecular mechanism that triggers NLRC4 activation remains poorly understood. In a recent issue of the journal *Nature*, Qu *et al.* identify the phosphorylation of NLRC4 as a critical step in the activation of the inflammasome (6).

A key question in the field of NLRs is the molecular mechanism that triggers their assembly and activation. To identify critical steps in NLRC4 activation, Qu *et al.* first generated knockin mice that express endogenous NLRC4 fused with a C-terminal Flag peptide tag. Taking advantage of this mouse system, Qu *et al.* characterized NLRC4 peptides by mass spectrometry analysis which revealed that *Salmonella* infection induces NLRC4 phosphorylation at Ser533. Using an antibody that specifically detects phosphorylated Ser533, Qu *et al.* showed that NLRC4 was phosphorylated in response to stimuli that trigger activation of NLRC4, but not of the NLRP3 or AIM2 inflammasome. In agreement with the

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role of flagellin and PrgJ in the activation of NLRC4, *Salmonella* mutants deficient in flagellin or PrgJ were impaired in the induction of Ser533 phosphorylation. Importantly, prevention of caspase-1 activation by specific caspase-1 inhibitors did not affect NLRC4 phosphorylation indicating that Ser533 phosphorylation is an early event upstream of caspase-1. Reconstitution of NLRC4-deficient macrophages with the NLRC4 mutant S533A abrogated activation of the inflammasome in response to *Salmonella* infection, which provides strong evidence that phosphorylation of S533 step is critical for NLRC4 activation. Using biochemical approaches, Qu *et al.* further identified PKC δ and PAK2 as the kinases capable of phosphorylating NLRC4 at Ser533. Further genetic studies suggested that PKC δ is the major NLRC4 kinase responsible for inflammasome assembly and activation. Collectively, the work by Qu *et al.* has moved the inflammasome field forward by providing strong evidence that PKC δ -mediated NLRC4 phosphorylation is a critical step in the activation of the NLRC4 inflammasome (Fig. 1)

As often happens in science, a discovery raises new questions. For example, how is PKC δ activated in response to bacterial infection? Previous work identified Naip2 and Naip5, two related members of the NLR family, as critical factors in the activation and assembly of the NLRC4 inflammasome through their direct interaction with flagellin and rod proteins (7, 8). The latter raises additional questions about how Naips are linked to PKC δ activation or whether NLRC4 phosphorylation plays a role in the recruitment of Naips to the NLRC4 inflammasome. In addition, the involvement of PKC δ opens the possibility that pathogens may target this kinase to inhibit the NLRC4 inflammasome. Qu *et al.* showed that the NLRC4 S533D mutation that mimics phosphorylated Ser 533 increases the ability of NLRC4 to induce macrophage cell death, suggesting that NLRC4 phosphorylation is sufficient to induce the activation of NLRC4. However, the molecular mechanism by which Ser533 phosphorylation promotes NLRC4 activation remains unclear. The Ser533 residue is evolutionarily conserved and located between the centrally located nucleotide-binding oligomerization domain and the C-terminal leucine-rich repeats (LRRs) of NLRC4. In the absence of any stimulation, NLR proteins are thought to be in an auto-inhibited state conferred by the N-terminal end of the LRR domain and relieved upon microbial stimulation (9, 10). Future studies will determine whether phosphorylation of Ser533 provides a positive activating signal or acts by relieving the self-inhibited state of NLRC4.

The study by Qu *et al.* highlights the importance of posttranslational modification as a mechanism for triggering the activation of intracellular NLRs in response to bacterial infection. Such an indirect mechanism for linking microbial molecules to receptor activation is not new as it has been previously described to be important for the activation of plant disease resistance nucleotide-binding-leucine rich repeat (NB-LRR) proteins that are structurally homologous to NLRs (11, 12). Like NLRC4, plant NB-LRRs mediate recognition of pathogen-derived molecules and subsequently activate host defense in plants (13). Many plant NBD-LRR proteins do not directly detect pathogen effectors, but monitor their presence via modification of accessory host proteins induced by pathogen effectors (11, 12). Future studies will determine whether modification of NLR proteins or the direct interaction between NLRs and their respective ligands is responsible for the activation of inflammasomes other than NLRC4.

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Fig. 1. Model for the activation of the NLRC4 inflammasome

Infection of macrophages with several Gram-negative bacteria including *Salmonella*, *Legionella*, *Pseudomonas* and *Shigella*, activates caspase-1 through NLRC4. A critical step is the cytosolic delivery of flagellin or PrgJ-like rod proteins via bacterial type III and IV secretion systems. Flagellin is recognized by Naip5 whereas PrgJ-like proteins are recognized by Naip2. *Shigella* activates the NLRC4 inflammasome independently of flagellin through an unknown microbial product. In addition, infection activates the kinase $\text{PKC}\delta$ and possibly other kinases like PAK2 that phosphorylate NLRC4 at Ser533, a step that is critical for inflammasome assembly and activation. Activation of caspase-1 via NLRC4 leads to processing and release of $\text{IL-1}\beta$ and IL-18 , the induction of pyroptosis, and processing and activation of caspase-7.