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The Inflammasome: First Line of the Immune Response to Cell Stress

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The NALP3-inflammasome is a protein complex that stimulates caspase-1 activation to promote the processing and secretion of proinflammatory cytokines. Recent work indicates that the NALP3-inflammasome can be activated by endogenous "danger signals" as well as compounds associated with pathogens (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006; Sutterwala et al., 2006). Here, we discuss new insights into the regulation of caspase-1 activity in the inflammatory response.

Caspase-1 regulates the processing and secretion of three interleukin (IL)-1 family members: IL-1β, IL-18, and IL-33. Both IL-1β and IL-18 are highly potent proinflammatory cytokines (Dinarello, 2002). IL-18 induces interferon γ expression and secretion from IL-12-primed naïve T cells to promote the differentiation of type 1 helper T cells. IL-33 has recently been identified as the ligand of the IL-1 receptor family protein ST2 and promotes responses mediated by type 2 helper T cells (Schmitz et al., 2005). Upon cleavage of their proforms by caspase-1, these cytokines become active and are secreted. Thus, caspase-1 activity is critical for the inflammatory response.

NALP3, also known as *cryopyrin* or *CIAS*, encodes a protein that regulates the activity of caspase-1 (Figure 1). The importance of caspase-1 in inflammation is demonstrated by the fact that gain-of-function mutations in the *NALP3* gene cause three autoinflammatory diseases: Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and neonatal-onset multisystem inflammatory disease. Collectively, these diseases are called cryopyrin-associated periodic syndromes (CAPS). Both CAPS and rheumatoid arthritis have been effectively treated by the administration of an IL-1 receptor antagonist, supporting the central role of IL-1 in the pathogenesis of these diseases. Thus, learning how caspase-1 activation is regulated is crucial for understanding the pathogenesis of these autoinflammatory disorders.

Similar to the activation of caspase-8 or -9 by the Apaf-1-apoptosome or Fas/CD95-DISC, respectively, it has been speculated that caspase-1 may be activated by a molecular platform termed an "inflammasome." Tschopp and colleagues have proposed three types of inflammasome based on biochemical analysis of three Apaf-1-like proteins, NALP1, NALP2, and NALP3. In the case of the NALP3-inflammasome, NALP3 binds to two adaptor proteins, ASC and CARDINAL, both of which in turn bind to caspase-1, so that the complex can attract two caspase-1 molecules (Agostini et al., 2004). It is assumed that a specific ligand induces assembly and activation of the NALP3-inflammasome, but its identity is unknown. Because other Apaf-1-like proteins such as Ipaf, NALP6, NALP10, and NALP12 modulate caspase-1 activity, additional types of caspase-1-activating platforms are likely to exist, each being activated by its own specific ligand.

Activation of Caspase-1 via ASC and Ipaf

Macrophages are known to secrete IL-1β in response to serial stimulation with lipopolysaccharide (LPS) and subsequent treatment with a high concentration of ATP (Ferrari et al., 2006). LPS is required both to induce expression of pro-IL-1β and to prime cells for caspase-1 activation in response to ATP. The effect of ATP is mediated by an ionotropic ATP receptor, $P2X_7$, which upon activation causes a rapid K^+ efflux from the cytosol. Because release of IL-1β is also triggered by potassium ionophores, membrane-permeabilizing agents, pore-forming agents, and depletion of K^+ from cell culture media, the efflux of cytosolic K^+ is thought to be a trigger of caspase-1 activation.

The bacterium *Salmonella typhimurium* is also known to activate caspase-1 in macrophages. *S. typhimurium* possesses machinery to hijack mammalian epithelial cells, called the type-III secretion system (TTSS). The TTSS injects a series of bacterial products into mammalian cells creating a hospitable environment for bacterial entry and growth. When *S. typhimurium* is phagocytosed by macrophages, the TTSS induces rapid caspase-1-dependent cell death, which is thought to be a strategy for *Salmonella* to escape the host phagocytes. Although the molecular mechanism by which the TTSS activates caspase-1 is not fully understood, bacterial compounds that are injected into the cytosol of macrophages are speculated to play a role.

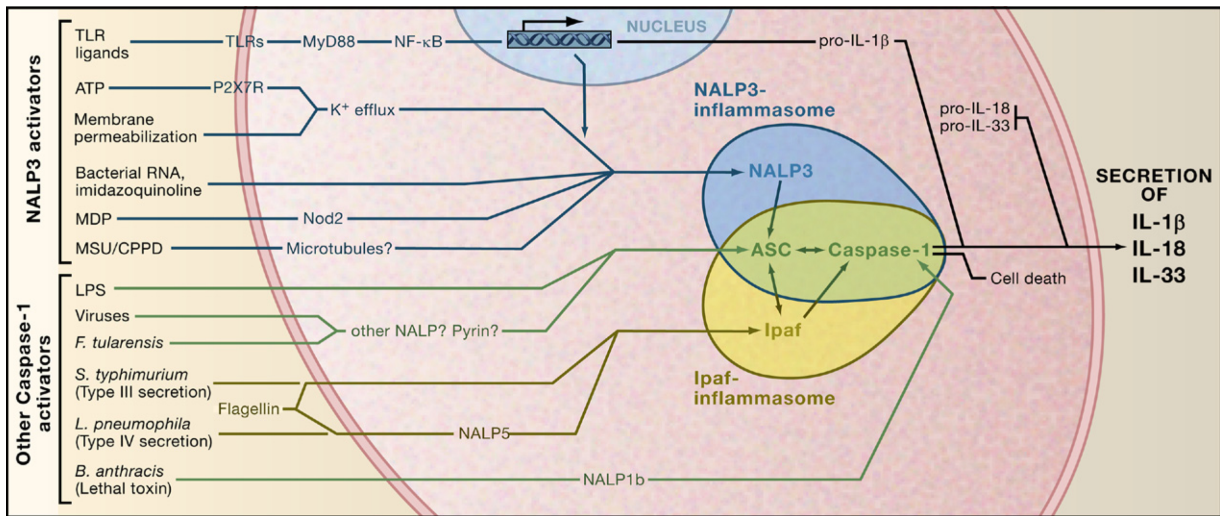

Figure 1. Multiple Pathways Activate Caspase-1

Various bacterial compounds and endogenous molecules that are released from injured cells activate caspase-1 through the NALP3-inflammasome. Serial stimulation with TLR ligands plus ATP or pore-forming agents activate the NALP3-inflammasome (Mariathasan et al., 2006; Martinon et al., 2006; Sutterwala et al., 2006). TLR ligands prime cells to respond to ATP or pore-forming agents possibly by inducing expression of an unidentified gene downstream of NF-κB activation. ATP or pore-forming agents induce a rapid K^+ efflux from cells, which triggers the activation of the NALP3-inflammasome. Previous pharmacological studies suggest that this pathway is mediated by Ca^{2+} release from intracellular stores, calcium-independent phospholipase A2, and AG126-sensitive protein tyrosine kinases. Bacterial RNA and imidazoquinoline compounds activate caspase-1 in a manner dependent on NALP3, but this activation is independent of TLRs (Kanneganti et al., 2006). LPS has been shown to activate caspase-1 in an ASC-dependent manner (Yamamoto et al., 2004), but it is not dependent on NALP3. A moiety of bacterial peptidoglycan, MDP, was shown to activate caspase-1 in human monocytic cells (Martinon et al., 2004) perhaps through Nod2. Monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD), associated with gout and pseudogout, respectively, activate the NALP3-inflammasome in a manner dependent on a colchicines-sensitive process (Martinon et al., 2006). Overlap between the MSU/CPPD pathway and the pathway for K^+ efflux is still unclear. In addition to the NALP3-inflammasome other NLR/CATERPILLER proteins appear to activate caspase-1 in response to various pathogens. Viral infection may activate ASC through an unknown pathway (Johnston et al., 2005). The bacterium *Francisella tularensis* activates caspase-1 through ASC but not through NALP3 or Ipaf. Instead this may involve another NLR/CATERPILLER protein (Mariathasan et al., 2006). The *Salmonella typhimurium* type-III secretion system activates caspase-1 through Ipaf (Mariathasan et al., 2004), whereas the *Legionella pneumophila* type-IV secretion system activates caspase-1 through Birc1e/NAIP5$^{C57BL/6}$ and Ipaf (Zamboni et al., 2006). Recently, intracellular flagellin was shown to play a role in the Ipaf and/or NAIP5-mediated pathways (Franchi et al., 2006; Miao et al., 2006; Molofsky et al., 2006; Ren et al., 2006). *Bacillus anthracis* lethal toxin can activate caspase-1 via NALP1b^{129S1} (Boyden and Dietrich, 2006).

Using mice lacking either ASC or Ipaf, Dixit and colleagues have demonstrated that the induction of caspase-1 activation by LPS plus ATP or by *S. typhimurium* are mediated by two distinct signaling pathways (Mariathasan et al., 2004). They showed that macrophages lacking ASC are unable to activate caspase-1 in response to both LPS plus ATP and *S. typhimurium*, whereas Ipaf-deficient macrophages are impaired only in the caspase-1 activation induced by *S. typhimurium*. This finding implies that ASC shares a role in both pathways of caspase-1 activation, and that Ipaf mediates only the signal from *Salmonella* as a factor upstream of ASC.

Activators of the NALP3-Inflammasome

ATP and Bacterial Pore-Forming Toxins

NALP3 is required by murine macrophages for caspase-1 activation in response to LPS plus ATP, but not in response to infection by *S. typhimurium* (Mariathasan et al., 2006; Martinon et al., 2006; Sutterwala et al., 2006). Moreover, other Toll-like receptor (TLR) ligands (lipid-A, lipoteichoic acid, lipoprotein, CpG oligodeoxyribonucleotides, and imidazoquinoline compounds) also prime the cells to activate caspase-1 in response to ATP; other inducers of K^+ efflux, such as nigericin and maitotoxin, also promote caspase-1 activation in a NALP3-dependent manner. Thus, the NALP3-inflammasome seems to be activated by serial stimulation by TLR ligands followed by K^+ efflux. Infection by *Listeria monocytogenes* and *Staphylococcus aureus* also induces caspase-1 activation in LPS-primed macrophages in a manner dependent on NALP3 and ASC (Mariathasan et al., 2006). Because these bacteria possess toxins that cause pore formation in the cytoplasmic membrane of host cells, the effect of these bacteria on the NALP3-inflammasome may be explained by K^+ efflux caused by these toxins. Indeed, Dixit's group has shown that *Listeria* lacking the pore-forming toxin listeriolysin O are incapable of activating caspase-1.

It is unclear which ligand directly activates the NALP3-inflammasome. However, it is likely that TLR stimulation leads to the induction of genes, such as NALP3 and caspase-11, that are required for caspase-1 activation, and that K^+ efflux is an upstream trigger of NALP3 activation (Ferrari et al., 2006; Mariathasan et al., 2006; Sutterwala et al., 2006). Pharmacological studies have revealed that IL-1β secretion induced by ATP is mediated by K^+

efflux, release of intracellular Ca^{2+} stores, and the activities of protein tyrosine kinases and calcium-independent phospholipase A2. The relationship between these events and the processing and release of IL-1β are still not clear, although NALP3 may recognize an endogenous molecule generated by these processes.

Uric Acid and Calcium Pyrophosphate Dihydrate

In addition to ATP, Tschopp and colleagues found that crystals of monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD), the causative agents of gout and pseudogout, respectively, induce caspase-1 activity in a manner dependent on the NALP3-inflammasome (Martinon et al., 2006). MSU was identified as an active component of the adjuvant activity of UV-injured mammalian cells and has been demonstrated to activate dendritic cells in vitro (Shi et al., 2003). Thus, MSU might be a "danger signal" released from damaged cells that results in the activation of the immune system (Matzinger, 2002).

Because caspase-1 activation by MSU and CPPD crystals does not require TLR signaling and was not blocked by antagonists of the $P2X_7$ receptor, the MSU/CPPD pathway for activating the NALP3-inflammasome may act in parallel with the ATP pathway. Interestingly, caspase-1 activation by MSU and CPPD, but not by ATP, was blocked by colchicine, an inhibitor of tubulin polymerization that is used for the treatment of gout and pseudogout. These findings suggest that the MSU/CPPD pathway for caspase-1 activation may be mediated by microtubule formation, and that blocking this pathway may be important for the therapeutic effect of colchicine. Where the ATP and the MSU/CPPD pathways merge is an important question that awaits resolution.

Pathogens

Compounds derived from pathogens can also activate NALP3. Tschopp and colleagues previously reported that the minimal biologically active moiety of bacterial peptidoglycan, muramyl dipeptide (MDP), can activate caspase-1 in a NALP3-dependent manner in a human monocytic cell line (Martinon et al., 2004). However, recent studies using macrophages from mice lacking NALP3 have not been able to confirm these findings (Kanneganti et al., 2006; Mariathasan et al., 2006; Sutterwala et al., 2006). Unlike human monocytes, murine macrophages did not secrete IL-1β in response to MDP alone, and MDP is dispensable for the activation of the NALP3-inflammasome in murine macrophages.

Núñez and colleagues have reported that NALP3 and ASC are essential for caspase-1 activation by bacterial RNA and the imidazoquinoline compounds, R837 and R848, but not by LPS, lipoteichoic acid, or bacterial lipoprotein, suggesting an important role for the NALP3-inflammasome in recognition of non-host RNA molecules (Kanneganti et al., 2006). However, it should be noted that their findings are in marked contrast to those of other studies (Mariathasan et al., 2006; Martinon et al., 2006; Sutterwala et al., 2006). Firstly, Núñez's group reported that TLR ligands alone (i.e., LPS, lipoteichoic acid, bacterial lipoprotein, or imidazoquinoline compounds) could activate caspase-1 in wild-type murine macrophages without the addition of a second stimulus such as ATP, whereas the other groups did not observe significant activation of caspase-1 by TLR ligands alone. Secondly, in Núñez's study, wild-type macrophages only minimally increased IL-1β secretion in response to ATP following stimulation with TLR ligands. These differences may be due to subtle differences in the protocol used for preparing or stimulating the macrophages. Because bacterial RNA, R837, and R848 seem to activate caspase-1 in a manner that is distinct from that of ATP and MSU/CPPD, an additional pathway leading to activation of the NALP3-inflammasome may exist in macrophages under certain conditions.

Does the NALP3-Inflammasome Sense Danger Signals?

ATP, MSU, and CPPD may be endogenous signals derived from damaged or dead cells that are sensed by the NALP3-inflammasome. The danger signal theory proposes that in addition to foreign objects, the immune system senses and responds to damaged cells or tissues (Matzinger, 2002). Assuming that the NALP3-inflammasome is a sensor of danger signals, two recent findings on the function of IL-1β can be explained in a unified way.

We reported that the NALP3- inflammasome is essential for the establishment of contact hypersensitivity to the hapten trinitrophenylchloride (Sutterwala et al., 2006). In these experiments NALP3 and ASC play a role primarily at the sensitization phase, although the exact mechanism and cell type involved remain unknown. This result is consistent with previous reports demonstrating a critical role for IL-1β in the migration of Langerhans' cells to draining lymph nodes in response to the hapten fluorescinisothiocyanate. This in turn initiates the adaptive immune response (Shornick et al., 2001). It has been suggested that chemical damage to cells or tissues by the hapten is required for the establishment of allergic contact dermatitis in addition to the antigenicity of the hapten (Smith et al., 2002). This suggests that the NALP3-inflammasome and IL-1β may play a role in establishing contact hypersensitivity through their ability to recognize and respond to a danger signal caused by inflammatory haptens.

Mice lacking MyD88, an adaptor protein recruited to the cytoplasmic domain of TLRs and the IL-1 receptor, are susceptible to cutaneous infection by *S. aureus.* Miller et al. reported that this susceptibility is more dependent on the loss of signaling by the IL-1 receptor than by the loss of signaling by TLR2. Moreover, recruitment of neutrophils to the lesion, which is caused by IL-1-receptor signaling in the skin, plays a critical role for the clearance of *S. aureus* (Miller et al., 2006). Although the source and mechanism of IL-1β secretion in these studies was not determined, IL-1β secretion by macrophages in response to *S. aureus* was shown to be NALP3 dependent by Mariathasan et al. (2006). By combining these findings, one can speculate that the cytotoxic activity of *S. aureus* rec-

ognized by the NALP3-inflammasome may potentially be a more important determinant for the host immune system to recruit neutrophils to the infected lesion than is the recognition of bacteria by TLRs.

Although caspase-1 clearly plays a role in the pathogenesis of autoinflammatory disorders such as rheumatoid arthritis and CAPS, its role in host defense against pathogens is not fully understood. The hypothesis that IL-1β and caspase-1 are mediators of danger signals may provide new insights into understanding the function of these factors in innate immune responses.

Multiple Pathways Activate Caspase-1

Recent studies have provided clues as to the mechanism of caspase-1 activation by *S. typhimurium*, *Legionella pneumophila*, *Francisella tularensis*, and *Bacillus anthracis* (Boyden and Dietrich, 2006; Franchi et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). Importantly, many of these involve NLR/CATERPILLER family proteins, which include NALP3 and Ipaf (for a complete review on NLR/CATERPILLER proteins see Ting et al., 2006). In addition to bacterial pathogens, viral infection also induces caspase-1 through an unknown mechanism. Recently, Johnston et al. found that Myxoma virus, a poxvirus, carries a protein that inhibits ASC/caspase-1 activation and subsequent cell death after virus infection. Given that poxviruses have evolved specific machinery to evade this pathway, ASC may also be important for viral elimination (Johnston et al., 2005). Thus, multiple types of signals appear to converge at caspase-1 through multiple "inflammasomes" (Figure 1).

It remains unclear as to how mature IL-1β, IL-18, and IL-33 are released from the cytosol to the extracellular space. Two models have been proposed for their secretion: through secretory lysosomes or microvesicle shedding, although no single theory has emerged to unify these two models. Because the release of mature IL-1β is linked to caspase-1 activation, the inflammasome may function as a part of the secretion machinery. The role of the inflammasome in cytokine release is an important issue that remains to be addressed. The exact role of caspase-1 in programmed cell death is also elusive. Depending on the stimulus, the activity of caspase-1 sometimes causes cytokine secretion and other times cell death, suggesting that there is a cellular machinery that regulates the events downstream of caspase-1. The specificity of the effects of caspase-1 may be explained by the involvement of different types of NLR/CATERPILLER proteins in the formation of the activation platform. Elucidating the composition and subcellular localization of the inflammasomes may provide a clearer insight into the mechanisms leading to cytokine secretion and cell death caused by caspase-1.

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