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Abbreviations: 7BIO: 7-bromoindirubin-3′-oxime · **DAMP**: danger-associated molecular pattern · **NLR**: nucleotide-binding domain leucine-rich repeat containing receptor

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Inflammasomes as microbial sensors

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Members of the Nod-like receptor family and the adaptor ASC assemble into multiprotein platforms, termed inflammasomes, to mediate the activation of caspase-1 and subsequent secretion of IL-1β and IL-18. Recent studies have identified microbial and endogenous molecules as well as possible mechanisms involved in inflammasome activation.

Eukaryotic hosts deploy an arsenal of defense mechanisms to counter invading microbes. Upon microbial invasion, sensing of pathogenic organisms and rapid induction of anti-microbial defenses are mediated by several classes of germline-encoded PRR. These include membrane-bound TLR and C-type lectin receptors as well as cytosolic Nod-like receptors (NLR) and RIG-like helicases [1]. Because PRR recognize pathogen-associated molecular patterns shared by large classes of microbes, the encounter with individual pathogens triggers the activation of multiple PRR and host defense signaling pathways [1]. The latter include the activation of NF-κB and MAPK which results in transcriptional induction of a large number of anti-microbial and proinflammatory molecules including TNF-α and IL-1β.

Discovered more than 25 years ago [2], IL-1β acts through the IL-1 receptor to transcriptionally regulate multiple biological functions including fever, infiltration of inflammatory cells from the circulation into the tissues and angiogenesis [3]. IL-1β is normally not expressed in phagocytic cells but, upon stimulation with a variety of microbial stimuli, IL-1β is rapidly synthesized as an inactive proform *via* transcriptional activation. Unlike most cytokines, the secretion of mature IL-1β requires processing of its pro-IL-1β form by caspase-1, a cysteine protease. Another substrate of caspase-1 is pro-IL-18, a cytokine that plays a role in the production of interferon-γ by T lymphocytes cells and NK cells [3]. Caspase-1 is present

in the cytosol of phagocytic cells as an inactive zymogen [4, 5]. Upon stimulation of phagocytic cells by pro-inflammatory signals, the procaspase-1 zymogen is activated by self-cleavage at aspartic residues to generate the enzymatically active homodimer of catalytic domains, consisting of a p20 and a p10 subunit [6, 7]. Although it has long been recognized that microbial stimuli elicit the secretion of mature IL-1β, the cellular machinery mediating the activation of caspase-1 was only identified in 2002 when Tschopp and colleagues described the inflammasome, a multi-protein complex that induces robust processing of proIL-1β [8]. Here we discuss recent findings about caspase-1 activation with an emphasis on the regulation of the NLRC4 and NLRP3 inflammasomes by microbial stimuli.

NLR family members are critical regulators of the inflammasome

The NLR family is composed of more than 20 family members in mammals which share a tripartite structure consisting of a variable N-terminal domain, a centrally located nucleotide-binding oligomerization domain (NOD) and a C-terminal leucine-rich repeat for upstream sensing. While NOD1 and NOD2 activate NF-κB and MAPK in response to peptidoglycan fragments, a class of NLR including NLRC4, NLRP1 and NLRP3 function as caspase-1 activators [9]. These NLR contain N-terminal CARDs or PYRIN domains that mediate the assembly of the inflammasome through NOD-mediated oligomerization and interaction with caspase-1 *via* the adaptor ASC [6]. Human NLRP1 senses bacterial muramyl dipeptide whereas mouse Nlrp1b recognizes lethal toxin, which is secreted by *Bacillus anthracis* [6]. Recently, the HIN-200 family member AIM2 has been shown to be a crucial molecule linking cytosolic double strand DNA to caspase-1 activation [10]. AIM2 regulates the host response to vaccinia viruses, but further work is needed to understand the role of AIM2 in microbial recognition [10]. We discuss in more detail in the following two sections the NLRC4 and NLRP3 inflammasomes.

The NLRC4 inflammasome

Several Gram-negative bacteria, including *Salmonella enterica* serovar Typhimurium, *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Shigella flexneri* induce caspase-1 activation *via* the NLRC4 inflammasome [11–18]. Although NLRC4 contains a CARD that presumably associates directly with that present in pro-caspase-1 [19], the adaptor ASC is still required for caspase-1 activation and IL-1β secretion in response to bacterial infection [12, 20]. The role of ASC in the NLRC4 inflammasome is still unclear, but it may promote the recruitment and/or dimerization of caspase-1 directly or through unknown factors. Several Gram-negative bacteria that activate the NLRC4 inflammasome require a functional type III secretion system or type IV secretion system to induce caspase-1 activation [6]. These bacterial secretion systems form pores in host membranes to inject virulence factors into the host cell cytosol [6]. Notably, NLRC4 senses cytosolic flagellin that is presumably delivered to the host cytosol through the type III secretion system/type IV secretion system to activate caspase-1 [11–13, 18]. Recognition of flagellin by NLRC4 is likely indirect and mediated through host cellular factors, which trigger inflammasome activation since there is no evidence to date for a direct interaction between NLRC4 and flagellin. NLRC4 can sense additional molecules besides flagellin as certain aflagellated bacteria including *S. flexneri* [14] and *Mycobacterium tuberculosis* [21] activate caspase-1 *via* NLRC4.

The NLR protein Naip5 is also critical for the sensing of a conserved C-terminal portion of flagellin from *L. pneumophila* and for NLRC4-dependent caspase-1 activation [22]. Remarkably, Naip5 is not required for caspase-1 activation triggers by *S. typhimurium* or *P. aeruginosa* infection [22]. The mechanism by which Naip5 regulates the NLRC4 inflammasome activated by *L. pneumophila* remains unclear [23]. Because caspase-1 is critical for restricting the replication of *L. pneumophila* in the host cytosol, these studies suggest that both Naip5 and NLRC4 control the susceptibility to *L. pneumophila* through the sensing of flagellin and caspase-1 activation. Alternatively, Naip5 may have additional NLRC4-independent roles that are important in restricting the growth of *L. pneumophila* in macrophages. Recent studies suggest that caspase-7 which is activated by the NLRC4 inflammasome is an important factor in restricting *L. pneumophila* replication, although the mechanism involved remains elusive [24].

The NLRP3 inflammasome

While the NLRC4 inflammasome is activated primarily by cytosolic flagellin, a plethora of microbial and non-microbial stimuli have been reported to activate caspase-1 *via* NLRP3. These include multiple TLR agonists and the Nod2 agonist, MDP [25, 26]. In addition, large particles including urate crystals, silica, asbestos, β-amyloid and aluminum hydroxide activate the NLRP3 inflammasome in phagocytes pre-stimulated with microbial ligands such as LPS [6]. Unlike TLR ligands, these particulate and crystalline molecules can activate the inflammasome in the absence of extracellular ATP [6]. Although the critical cellular events remain poorly understood, disruption of the lysosomal membrane and/or production of ROS [27] have been suggested to be important for particulate matter-induced NLRP3 activation [28].

The ability of multiple pathogen-associated molecular patterns to activate the NLRP3 inflammasome is puzzling because most of the molecules including TLR ligands are structurally unrelated. Recent findings suggest that most or all TLR agonists as well as MDP do not activate the NLRP3 inflammasome directly. Instead, they prime the inflammasome *via* NF-κB to promote caspase-1 activation [29, 30], which is

consistent with previous results [31]. Consistently, TNF-α and IL-1 are as effective as TLR agonists in promoting caspase-1 activation in response to ATP or silica [29]. Because enforced expression of NLRP3 can bypass the requirement for LPS or cytokine stimulation, priming of the inflammasome appears to be mediated at least in part through NLRP3 induction, which is regulated *via* NF-κB [30]. Extracellular ATP activates the ATP-gated P2X7 receptor (P2X7R), which acts as a cation channel to rapidly induce potent K^+ efflux and a complete collapse of normal ionic gradients [32]. P2X7R activation also recruits pannexin-1 which mediates the formation of a pore that has been implicated in inflammasome activation [33]. However, the concentration of ATP that is required for activation of the NLRP3 inflammasome *in vitro* far exceeds that found physiologically in the extracellular milieu. Thus, the relevance of the ATP-mediated pathway for inflammasome activation *in vivo* is unclear.

The NLRP3 as a sensor of pathogens

Several pathogenic microorganisms including certain viruses, fungi and bacteria induce the activation of the NLRP3 inflammasome. For example, NLRP3 regulates IL-1β production in response to influenza A, Sendai virus and vaccinia virus Ankara [34–38]. In the case of influenza A virus, dsRNA production has been suggested to mediate inflammasome activation, although this remains controversial [34, 39, 40]. One possibility is that dsRNA primes the NLRP3 inflammasome [29, 30]. The importance of NLRP3 in host defense against influenza A virus is also unclear because conflicting findings have been observed regarding its role in the control of viral burden, lung pathology and adaptive immune responses [34–36].

The NLRP3 inflammasome is also critical for the regulation of IL-1β in response to the fungus *Candida albicans* [41, 42]. Importantly, the NLRP3 inflammasome regulates fungal burden and survival in mice infected with *C. albicans*, which may be explained through IL-1β production and IL-1R signaling [41, 42]. How fungal infection leads to inflammasome activation is unclear, but Syk, a tyrosine kinase acting downstream of multiple ITAM-coupled fungal PRR, was found to be important in both pro-IL-1β induction and caspase-1 activation [42]. Caspase-1 activation was impaired in LPS-stimulated macrophages infected with the *C. albicans*, suggesting that Syk can direct the activation of NLRP3 independently of priming. One possibility is that Syk mediates ROS production [42] to induce inflammasome activation. Clearly more work is needed to understand the link between Syk and the activation of the NLRP3 inflammasome.

The role of the NLRP3 inflammasome in the host defense response against *Plasmodium berghei,* a mouse model of malaria induced by *Plasmodium falciparum*, is controversial. β-hematin, a synthetic compound of hemozoin, a polymer resulting from the degradation of erythrocyte hemoglobin by the parasite, induces caspase-1 activation and IL-1β production through NLRP3 [43–45]. β-hematin activation of the NLRP3-inflammasome may involve the tyrosine kinases Syk and Lyn [43]. Interestingly, NLRP3-deficient mice show mild protection against plasmodium infection when compared to WT mice [44, 45]. However, mice deficient in caspase-1, ASC or IL-1R are as susceptible to plasmodium infection as the WT, suggesting that the role of NLRP3 in cerebral malaria is independent of the inflammasome [44].

Several pathogenic bacteria including *Staphylococcus aureus*, *Klebsiella pneumonia* and *Streptococcus pyogenes* also activate caspase-1 *via* NLRP3 [46–48]. Exotoxins acting as pore-forming or membrane-damaging factors are important in mediating activation of the NLRP3 inflammasome [49, 50]. For example, *S. aureus* hemolysins and *S. pyogenes* streptolysin O are critical for NLRP3 activation [46, 47]. Although TLR stimulation contributes to NLRP3 activation *via* priming, *S. aureus* and *S. pyogenes* can activate caspase-1 independently of MyD88/TRIF, the critical adaptors required for all TLR signaling [46, 47]. One possibility is that pathogenic bacteria induce priming of the NLRP3 inflammasome *via* TLR-independent mechanisms. Alternatively, exotoxins may mediate the delivery of microbial molecules for NLRP3 activation. Unlike that triggered by TLR ligands, NLRP3 activation induced by bacterial or fungal infection is independent of the P2X7R [46, 47]. Thus, the role of ATP-induced P2X7R signaling in microbial activation of the NLRP3 inflammasome *in vivo* is unclear.

Mechanism of NLRP3 inflammasome activation

Recent studies suggest a model of NLRP3 activation that is mediated by two signals. The first, signal one, is provided by microbial molecules such as TLR ligands or by certain cytokines that induce priming of the inflammasome at least in part by NF-κB and NLRP3 induction (Fig. 1) [29, 30]. The second signal directly triggers caspase-1 activation, and can be mediated by at least four separate pathways that include ATP-P2X7R-pannexin-1, Syk signaling, lysosomal membrane rupture and bacterial exotoxins (Fig. 1). It is likely that these different pathways culminate in a common step that leads to NLRP3 activation. However, the identification of a unifying mechanism of NLRP3 activation remains elusive. The mechanisms regulating NLRP3 activation are discussed in more detail in accompanying articles of this issue [51, 52]. A possible common link is provided by the ROS because NLRP3 activation is blocked by ROS inhibitors [27]. However, most of these studies rely on pharmacological inhibitors that are used at high concentrations and exhibit variable effects or RNA interference, which is artifact prone. Nonetheless, Tschopp and colleagues have identified thioredoxin-interacting protein (TXNIP) as an NLRP3-interacting protein [53]. Although, it remains to be determined whether TXNIP is an essential activator

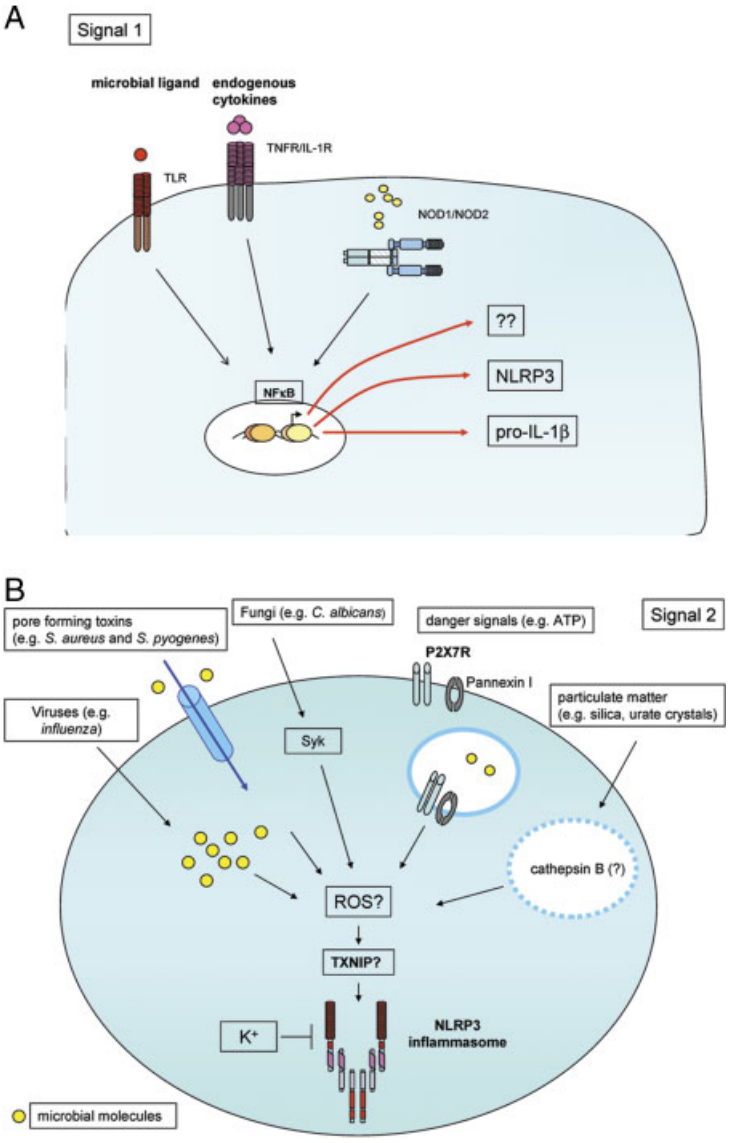

Figure 1. NLRP3 activation is mediated by two signals. Microbial and cytokines provide signal 1 for pro-IL-1β induction and priming of the NLRP3 inflammasome *via* NF-κB (A). Several stimuli (including viruses, bacteria, fungi, danger signals and particulate matter) and signaling pathways induce activation of the NLRP3 inflammasome (signal 2) in primed phagocytic cells (B). The role of ROS and TXNIP in NLRP3 activation is currently not well understood.

or just a regulator of the NLRP3 inflammasome.

Concluding remarks

There has been a remarkable growth in our knowledge about the regulation, activation and biological role of the inflammasome. However, many important questions remain. They include identifying the link between microbial stimulation and inflammasome activation given that recognition of NLRC4/NLRP3 appears indirect. The identification of TXNIP as a possible link between ROS and NLRP3 is important, but more work is needed to understand its precise role in inflammasome activation. Another unanswered question is how bacterial toxins promote inflammasome activation. Finally, the role of the inflammasome in host defense (*e.g.* influenza) and disease pathogenesis (*e.g.* cerebral malaria, Alzheimer's disease, diabetes) remains poorly understood.

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Abbreviations: NLR: Nod-like receptor · **NOD**: nucleotide-binding oligomerization domain · **TXNIP**: thioredoxin-interacting protein

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