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Sensing and Reacting to Microbes via the Inflammasomes

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Abstract

Inflammasomes are multi-protein complexes that activate Caspase-1 which subsequently leads to the maturation of the proinflammatory cytokines IL-1 β and IL-18 as well as pyroptosis, a form of cell death induced by bacterial pathogens. Members of the Nod-like receptor family including NLRP1, Nlrp3 and Nlrc4 as well as the HIN-200 family member AIM2 are critical components of the inflammasome and link microbial and endogenous danger signals to Caspase-1 activation. In response to microbial infection, activation of the inflammasomes contribute to host protection by inducing immune responses that limit microbial invasion, but deregulated activation of inflammasomes is associated with autoinflammatory syndromes and other pathologies. Thus, understanding inflammasome pathways will provide insights into the host defense response system against microbes and the development of inflammatory disorders.

Introduction

The innate immune system is the first line of defense against microbial infection and is activated by the engagement of germ-line encoded pattern-recognition receptors (PRRs) in response to microbes¹. PRR recognize the presence of unique microbial components called pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular patterns (DAMPs) generated in the setting of cellular injury or tissue damage². In response to infection, PRR activation initiates signal transduction pathways that ultimately culminate in host defense responses that eliminate microbial invasion. A major inflammatory pathway downstream of PRRs is the activation of the inflammasome, a multi-protein platform that activates Caspase-1³. Once activated, Caspase-1 proteolytically cleaves pro-IL1 β and pro-IL-18, which is critical for secretion of their biologically active forms and triggering pro-inflammatory and anti-microbial responses⁴. In addition, active Caspase-1 can cleave less defined protein substrates to regulate the induction of pyroptosis, autophagy and bacterial degradation via mechanisms that remain poorly understood⁴. To date, four bonafide inflammasomes named by the PRR that regulates their activity have been identified: the NLRP1, Nlrp3, Nlrc4 and AIM2 inflammasomes. With the exception of AIM2, the other inflammasomes contain a PRR that belongs to the Nod-like receptor (NLR) family. NLRs are intracellular PRRs that are defined by a tripartite structure, namely, an N-terminal caspase recruitment domain (CARD), pyrin domain (PYD), acidic transactivating domain, or baculovirus inhibitor repeat (BIR) that mediate downstream protein-protein interactions, a central nucleotide-binding oligomerization (NOD) domain which mediates self-oligomerization, and a C-terminal leucine-rich repeats (LRRs) that dictate ligand specificity⁴. In this review, we will focus on the activation, regulation, and function of NLR inflammasomes with an emphasis on their interaction with microbes and their role in host defense.

The Nlrc4 inflammasome

Mechanism of microbial recognition and activation

Initial experiments showed that Nlrc4 is important for the activation of Caspase-1 in macrophages infected with several pathogenic bacteria including *Salmonella enterica* serovar Typhimurium (*Salmonella*)^{5, 6}, *Legionella pneumophila* (*Legionella*)^{7–9}, and *Pseudomonas aeruginosa* (*Pseudomonas*)^{10, 11}. The activation of Caspase-1 by these pathogenic bacteria requires a functional bacterial secretion system which suggested a link between bacterial pathogenicity and Nlrc4 activation^{3, 12}. These secretion systems, which include the type III (T3SS) and type IV (T4SS), act as molecular needle-like structures to inject effector proteins into the cytosol of host cells and are critical for pathogen colonization. Early studies revealed that flagellin, the main component of the flagellum, was important for activation of the Nlrc4 inflammasome^{5, 6}. Because delivery of purified flagellin to the macrophage cytosol triggered Caspase-1 activation via Nlrc4^{5, 6}, it is thought that Nlrc4 is activated in macrophages through the leakage of small amounts of flagellin via the T3SS (e.g., *Salmonella* and *Pseudomonas*) or the T4SS (e.g., *Legionella*) during infection¹³. However, *Shigella flexneri* (*Shigella*), an aflagellated pathogenic bacterium, induces the activation of the Nlrc4 inflammasome via the T3SS¹⁴. Furthermore, flagellin-deficient *Salmonella* and *Pseudomonas* can activate Nlrc4 at high bacterial-macrophage ratios further suggesting that factors other than flagellin can induce the activation of the Nlrc4 inflammasome^{15, 16}. Initial insights into a flagellin-independent pathway came from the observation that proteins forming the basal body rod component of the secretion system, such as PrgJ, can activate the Nlrc4 inflammasome. Remarkably, PrgJ-like proteins contain regions structurally homologous to the C-terminal portion of flagellin¹⁵, which is the critical portion of flagellin that is sufficient to trigger Nlrc4 inflammasome activation^{6, 17}. It must be noted, however, that the contribution of T3SS rod proteins to the activation of the Nlrc4 inflammasome is difficult to evaluate because bacterial pathogens without a functional secretion system are impaired in the secretion of effector proteins and therefore highly attenuated.

How does Nlrc4 sense different structures such as flagellin and PrgJ-like proteins? Initial studies showed a link between Nlrc4 and Naip5, another NLR family member, in that Naip5 sensed the C-terminal region of flagellin and was required for the activation of Nlrc4 in response to *Legionella*¹⁷. In contrast, Naip5 was dispensable for Nlrc4 activation in response to *Salmonella*, *Pseudomonas* or flagellin purified from *Salmonella*¹⁷. These puzzling results have been recently clarified by the observation that distinct Naip proteins link flagellin and PrgJ-like proteins to Nlrc4. While flagellin binds Naip5 and Naip6, PrgJ-like proteins interact with Naip2^{18, 19}. In addition, CprI, a subunit of the secretion system of *Chromobacterium violaceum*, binds to human NAIP¹⁸. Additional studies suggest that Naip proteins act upstream of Nlrc4 to promote inflammasome activation. Although the mechanism by which Naips activate Nlrc4 remains unclear, a proposed model is that flagellin or PrgJ-like proteins bind to the LRRs of Naip proteins to induce a conformational change in Naips, which in turn induces the activation of Nlrc4. The activation of the Nlrc4 inflammasome is summarized in Figure 1. A better understanding of the link between Naips and Nlrc4 is needed to explain why Naip5 is essential for the activation of Caspase-1 in response to *Legionella*, but dispensable in *Salmonella* infection despite the fact that flagellin from both pathogens bind to Naip5^{17–19}. Because the N-terminus of flagellin can inhibit the interaction of flagellin with Naip5, one possibility is that the differential role of Naip5 in the recognition of *Legionella* and *Salmonella* depends on other factors that regulate the exposure or conformation of the C-terminus, the portion of flagellin that is sufficient for Nlrc4 activation.²⁰ Finally, it should be mentioned that *Salmonella* is a potent inducer of Caspase-1 activation in human cells²¹, but flagellin is not sensed by human NAIP¹⁸,

raising the question about the molecular mechanism that induces Caspase-1 activation in human cells.

Role of the Nlrc4 inflammasome in host defense

The Nlrc4 inflammasome regulates host defense through the regulation IL-1 β and IL-18 secretion, bacterial degradation and pyroptosis. IL-1 β and IL-18 play an important role in the host defense response to *Shigella* ²², but it is not known whether the production of these cytokines is dependent on Nlrc4 in vivo. In the case of *Salmonella* infection, Nlrc4, and Caspase-1 as well as IL-1 β and IL-18 play a minor or no significant role in host defense after orogastric infection in C57BL/6 mice ^{23, 24}, but confer host protection in the Balb/c background (LF and GN, unpublished observations). It was initially observed that activators of the Nlrc4 inflammasome induce pyroptosis, a form of caspase-1-dependent cell death with features of both apoptosis and necrosis ²⁵. It was recently shown that Nlrc4-dependent pyroptosis plays role in host defense. In fact, mutants of *Salmonella* ²⁶ or *Listeria* ^{27, 28} that ectopically express flagellin cannot evade detection by the Nlrc4 inflammasome and are highly attenuated. Furthermore, Nlrc4 restricts *Salmonella* strains overexpressing flagellin independently of IL-1 β and IL-18 by promoting the release of intracellular *Salmonella* from pyroptotic macrophages ²⁶. Consequently, extracellular bacteria are engulfed and killed by neighboring neutrophils ²⁶. However, the contribution of this protective mechanism to host defense during physiological infection with intracellular bacteria remains to be determined.

Nlrc4 can also promote the degradation of pathogens inside macrophages. This is best exemplified by studies performed with *Legionella*, a Gram-negative intracellular bacterium that causes an acute form of pneumonia called Legionnaires' disease. After infection, *Legionella* replicates within specialized vacuoles inside macrophages. Notably, Nlrc4-dependent Caspase-1 activation was found to restrict the intracellular growth of *Legionella*, at least in part, by promoting the fusion of bacteria-containing vacuoles with lysosomes ⁹. The ability to restrict *Legionella* growth by Nlrc4 required expression of flagellin and host caspase-7, a proteolytic substrate of Caspase-1, but not IL-1 β and IL-18 ^{9, 29}. However, the mechanism by which Caspase-7 promotes fusion of the *Legionella*-containing vacuoles with lysosomes and bacterial degradation remains unclear. Naip5 also plays a critical role in restricting the growth of *Legionella* inside macrophages which can be partially explained by a role for Naip5 in linking cytosolic flagellin to Nlrc4 activation ^{9, 29}. However, analysis of mice expressing mutant Naip5 protein from A/J mice that support *Legionella* replication and Naip5-null mice suggest that Naip5 may also act independently of Nlrc4 to regulate *Legionella* replication ³⁰. While both mutant mice show increased bacterial replication, the amino acids of Naip5 substituted in A/J mice are not important for flagellin binding or activation of the Nlrc4 inflammasome ¹⁸, suggesting that Naip5 plays a role in restricting bacterial replication that is distinct from its role in activating the Nlrc4 inflammasome. In addition to promoting bacterial degradation, Nlrc4 and Naip5 can also control *Legionella* growth through the induction of pyroptosis, a phenotype that is evident under high multiplicity of infection. While experiments with macrophages deficient in Naip5, Nlrc4, Caspase-1 or Caspase-7 showed a dramatic phenotype in regulating *Legionella* replication in vitro, the role of the flagellin/Naip5/Nlrc4 signaling pathway in lung infection is more modest, suggesting that other signaling pathways can compensate for inflammasome deficiency in vivo ^{9, 29, 31}.

The NLRP1 inflammasome

The initial description of the inflammasome was based on the assembly of the human NLRP1 inflammasome. Although the role of NLRP1 in immune responses remains poorly understood, its relevance is underscored by the association of genetic variations of NLRP1 with generalized vitiligo, vitiligo-associated type I diabetes, Addison's disease and

rheumatoid arthritis³. The domain structure of human NLRP1 consists of an N-terminal PYD, a centrally located NOD and LRRs, and C-terminal FIIND and CARD domains. Thus, NLRP1 is different from other NLR proteins in that it possesses two signal transduction domains, that is, PYD and CARD. Using crude extracts from THP1 cells, pioneering experiments showed that NLRP1 forms a multi-protein complex containing Asc (Apoptosis-associated speck-like protein containing a CARD), CARD8, Caspase-5 and Caspase-1 that exhibits IL-1 β processing activity³². Later reconstitution of the NLRP1 inflammasome using purified components demonstrated that the minimal elements of the NLRP1 inflammasome are NLRP1, a nucleotide triphosphate (NTP) and Caspase-1³³. The activity of the reconstituted NLRP1 inflammasome was induced by muramyl dipeptide (MDP) and based on these initial studies, it was proposed that Caspase-1 is activated via a two-step mechanism: first, microbial MDP binds NLRP1 and changes its conformation allowing it to bind an NTP, which, in turn, induces NLRP1 oligomerization through its NOD, thus creating a platform for Caspase-1 activation. The adaptor molecule Asc was not essential for Caspase-1 activation, most likely because human NLRP1 can bind to Caspase-1 directly through a CARD-CARD interaction, but the presence of Asc augmented NLRP1-mediated Caspase-1 activation³³. Consistent with these results, macrophages stimulated with MDP and TiO₂ activated the NLRP1 inflammasome in an Asc independent manner³⁴. However, there is no direct evidence that MDP binds to NLRP1. Thus, further work is needed to understand the activation of NLRP1 by MDP and the role of CARD8 and Caspase-5 in the function of the NLRP1 inflammasome.

Unlike humans that possess a single *Nlrp1* gene, in mice, three tandem paralogs, *Nalp1a*, *Nalp1b* and *Nalp1c*, are present. Furthermore, different strain-specific alleles exist for *Nlrp1b* and these genetic variants led to the identification of *Nlrp1b* as the sensor of *Bacillus anthracis* lethal toxin (LT)³⁵. LT, a metalloproteinase, is a bipartite toxin that consists of Protective Antigen (PA), a pore-forming molecule that mediates the translocation of lethal factor (LF) into the host cytosol where it activates Caspase-1. LT-induced Caspase-1 activation, IL-1 β production and pyroptosis require the *Nalp1b* susceptible allele³⁵ while Asc is required for LT-induced IL-1 β production, but is dispensable for pyroptosis³⁶. In vivo experiments showed that the *Nlrp1b* allelic variation plays a protective role against *B. anthracis* infection and, consistent with a role for Nlrp1b in activating the inflammasome, caspase-1^{-/-} and IL-1 β ^{-/-} mice are more susceptible to *B. anthracis* infection³⁷. Thus, the host protective response mediated via the Nlrp1b inflammasome is dependent on the production of IL-1 β rather than the induction of pyroptosis. Although much progress has been made regarding the recognition of *B. anthracis* via the Nlrp1b inflammasome, the mechanism by which LF triggers the activation of the Nlrp1b inflammasome remains elusive.

The Nlrp3 inflammasome

Mechanism of activation

Initial studies identified Caspase-1 as the protease responsible for IL-1 β maturation and secretion in response to ATP. A deeper understanding of this process came with the discovery that Nlrp3³⁸ and the adaptor Asc¹² are required to activate Caspase-1 in response to ATP and certain bacterial pore-forming toxins. Consistent with the important role of Nlrp3 in IL-1 β production, Nlrp3 gain-of-function mutations lead to Cryopyrin-associated periodic syndromes, which are efficiently treated with inhibitors of IL-1 β -mediated signaling³. Subsequent studies revealed that the Nlrp3 inflammasome is activated by a plethora of microbial stimuli including MDP^{39, 40}, bacterial RNA⁴¹, the dsRNA analog polyI:C⁴¹, LPS, microbial lipopeptide, and the antiviral imidazoquinolines R837 and R848⁴¹. In addition to microbial products, Nlrp3 can also be activated by endogenous stimuli and particulate matter such as uric acid, cholesterol and hydroxyapatite crystals,

silica, aluminum salts, asbestos, malarial hemozoin, amyloid deposits, and fatty-acids⁴. Given the chemical and structural diversity of the Nlrp3 activators, it has been hypothesized that Nlrp3 does not interact directly with its activators; rather, its activation is triggered via an intermediate cellular signal elicited by all these stimuli. Recent evidence indicates that most, if not all, TLR agonists and MDP do not activate directly the Nlrp3 inflammasome. Instead, these microbial stimuli prime the activation of Nlrp3 through the induction of Nlrp3 expression in macrophages⁴², a prerequisite for inflammasome activation^{42–44}. Consistently, the activation of the Nlrp3 inflammasome by ATP, bacterial pore-forming toxins and particulate matter require pre-stimulation with TLR agonists to induce Nlrp3 expression^{42–45}. While the adaptor Trif plays a minor role in the priming process in response to stimulation with LPS⁴⁶, it plays a major role in response to bacterial RNA⁴⁷. Because Nlrp3 induction is mediated via NF- κ B, endogenous cytokines such TNF- α and IL-1 β are also effective in inducing Nlrp3 expression and promoting Caspase-1 activation in response to Nlrp3 activators⁴⁴. Thus, the activation of the Nlrp3 inflammasome requires two signals in mouse macrophages. The first signal is provided by microbial or endogenous molecules that activate NF- κ B and induce Nlrp3 expression (Figure 2). The second signal directly activates Nlrp3 and is provided by ATP, certain bacterial toxins, or particulate matter (Figure 2). In human monocytes and microglia cells, stimulation with TLR ligands induces the release of IL-1 β in the absence of exogenous stimulation with ATP^{48–50}. It has also been recently proposed that TLR stimulation of human monocytes and microglia cells induces the release of endogenous ATP that act in an autocrine fashion to activate the P2X₇ receptor (P2X₇R)^{48–50}. Interestingly, under conditions in which the autophagic pathway is compromised, PAMP stimulation induces the activation of the Nlrp3 inflammasome⁵¹ and increased production of pro-IL-1 β ^{52, 53}, suggesting that in physiological conditions, autophagy plays an important role in controlling the production of IL-1 β .

Several theories have been proposed regarding the identity of the cellular signal responsible for Nlrp3 activation including a change in the intracellular concentration of K⁺ and Na⁺, formation of a large pore in the cell membrane, release of cathepsins from damaged lysosomes, production of reactive oxygen species (ROS) and damage in the mitochondria^{3, 54}. The involvement of K⁺ efflux in Nlrp3 activation is supported by the fact that some Nlrp3 activators including ATP, nigericin and pore-forming toxins induce a drop in the intracellular concentration of K⁺ and high extracellular concentrations of K⁺ prevent Nlrp3 activation by all known stimuli^{55, 56}. However, there is no evidence that particulate matter triggers an efflux of K⁺. Furthermore, the interpretation of experiments replacing extracellular Na⁺ with K⁺ is complicated by the fact that extracellular Na⁺ has also been reported to be required for Nlrp3 activation independently of K⁺ efflux⁵⁷. Hence, it is difficult to discern if the inhibitory effect of isotonic high K⁺ medium on Nlrp3 activation is due to the high concentration of K⁺ or the low concentration of Na⁺. In addition, very high extracellular concentrations of K⁺ also block the activation of the NLRP1, Nlrp4, and AIM2 inflammasomes⁵⁸. Therefore, further studies are required to clarify the role of changes in cytosolic ionic concentrations in the activation of the Nlrp3 inflammasome.

Extracellular ATP activates Nlrp3 through the opening of ATP-gated P2X₇R. In contrast, bacterial pore-forming toxins activate Nlrp3 independently of P2X₇R^{43, 59}. The P2X₇R is unique among ion channels in that its activation not only opens a cation channel, but also leads to opening of a larger pore permeable to molecules up to 900 Da. Previous work suggested that the opening of a large pore formed by the hemichannel pannexin-1 upon P2X₇R stimulation was necessary for Nlrp3 activation independently of K⁺ efflux⁶⁰. However, a recent study did not find any defect in Nlrp3 activation or opening of the large pore in pannexin-1^{-/-} macrophages stimulated with ATP and nigericin⁶¹. While these results suggest that pannexin-1 is not the molecular component of the large pore opened by ATP, it is still unknown whether the opening of a large pore is required to activate Nlrp3.

The activation of Nlrp3 by particulate matter requires endocytosis in that pretreatment of macrophages with drugs that interfere with cytoskeletal dynamics such as colchicine and cytochalasin B inhibit Nlrp3 activation by uric acid crystals, silica and aluminum salts but not by ATP⁴. Furthermore cathepsin B inhibitors can prevent the activation of caspase-1 induced by certain microbes^{62, 63}. However, cathepsin B^{-/-} mice display a modest or no defect in Nlrp3 activation by particulate matter⁶⁴. Therefore, it is possible that the observed impairment of Nlrp3 activation by cathepsin B inhibitor is due to off-target effects. Alternatively, given the high redundancy among the members of the cathepsin family, several lysosomal proteases may be able to trigger Nlrp3 activation. Genetic studies using double-knockouts will help clarify the role of cathepsins in Nlrp3 activation and the mechanism involved.

The production of ROS has also been suggested to act as a common cellular signal upstream of Nlrp3 triggered by ATP and particulate matter⁶⁵. Nlrp3 activation is blocked by ROS scavengers and NADPH oxidase inhibitors⁶⁵. In line with these findings, Zhou and collaborators have proposed that thioredoxin-interaction protein (TXNIP) binds and activates Nlrp3 following the production of ROS by Nlrp3 activators⁶⁶. However, these results could not be independently confirmed in a different study^{28, 67}. Furthermore, a recent report suggested that ROS inhibitors interfere with Nlrp3 priming rather than its activation⁴⁵. The fact that the mitochondria are a major cellular site of ROS production led Zhou and collaborators to investigate whether mitochondria ROS production is responsible for Nlrp3 activation in a separate study. In line with their previous work⁶⁶, inhibition of the respiratory chain by rotenone and antimycin A was sufficient to activate the Nlrp3 inflammasome⁶⁸. However, a previous study investigating the effect of rotenone in Nlrp3 activation failed to see this effect⁶⁵. While none of the aforementioned results provide a satisfactory explanation for the mechanism of Nlrp3 activation, it is possible that the combination of several cellular signals is required to activate Nlrp3. Indeed, it has been proposed that Nlrp3 acts as an integrator of cellular signals that indicate cellular damage or stress, including membrane permeation, lysosomal damage, ROS production and mitochondrial damage.

Nlrp3 activation by microbes

Numerous bacterial pathogens activate the Nlrp3 inflammasome through the secretion of pore-forming toxins. *S. aureus* α -hemolysin activates the Nlrp3 inflammasome³⁸ in combination with TLR2 stimulation by bacterial lipopeptides released during growth⁵⁹. Analysis of isogenic single, double and triple mutant *S. aureus* strains defective in α -, β - and γ -hemolysins revealed that they play a redundant role in Nlrp3 activation^{47, 59}. In vivo experiments using a *S. aureus* subcutaneous abscess model revealed a critical role of Asc^{-/-} and IL-1 β signaling in neutrophil recruitment and control of the infection^{69, 70}. Similar to the redundant role of *S. aureus* hemolysins in Nlrp3 activation, *V. cholera* secrete the hemolysins HlyA and MARTX to activate Nlrp3⁷¹. Furthermore, in vivo studies using mice strains deficient in inflammasome components revealed that Caspase-1 and Asc, but not Nlrp3, play an important protective role in the immune response against *V. cholera*⁷¹. These results suggest that multiple inflammasomes contribute to host defense against *V. cholera* in vivo.

S. pneumoniae, which colonizes the upper respiratory tract, is a leading cause of pneumonia and meningitis and activates Nlrp3 through the secreted pore-forming toxin pneumolysin^{72, 73}. In a *S. pneumoniae* lung infection model^{72, 73}, Nlrp3 elicited a protective immune response as Nlrp3^{-/-} mice exhibited higher bacterial loads and increased mortality. In contrast, in a pneumococcal meningitis model⁷⁴, Nlrp3^{-/-} and Asc^{-/-} mice showed decreased brain inflammation and improved clinical outcome. In addition, blockage of Caspase-1-mediated signaling using a combined regime of rIL-1RA and rIL-18BP led to

significant amelioration of disease severity and brain pathology suggesting that interfering with inflammasome activation might be a strategy for pneumococcal meningitis therapy.

Several other bacterial toxins have been reported to induce the activation of the Nlrp3 inflammasome, including cholera toxin B (CTB), adenylcyclase toxin⁷⁵, and *Clostridium difficile* toxin A and toxin B⁷⁶. These toxins have a different mechanism of action, and it remains unclear how they mediate the activation of the Nlrp3 inflammasome. Recent evidence indicates that the activation of the Nlrp3 inflammasome mediated by CTB, but not by adenylcyclase toxin, *C. difficile* toxin B or pore-forming toxins, is dependent on Caspase-11⁷⁵. Remarkably, Caspase-11 is dispensable for the activation of Caspase-1 by most stimuli that activate the Nlrp3 inflammasome and known triggers of the Nlrp4 and AIM2 inflammasome, but plays a role in the induction of pyroptosis and secretion of DAMPs⁷⁵. Some T3SS effector proteins have been reported to induce the activation of the Nlrp3 inflammasome. For example, activation by *Y. pestis* has recently described for the subspecies Kim through the effector protein YopJ⁷⁷, an acetyl transferase that causes apoptosis through inactivation of MAPK and IKK kinases⁷⁸. The role of Nlrp3 in *Y. pestis* infection in vivo, however, remains to be determined.

Several studies have reported a role for Nlrp3 in the innate immune response against viruses. Initial studies revealed that the Nlrp3 inflammasome can be activated in vitro by Sendai virus⁷⁹, Influenza A virus⁷⁹ and adenovirus⁸⁰. Influenza A virus activates Nlrp3 through the proton-selective M2 channel⁸¹ and elicits a protective inflammatory response^{82–84}. However, conflicting evidence exist regarding the contribution of Nlrp3 to the control of viral burden, host survival and the generation of adaptive immunity upon influenza infection^{82–84}. In an initial analysis⁸², mice deficient in Asc, Caspase-1 and IL-1R, but not in Nlrp3, had higher mortality accompanied by decreased immunoglobulin responses following influenza virus infection. However, in two later studies^{83, 84} Nlrp3^{-/-}, Asc^{-/-} and caspase-1^{-/-} mice exhibited higher mortality, but no defect in the generation of adaptive immunity against influenza⁸³. The reason for these contradictory results is not clear.

Candida albicans, a fungal pathogen that can cause severe opportunistic infections in immunocompromised hosts, can activate the Nlrp3 inflammasome⁸⁵. Experiments using *C. albicans* at different morphological stages^{86, 87} and mutants incapable of forming hyphae revealed that the yeast form is a more potent activator of Nlrp3 than the hyphal form; furthermore, the transition from the yeast to the hyphal form is an important step to elicit Nlrp3 activation⁸⁶. *C. albicans* requires TLR2, the Dectin-1 receptor, the tyrosine kinase Syk and its downstream adaptor CARD9 for the priming step, whereas Syk but not CARD9 is required for the activation of the Nlrp3-inflammasome^{85, 87}. In vivo experiments performed in TLR2^{-/-}, Dectin-1^{-/-}, Nlrp3^{-/-}, Asc^{-/-}, Caspase-1^{-/-}, and IL-1R^{-/-} mice revealed a protective role for the Nlrp3 inflammasome in a model of disseminated candidiasis⁸⁷.

Malaria is caused by Plasmodium parasites, which feed on erythrocyte hemoglobin and use a heme detoxification mechanism that results in the formation of dark-brown crystals called hemozoin (Hz). Similar to other particulate matter, there is evidence that Hz crystals activate the Nlrp3 inflammasome upon phagocytosis⁶⁴. While an study found a modest, but significant role of Nlrp3 in promoting cerebral malaria⁸⁸, subsequent studies found no evidence for Nlrp3, Asc, Caspase-1, IL-1 β or IL-18 in the development of cerebral malaria⁸⁹.

Evidence for redundancy in inflammasome activation in vitro and in vivo

Bacterial infection can trigger the activation of several inflammasomes. The clearest example is infection by the intracellular pathogen *Listeria monocytogenes*. Initial studies suggested that *Listeria* induces the activation of the Nlrp3 inflammasome^{38, 90} while other

studies found Nlrp3 to be dispensable^{55, 91}. This apparent discrepancy was reconciled by the observation that *Listeria* can engage multiple inflammasomes and that the contribution of each inflammasome (i.e., Nlrp3, Nlrc4 or AIM2) is dependent in part on the experimental condition of infection^{90, 92–94}. Although controversial, it has been reported that Caspase-1^{-/-} and Asc^{-/-} mice are more susceptible than wild-type mice to *Listeria* infection^{27, 95, 96}. However, it remains unclear whether a particular inflammasome predominates or whether multiple inflammasomes are redundantly activated in vivo. Similar to *Listeria*, several inflammasomes can be activated, depending on the experimental conditions, by *Salmonella*^{5, 6, 97}, *Shigella*^{14, 15, 98}, and other bacteria (see also Supplemental Table 1). However, with the exception of *Salmonella*, the specific contribution of each inflammasome to host defense in vivo remains largely unknown. Mice deficient in both Nlrc4 and Nlrp3, but not in either inflammasome alone, are slightly more susceptible to *Salmonella* infection than wild-type mice which correlated with a 5 to 10-fold increase in pathogen burden⁹⁷. Consistently, the phenotype of Nlrc4^{-/-} Nlrp3^{-/-} mice is comparable to that observed with Caspase-1^{-/-} mice^{24, 97}. The role of the adaptor protein Asc in host defense against *Salmonella* infection is more complex. Asc is composed of a PYD and a CARD domain, and is thought to be an essential adaptor that bridges Nlrp3 to Caspase-1⁵⁴. Experiments with mice deficient in Asc showed that Asc was necessary for the activation of Caspase-1 and the maturation of IL-1 β in mice infected with *Salmonella*, *Pseudomonas* or *Legionella*⁹⁹. Notably, Asc was dispensable for the induction of pyroptosis^{6, 11, 36}, which, in these infection models, is dependent on Caspase-1, but independent of Caspase-11³⁶. Recent studies revealed that induction of pyroptosis does not require the proteolytic maturation of Caspase-1 and suggested that phagocytes can assemble two different inflammasomes in response to *Salmonella* infection³⁶. One inflammasome containing Nlrc4 and Caspase-1 is responsible for the induction of pyroptosis whereas the other inflammasome containing Nlrc4, Asc and Caspase-1 mediates the maturation of IL-1 β and IL-18³⁶. These data suggest that microbial infections can activate different Nlrc4-containing inflammasomes that exert a different function. The relevance of these findings awaits more detailed analysis of the composition and biochemical properties of the different protein complexes within the Nlrc4 inflammasome.

Role of the inflammasome in the discrimination of pathogenic and non-pathogenic bacteria

Commensal microorganisms abundant in the skin and intestines continuously challenge the immune system without eliciting an inflammatory response. TLRs detect microbial ligands present in the extracellular environment and are activated by both commensal and pathogenic bacteria. However, the keratinized epithelium of the skin and the mucus layer of the gut form a physical barrier that prevents noninvasive microbes from engaging TLRs. In contrast to TLRs, NLR proteins sense the presence of microbial ligands in the cytosol making this class of PRRs ideal sensors of pathogenic bacteria because bacterial secretion systems or pore-forming toxins, which are features of pathogenic bacteria, can promote the delivery of microbial ligands to the host cytosol. The presence of bacterial secretion systems and pore-forming toxins has been shown to be important for the activation of the inflammasome and the production of IL-1 β . For example, the activation of the Nlrc4 inflammasome by several pathogenic bacteria requires a functional T3SS or T4SS⁹⁹. Similarly, activation of the Nlrp3 inflammasome by *S. aureus*, *V. cholera* and *S. pyogenes* requires bacterial pore-forming toxins^{4, 99}. Unlike phagocytic cells located in peripheral tissues, the intestine is populated with a specialized population of resident phagocytes that are hyporesponsive to microbial stimulation¹⁰⁰. Recent studies in our laboratory indicate that intestinal phagocytes are capable of sensing the presence of pathogenic bacteria, such as *Salmonella* and *Pseudomonas*, by selectively inducing the activation of the Nlrc4

inflammasome. Consistent with a role for the inflammasome in the detection of pathogenic bacteria in the gut, Nlrp4^{-/-} and IL-1R^{-/-} mice in the Balb/c background are more susceptible than wild-type mice to orogastric, but not to systemic, infection with *Salmonella* (L. Franchi and G. Nunez, unpublished). These data suggest that the Nlrp4 inflammasome in particular plays a major role in the intestine where it can function to discriminate commensal from pathogenic microbes and initiate a host defense response against harmful microbes.

Conclusions and Future Perspectives

Over the past decade, much progress has been made in understanding the activation, regulation and function of the inflammasomes in response to microbes (Supplemental Table 1). The microbial sensors responsible for the activation of Caspase-1 have been identified, as well as the role of Naip5 in binding flagellin and PrgJ-like proteins and promoting Nlrp4 activation. Moreover, there is conclusive evidence that inflammasomes contribute to host defense against a variety of pathogens. Yet, the molecular mechanism by which Naips activate Nlrp4 and microbial stimuli induce the activation of the Nlrp3 inflammasome remains largely unknown. Furthermore, we know little about the protein substrates cleaved by Caspase-1 and/or Caspase-11 and their role in executing pyroptosis. Another unresolved question is how inflammasomes and other signaling pathways cooperate in vivo to orchestrate innate and adaptive immune responses. Clearly, much remains to be learned about the inflammasomes and their role in the recognition and host defense against microbes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The Nlrc4 inflammasome. Infection of macrophages with several Gram-negative bacteria including *Salmonella*, *Legionella* and *Pseudomonas* activates Caspase-1 through Nlrc4. A critical step is the cytosolic delivery of flagellin or PrgJ-like proteins via bacterial T3SS or T4SS. Flagellin is recognized by Naip5 or Naip6 (not depicted) whereas PrgJ-like proteins are recognized by Naip2. *Shigella* activates the Nlrc4 inflammasome independently of flagellin through an unknown microbial product. Activation of Caspase-1 via Nlrc4 leads to processing and secretion of IL-1 β and IL-18 as well as other cellular activities that are poorly understood.

Figure 2.

The Nlrp3 inflammasome. Activation of Caspase-1 through Nlrp3 requires two signals. Signal 1 is represented by microbial molecules or endogenous cytokines and is required for the upregulation of Nlrp3 and pro-IL-1 β . Signal 2 activates the Nlrp3 inflammasome. Activation by *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Vibrio cholere* is mediated by pore-forming toxins. In addition, other bacterial toxins can induce the activation of the Nlrp3 inflammasome, such as Cholera Toxin (CT) or *Clostridium difficile* toxins TcdA and TcdB (not depicted); *Candida albicans* induces the activation of the Nlrp3 inflammasome through the kinase Syk, although the mechanism involved is unclear. Influenza virus can induce the activation of the Nlrp3 inflammasome, but whether this is due to a pore-forming activity mediated by M protein or to sensing of viral RNA species in the cytosol remains controversial. Cytosolic bacterial RNA has been reported to induce the activation of the Nlrp3 inflammasome.