Published in final edited form as:

Semin Immunol. 2009 August ; 21(4): 194–198. doi:10.1016/j.smim.2009.05.002.

The NLRP3 inflammasome: a sensor of immune danger signals

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Abstract

The innate immune system senses danger signals via evolutionary conserved receptors. The nucleotide-binding domain leucine-rich repeat containing receptors (NLR) family is a group of intracellular receptors that drive a wide variety of inflammatory responses. A number of the NLR family members can form inflammasomes, which are multiprotein complexes that can activate caspase-1 and ultimately lead to the processing and secretion of interleukin (IL)-1 β , IL-18 and IL-33. One of the best-studied members of the NLR family is NLRP3 for which a number of divergent activators have recently been described. These and other studies examining the NLRP3 inflammasome will be discussed in this review.

Keywords

Inflammasome; NLRP3; Caspase-1; Interleukin-1 β

Introduction

The success of the mammalian immune system lies in the complementation of its two arms, the innate and adaptive systems. While the adaptive immune system has the ability to refine its response through exposure to challenges, these modifications and expansions are relatively slow to develop. Thus, were the adaptive immune system to exist independently, an invasive organism could compromise the host before the creation of a controlling immune response. The innate immune system fills this void with a more immediate response that is genetically programmed, or “hardwired”, and thus does not change in response to repeated activation. The advantage to the innate immune system is its ability to respond rapidly and consistently to adverse conditions. In addition, activation of the innate immune system acts as a direct trigger for the adaptive immune system, and is thereby critical, not only in providing early management for infections, but also for driving the subsequent acquired immune response.

Recognition by the innate immune system occurs via families of germline encoded receptors [1]. These receptors include Toll-like receptors (TLRs), nucleotide-binding domain leucine-rich repeat containing receptors (NLRs), RIG-I-like RNA helicases (RLHs) and C-type lectin

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receptors (CLRs). These receptors, known collectively as pattern recognition receptors (PRRs) are specific for conserved moieties associated with cellular damage (DAMPs; Danger Associated Molecular Patterns) or invading organisms (PAMPs; Pathogen Associated Molecular Patterns), which are composed of a wide and divergent group of molecules including proteins, lipids, carbohydrates and nucleic acids. Activation of these receptors ultimately leads to the production of molecules that drive the inflammatory response.

While each receptor has a unique ligand, the accessibility of these ligands to their receptors shares an invariant association with either an infecting microbe in the case of PAMPs or cellular damage for DAMPs. PAMPs are generally molecules critical for the function of the microbe; in the case of cellular damage, DAMPs are modified host molecules or endogenous molecules normally found in a sequestered location. The presence of a DAMP in an atypical location is a reliable alarm signal for cellular dysfunction. As intracellular receptors, the NLR family is in a prime location to detect danger signals and therefore play a critical role in recognizing a wide variety of molecules associated with host stress. The NLR family is found within the cytoplasm of the cell and contains 22 members including 14 NLRP members, 5 members of the NLRC subfamily, NAIP, NLRX, and CIITA [2–4]. Recent work has elucidated the conditions under which some of these family members are activated, which will be discussed below.

The NLR family

The NLR family members have similar structures with an effector domain at the amino terminus consisting of either a pyrin domain (PYD), a caspase activation and recruitment domain (CARD) or a baculovirus inhibitor of apoptosis protein repeat (BIR). NLR family members also contain a ligand binding leucine rich repeat domain (LRR) at the carboxy terminus and a central nucleotide binding and oligomerization domain (NACHT). The NLR domain structure is reminiscent of plant R proteins, which are known to be involved in pathogen recognition [5,6]. Similarly, the NLR molecules have been shown to have a crucial role in alerting the mammalian immune system to the presence of pathogens and danger conditions.

A number of NLR molecules have been demonstrated to activate caspase-1 within multiprotein complexes termed inflammasomes. The NLRP1 (also known as NALP1 or DEFCAP), NLRP3 (also known as Cryopyrin, CIAS1 and NALP3) and NLRC4 (also known as CARD12 or IPAF) inflammasomes are amongst the best characterized and have all been shown to have clear physiological roles *in vivo*. However NLRP2, NLRP6, NLRP7, NLRP10 and NLRP12 have also been demonstrated to modulate caspase-1 activity *in vitro* although the significance of these interactions remains to be elucidated [7].

The NLRP1 inflammasome (a complex comprising of NLRP1, caspase-1, caspase-5 and the adaptor protein ASC) [8] has been revealed as the key mediator of cell death due to anthrax lethal toxin. Lethal toxin activates the inflammasome, which in turn activates caspase-1 leading to IL-1 β processing and secretion and rapid cell death [9,10]. Additionally, autoimmune and autoinflammatory diseases associated with vitiligo have been mapped to the NLRP1 genetic locus [11]. The NLRC4 inflammasome can be activated by numerous Gram-negative bacteria that possess either a type III or type IV secretion systems [12–14]. NLRC4 activation leads to the production of pro-inflammatory cytokines and similarly to NLRP1, ultimately results in cell death. The mechanism by which the NLRC4 inflammasome is activated remains controversial. NLRC4 has been shown to be activated in response to cytosolic delivery of bacterial flagellin [15,16]. The NLRC4 inflammasome can also be activated independently of flagellin. The non-flagellated bacterium *S. flexneri* [17] and the *P. aeruginosa* mutant PAK Δ *flhC* [14], which is deficient in flagellin, are still capable of activating caspase-1 in an NLRC4-dependent manner. Furthermore, NLRC4 may possibly interact with another cytosolic

NLR, Naip5 that may play a role in NLRC4 inflammasome activation [13]. A recent study by Lightfield and colleagues suggests that activation of the NLRC4 inflammasome by flagellin requires the carboxy terminus of flagellin and is also dependent on Naip5 [18].

The NLRP3 inflammasome

One of the best characterized NLR family members is NLRP3 which is linked to the human diseases Muckle-Wells syndrome, familial cold autoinflammatory syndrome and NOMID [19–22]. These disorders share similar clinical findings of skin rashes and a spectrum of other symptoms associated with generalized inflammation. These multi-system syndromes involve the musculoskeletal system with the development of arthralgias ranging from mild to debilitating joint damage, the central nervous system leading to headaches, elevated spinal fluid pressure, cognitive deficits and sensorineural deafness, and renal amyloidosis. These disorders are associated with mutations within the NLRP3 gene leading to a constitutively active form of the molecule. This in turn results in uncontrolled IL-1 β production, which is the mediator of the clinical syndrome.

NLRP3 has been shown to act in a multiprotein complex termed the NLRP3 inflammasome, composed of NLRP3, the adaptor molecule ASC as well as the cysteine protease caspase-1 [23]. Cardinal is part of the NLRP3 inflammasome complex in humans [23] although no mouse homologue of Cardinal has been identified to date and its specific function is unknown. The recruitment of the molecules to form the inflammasome is regulated by their effector domains. It is believed that activation of NLRP3 leads to the association of the PYD of NLRP3 with the PYD of ASC. This causes the CARD of ASC to associate with the CARD of caspase-1, which completes the assembly of the NLRP3 inflammasome (Figure 1). This association and resultant activation of the inflammasome leads to the activation of caspase-1, heralded by its self-cleavage from the inactive precursor pro-caspase-1 to the active form. Active caspase-1 cleaves the pro-forms of the cytokines IL-1 β , IL-18 and IL-33 to their active and secreted forms (Figure 1). Recent studies have suggested caspase-1 may also have additional functions including regulation of glycolysis pathways [24] and unconventional protein secretion [25].

The NLRP3 inflammasome can be activated in response to a number of diverse stimuli including those of microbial, endogenous and exogenous origins. However, the common pathway at which these stimuli converge to activate NLRP3 remains unclear. Microbes that can activate the NLRP3 pathway include Sendai virus [26], influenza virus [26,27], adenovirus [28], *Staphylococcus aureus* [29], *Listeria monocytogenes* [29,30] and *Shigella flexneri* [31]. Bacterial pore forming toxins can also activate this pathway; nigericin (*Streptomyces hygroscopicus*), maitotoxin (*Gambierdiscus toxicus*), aerolysin (*Aeromonas hydrophila*) and listeriolysin O (*L. monocytogenes*) have all been shown to activate caspase-1 in a NLRP3 dependent manner [29,32]. The pannexin hemichannel has also been shown to play an important role in activation of the NLRP3 inflammasome particularly in response to ATP, through the P2X7 receptor, nigericin and maitotoxin [33,34]. However, the role for pannexin in NLRP3 activation in response to other stimuli remains unclear. DNA, bacterial RNA and the antiviral imidazoquinoline compounds R837 and R848 can also activate the NLRP3 inflammasome independently of TLRs and RIG-I [26,28]. Interestingly, various immune cells differentially regulate IL-1 β production. Netea et al. have shown that while macrophages required a two signal process (TLR ligand and NLRP3 stimuli), monocytes only require stimulation with TLR2 or TLR4 ligands since they possess a constitutively activated form of caspase-1 and release endogenous ATP [35]. Due to their obvious role in innate responses to invading microorganism, most studies have focused on the role of NLRP3 in immune cells, especially monocytes and macrophages. However, recent studies have examined the role of the NLRP3 inflammasome in other cell types and consequently other tissues and diseases. Indeed, skin keratinocytes have been shown to mediate a NLRP3-dependent response to

various exogenous sources of skin irritants such as UVB and chemicals inducing contact hypersensitivity [36–38]. The number of reports describing molecules that activate NLRP3 to generate inflammation is drastically increasing. Recently, exogenous as well as endogenous molecules have been implicated in activation of this pathway, which we will discuss in further detail below.

Particulate activators of the NLRP3 inflammasome

Uric acid

A number of recent studies have associated the activation of the NLRP3 inflammasome with exposure to crystalline particles. Uric acid was the first of these crystalline molecules demonstrated to activate the NLRP3 inflammasome [39]. Uric acid is not only the mediator of the inflammatory condition gout, but has also been postulated to be an endogenous danger signal released from dying cells [40]. Monosodium urate (MSU, uric acid) as well as calcium pyrophosphate dihydrate (CPPD), the causative agents of gout and pseudogout respectively, were both shown to activate the NLRP3 inflammasome and elicit IL-1 β release from macrophages [39]. Based on these findings a pilot study to examine the efficacy of blockade of this pathway in acute gout was carried out. Ten patients with acute gout who could not tolerate or had failed standard anti-inflammatory therapies all responded to the IL-1R antagonist Anakinra [41]. These encouraging preliminary studies suggest that treatment modalities aimed at IL-1 β and the NLRP3 inflammasome may be an avenue to provide effective new therapies for numerous inflammatory diseases, especially autoimmune diseases.

Acetaminophen-induced hepatotoxicity results in substantial necrosis of the liver. Inhibition of the NLRP3 inflammasome pathway either by neutralizing antibodies against IL-1 β or utilizing mice deficient in components of the NLRP3 inflammasome resulted in decreased hepatic injury and improved survival in response to acetaminophen challenge [42]. Overall this study underlines the importance of the NLRP3 pathway in mediating a response to sterile inflammatory insults. However in this particular context, the role of endogenous molecules such as uric acid or ATP in the activation of the NLRP3 inflammasome have yet to be demonstrated. In a recent study by Gasse and colleagues it was shown that bleomycin-induced pulmonary injury and fibrosis were dependent on the NLRP3 inflammasome [43]. Uric acid released from injured cells played a central role in mediating pulmonary injury and inhibitors of uric acid synthesis, such as allopurinol or uricase, decreased bleomycin-induced pulmonary damage *in vivo* [43].

Silica and asbestos

It is known that IL-1 β is a critical cytokine in the development of disease in response to inhaled silica or asbestos particles. Three recent studies [44–46] expanded this understanding and showed that asbestos and silica crystals activate the NLRP3 inflammasome to produce IL-1 β . Using *in vivo* models of asbestosis and silicosis respectively, these groups were also able to show an amelioration of the inflammatory diseases in NLRP3 deficient mice [44–45]. This was shown for asbestosis via a decrease in the immediate inflammatory cellular influx into the lungs following inhalation of asbestos and for silicosis in impairment in the chronic inflammation and fibrosis associated with inhaled silica crystals. In addition to the study demonstrating the role of the NLRP3 inflammasome in bleomycin-induced lung injury [43], these studies show that NLRP3 plays a central role in mediating a number of pulmonary diseases culminating in pulmonary fibrosis. It will be interesting to expand these studies and determine if other fibrotic diseases are also dependent upon the activity of the NLRP3 inflammasome.

Vaccine adjuvants

Alum, an aluminum vaccine adjuvant, has been an important agent for physicians and immunologists alike for many years. It is a key ingredient for the development of an inflammatory response to an adsorbed protein, but its mechanism of action has remained unknown. Recent studies have revealed that alum acts via activation of the NLRP3 inflammasome [46–49]. Eisenbarth et al. demonstrated that the adjuvant activity of alum requires the NLRP3 inflammasome using a mouse model of allergic airway disease in which alum is administered intraperitoneally along with the protein ovalbumin [47]. Deficiency in components of the NLRP3 inflammasome resulted in abrogation of the cellular influx into the airway as well as loss of specific IgG1 antibody production to ovalbumin [47]. Independently, Li et al. [49] as well as Kool et al. [48] also demonstrated a role for NLRP3 in the generation of specific antibody responses to antigen challenge with the adjuvant alum. Interestingly Li and colleagues found that the adjuvants QuilA and chitosan also activated the NLRP3 inflammasome *in vitro* suggesting that particulate adjuvants may share a common mechanism of action [49]. Recently, Sharp et al. demonstrated further that enhancement of IL-1 β secretion through NLRP3 in dendritic cells was likely a general property shared by particulate adjuvants [50]. In addition they demonstrated that contrary to results obtained using alum, the generation of a potent antigen-specific antibody response with PLG (poly (lactide-co-glycolide)) microparticles was not dependent on NLRP3. However, NLRP3-deficient mice presented defective antigen specific cell mediated immunity when PLG was used as an adjuvant [50].

Amyloid- β

Amyloid- β is an endogenous peptide that constitutes the main component of amyloid plaques in Alzheimer's disease. The insoluble fibrillar form, as opposed to non-fibrillar, has also been implicated in the activation of NLRP3 [51]. The authors showed that phagocytosis of amyloid- β by mouse microglia resulted in IL-1 β secretion via the NLRP3 inflammasome. Moreover, this study suggested that the presence of cytoplasmic cathepsin B, as a result of destabilization and dysfunction of amyloid- β containing lysosomes, was responsible for NLRP3 inflammasome activation. They further showed that secretion of neurotoxic mediators, such as TNF- α and nitric oxide, is associated with IL-1 β production elicited via NLRP3 activation. Injection of amyloid- β resulted in recruitment of microglia in wild type mice, but the migration was impaired in mice lacking either components of the NLRP3 inflammasome or the IL-1 receptor [51]. Amyloid and structurally similar fibrillar molecules can be found in deposits at various sites of inflammation. It is intriguing to consider that these amyloid deposits may not only be a consequence of chronic inflammation but also act as endogenous danger signal themselves.

Mechanism of NLRP3 inflammasome activation

NLRP3 has been implicated in activation of innate immune responses to a wide range of seemingly unrelated danger signals. Given the structural dissimilarity of these stimuli, it is unlikely that NLRP3 is directly interacting with all of them. It is however plausible that a single ligand interacts with NLRP3 and the identified stimuli in fact serve to facilitate the release, modification or recognition of that ligand. Determining the direct ligand of NLRP3 requires understanding the common signaling pathways utilized by these divergent molecules (Figure 1).

Multiple studies reveal that phagocytosis of particulate activators of the NLRP3 inflammasome is necessary for activation [39,44–47,51]. However, while this activity is clearly required for particulate activators, such as MSU, silica, asbestos, and alum, it is not required for ATP, which binds the P2X7 receptor and triggers the formation of a pannexin pore. Similarly, bacterial pore forming toxins that activate the NLRP3 inflammasome also do not require internalization

by the macrophage for their ability to activate caspase-1. One event that is required for NLRP3 inflammasome activation by all known activators is a shift in potassium down its concentration gradient and out of the cell [12,52]. This movement of potassium can be prevented by equilibrating intra- and extra-cellular potassium in the cell culture media. Under these conditions, the potassium shift is abrogated as is the activation of the inflammasome in response to silica, asbestos, alum and ATP [44,45,47]. The exact role of the potassium efflux has yet to be explained; however the assembly of the NLRP3 inflammasome may require a low potassium environment.

Another common cellular event shared by a number of NLRP3 inflammasome activators is the generation of reactive oxygen species (ROS). It has been shown that the activation of caspase-1 in response to ATP is dependent upon the intervening step of ROS generation [53]. The critical role for ROS in stimulating caspase-1 has been expanded to other activators of the NLRP3 inflammasome via the use of inhibitors [44,45,52]. The source of the ROS remains unclear; studies utilizing macrophages derived from mice deficient in components of the NADPH oxidase system retained the ability to activate caspase-1 in response to NLRP3 inflammasome activators [46,54]. These data suggest that the source of ROS required to activate the NLRP3 inflammasome may be of mitochondrial origin. A recent study by Meissner and colleagues examined the role for superoxide dismutase 1 (SOD1) in the regulation of caspase-1 activation [54]. Higher superoxide production in SOD1-deficient macrophages resulted in inhibition of caspase-1 activation by reversible oxidation and glutathionylation of the redox-sensitive cysteine residues in caspase-1 [54]. Although this study does not directly explain how ROS are required for NLRP3 inflammasome activation it does demonstrate that the redox potential of caspase-1 and possibly the NLRP3 inflammasome plays an important role in its activation.

The uptake of particulates, such as amyloid- β , silica or alum, by phagocytosis results in lysosomal disruption [46,51]. Release of the lysosomal protease cathepsin B into the cytosol has been shown to be necessary for IL-1 β release in response to a number of the phagocytosis dependent mediators [46,51]. This intriguing role of cathepsin B, though, is not required for all the activators of NLRP3, as ATP dependent release of IL-1 β is not impacted by inhibition of cathepsin B.

Conclusion

Despite extensive progress in our understanding of the assembly and activation of the NLRP3 inflammasome, a central molecule at the crossroads of each pathway implicated in NLRP3 inflammasome activation has yet to be identified. Common pathways uniting all activators remain elusive, which is not unexpected given the marked diversity of the individual activators. It is attractive to focus on the role of the phagosome and the theory of phagosome disruption as a common thread. The difficulty therein, though, is the inherent exclusion of activation by ATP, bacterial toxins, UVB or chemical irritants that occur independently from phagocytosis. The key seems to be held in one or both of the two aspects of activation which remain conserved to numerous classes of NLRP3 inflammasome activators, that is the potassium efflux and the generation of ROS. Studies on the molecular and signal transduction pathways leading to NLR activation and resulting downstream events are likely to emerge as a next step for the inflammasome research. The unfolding of this conundrum undoubtedly will be a focus of this field for a time to come.

Acknowledgments

This work was supported by National Institutes of Health grants K08 AI065517 (F.S.S.) and K08 AI067736 (S.L.C.).

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Figure 1.

Model of NLRP3 inflammasome activation. Endogenous and exogenous danger signals engage various cytosolic events towards the assembly of a multiprotein complex composed of the cytosolic nucleotide-binding domain leucine-rich repeat containing receptor NLRP3, the adaptor molecule ASC and the cysteine protease caspase-1. Once activated, the NLRP3 inflammasome results in caspase-1 activation leading to the processing and secretion of the proinflammatory cytokines IL-1 β , IL-18 and IL-33. The commonality between signals involved in pathways as distinct as cell surface mediated events and lysosomal disruption is yet to be discovered, however, a unique molecule at the crossroads of these pathways is likely involved.