

Modulation of Inflammasome Activity for the Treatment of Auto-inflammatory Disorders

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Received: 1 March 2010 / Accepted: 8 March 2010 / Published online: 1 April 2010
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Abstract

Introduction The innate immune system orchestrates inflammatory responses to microorganisms or danger-associated molecular patterns generated, for example, by the deposition of uric acid in the joints of gout patients. The innate immune system comprises multiple germ-line encoded receptors, of which the nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) are crucial for the maturation of pro-inflammatory cytokines. NLRs oligomerize to form large multi-protein complexes termed inflammasomes that generate active caspase-1 fragments leading to the cleavage and secretion of mature cytokines such as IL-1 β and IL-18.

The regulation of multiple inflammasomes At least four independent inflammasomes have been identified, NLRP1, NLRP3, IPAF, and AIM2. These inflammasomes assemble in response to different stimuli to confer specificity and are also subject to negative regulatory mechanisms to ensure that once a productive inflammatory response has been mounted, inflammatory cytokine production is restrained.

Treatment of auto-inflammatory disorders A number of human conditions are characterized by unrestrained inflammasome activation. As much is now known about how inflammasomes are regulated, it is hoped that this can be channeled into the development of novel therapeutics, for example, those that may block the upstream activation and assembly of inflammasomes.

Keywords Inflammasome · NLR proteins · auto-inflammatory disorder · caspases · DAMP · PAMP · IL-1 β

Components of the Innate Immune System Coordinate Inflammatory Responses

An inflammatory response consists of a multifactorial network of intracellular and chemical signals that initiate and maintain a host response designed to eliminate pathogens and heal the damaged tissue. This response is initiated by the innate immune system, which, unlike the adaptive system, does not depend on somatic recombination to generate a highly specific repertoire of receptors for pathogen recognition. Key sensors of these “danger” signals expressed on cells of the innate immune system such as macrophages and dendritic cells include germline-encoded receptors that are classified according to the presence of variable numbers and types of binding domains, cellular localization, and substrate specificity (Fig. 1). These include transmembrane Toll-like receptors (TLRs), RIG-1-like helicases (RLRs), and the nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs).

These receptor complexes detect pathogen-associated molecular patterns (PAMPs) that represent largely invariant microbial components, thus enabling a more rapid response to a broader range of stimuli. For example, mammalian cells detect unique nucleic acid structures or the presence of cell wall components that specify the “otherness” of the invading microorganism. In contrast, damage (self)-associated molecular patterns (DAMPs) perpetuate immune responses in a non-infectious manner to clear agents that have normal physiological roles but, as a consequence of a disease state, have become mislocalized or even released from the cell. Signaling downstream of these receptors engages common effector machinery, including NF κ B/AP-1-dependent transcription of pro-inflammatory cytokines and chemokines, interferon regulatory factor (IRF)-dependent induction of type I interferon-mediated antiviral response and assembly of a large multiprotein scaffold termed the

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“inflammasome” (Fig. 1). This review will focus on the inflammasome as an effector of pro-inflammatory cytokine production and how knowledge of the regulation of this signaling cascade can be exploited for therapeutic benefit.

Following an inflammatory stimulus, oligomerization of NLR components catalyzes the recruitment of inactive pro-caspase-1 either by direct, homotypic caspase activation and recruitment domain (CARD) interaction in the case of NLRP1 and IPAF1 or via binding to the adaptor protein ASC in the cases of NLRP3 and absent in melanoma 2 (AIM2) (Fig. 1). ASC is a central player in the formation of multiple inflammasome complexes and when abolished leads to a specific reduction in IL-1 β and IL-18 secretion without concomitant attenuation in the production of TNF- α and IL-6 following stimulation [1]. Caspases have long been established as executioners of the apoptotic response but a subclass of these cysteine proteases (mouse caspase-1, -11, and -12 and human caspase-1, -4, and -5) are now known to contribute to inflammation, as their main substrates are the pro-inflammatory cytokines proIL-1 β and proIL-18. Caspase-1 is synthesized as an inactive zymogen, and the induced proximity of multiple caspase-1 zymogens within the inflammasome complex leads to

autocatalytic activation and production of potent p10 and p20 caspase-1 fragments. As caspase-1^{-/-} animals are highly resistant to endotoxic shock due to defective maturation of these cytokines [2, 3], it is clear that this caspase is responsible for the maturation of IL-1 β and IL-18 by cleavage of their precursor forms. The pleiotropic activity of mature IL-1 β is responsible for increased infiltration of leukocytes into the site of injury to neutralize and phagocytose pathogens, induction of fever, and regulation of hematopoiesis. Due to the potentially deleterious effect of unrestrained IL-1 β secretion, IL-1 β is subjected to two levels of regulation: firstly, increased NF κ B-dependent transcription of the pro-form and secondly, proteolytic maturation via cleavage by caspase-1. In contrast, the inactive form of IL-18 is constitutively expressed and, therefore, only requires cleavage by caspase-1 to become active.

Conferring Specificity upon an Inflammatory Response

Genetic studies in mice have identified at least three independent inflammasomes that assemble in response to

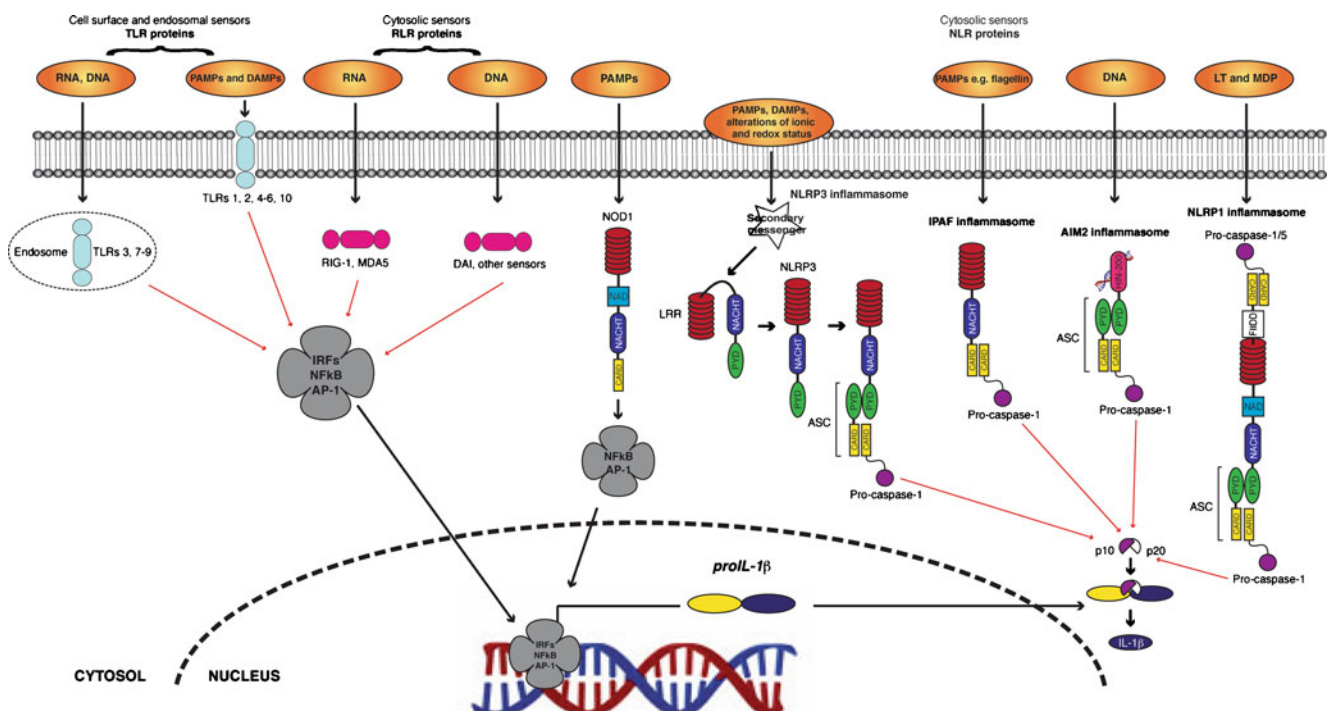


Fig. 1 The complexity of innate immune receptor surveillance and inflammasome activation. Detection of pathogens by the innate immune system is dependent on the function of multiple receptor classes that differ in localization, stimulus specificity, regulation, and function. TLRs are found on the cell surface and within endosomes, whereas the RLR and NLR receptors function within the cytosol. The production of mature IL-1 β requires NF κ B-dependent transcriptional upregulation followed by proteolytic maturation mediated by the p10 and p20 fragments of caspase-1. LT lethal toxin, MDP muramyl

dipeptide, CARD caspase activation and recruitment domain, PYD pyrin, NAD NACHT-associated domain, NLR Nod-like receptor, AIM2 absent in melanoma, PAMPs pathogen-associated molecular pattern, RIG-1 retinoid acid-inducible gene-1, RLR RIG-1-like receptor, TLR Toll-like receptor, IL-1 interleukin-1, AP-1 activator protein-1, DAMP danger-associated molecular pattern, MDA5 melanoma differentiation-associated gene-5, NF κ B nuclear factor κ B, NOD nucleotide-binding and oligomerization domain, LRR leucine repeat region, IRF interferon regulatory factor

different stimuli and are classified in terms of their requisite NLR protein. These are the NLRP3, NLRP1, and IPAF inflammasomes. Although numerous other NLR proteins have been identified, these are the only ones firmly established by genetic and biochemical experiments to have physiological roles. The two distinguishing features of these intracellular immune receptors are a NACHT nucleotide-binding domain and a C-terminal leucine-rich repeat region (LRR; Fig. 1). The NACHT domain belongs to a large superfamily of NTPase domains and is proposed to mediate ATP-dependent oligomerization of multiple NLR proteins. LRR regions form protein–protein interaction motifs found in a variety of proteins with diverse functions and consequently confer the sensor activity of the NLR protein. In addition to these NLR-dependent inflammasomes, double-stranded DNA was shown to trigger caspase-1 activation mediated by the HIN-200 protein AIM2.

The NLRP3/Cryopyrin Inflammasome

Of the three NLRP inflammasomes identified to date, NLRP3 remains the most extensively characterized owing to the diversity of its activating stimuli (Table 1). These include bacterial RNA, ATP, a range of PAMPs, pore-forming toxins, and structural aggregates such as monosodium urate and calcium pyrophosphate that are produced as by-products of a disease state such as gout. In addition, environmental exposure to crystalline agents such as asbestos and silica induce NLRP3-dependent inflammasome activation and consequently has been implicated in the etiology of asbestosis and silicosis. This observation has catalyzed much interest in the identification of how such structurally diverse ligands can activate a common inflammasome pathway. Three proposed models predominate: translocation of extracellular NLRP3 agonists into the cytosol to facilitate direct ligand–receptor interaction, lysosomal destabilization, and the existence of a common secondary messenger.

The efflux of potassium from the cell underpins the activity of the majority of NLRP3 agonists and represents a more general detection of perturbed cellular homeostasis. For example, ATP is a DAMP that triggers a sequence of events culminating in the opening of the hemichannel pannexin-1, which is recruited upon activation of the purinergic P2X7 receptor. Knockdown and pharmacological inhibition of pannexin-1 confirmed that this process is absolutely required for K⁺ efflux, efficient NLRP3-dependent caspase-1 activation, and IL-1 β secretion in response to LPS plus ATP. The formation of this pore also presumably enables the translocation of pathogens into the cytosol to activate the NLRP3 inflammasome by direct ligand–receptor interaction, although to date, no such interactions have been demonstrated. The lysosomal destabi-

Table 1 Inflammasome Activators

Inflammasome	Activators	Class
NLRP1	Anthrax lethal toxin	PAMP
	MDP	PAMP
NLRP3	Sendai	Virus
	Influenza A	Virus
	Adenovirus	Virus
	<i>Staphylococcus aureus</i>	Bacteria
	<i>Listeria monocytogenes</i>	Bacteria
	<i>Escherichia coli</i>	Bacteria
	<i>Mycobacterium marinum</i>	Bacteria
	<i>Neisseria gonorrhoeae</i>	Bacteria
	<i>Candida albicans</i>	Fungus
	MDP	PAMP
	Bacterial RNA	PAMP
	Lipopolysaccharide	PAMP
	poly(I:C)	PAMP
	Nigericin	PAMP
	Listeriolysin O	PAMP
	Aerolysin	PAMP
	Maitotoxin	PAMP
	α -Toxin	PAMP
	ATP	DAMP
	Hyaluronan	DAMP
Uric acid	DAMP	
Asbestos	DAMP	
Silica	DAMP	
β -Amyloid	DAMP	
Ipaf	Flagellin	PAMP
	<i>Salmonella typhimurium</i>	Bacteria
	<i>Legionella pneumophila</i>	Bacteria
	<i>Shigella flexneri</i>	Bacteria
	<i>Pseudomonas aeruginosa</i>	Bacteria
AIM2	Double-stranded DNA	PAMP

lization model posits that internalization of particulate matter by phagocytes causes rupture and cytosolic release of these agents, which are subsequently detected by the NLRP3 inflammasome [4].

However, in terms of accounting for the diversity of NLRP3 activators, the most attractive model is the existence of a common secondary messenger. It can be envisaged that this messenger would have similar properties to classical messengers such as cAMP, including rapid spatial and temporal regulation, diffusibility, and sensitivity to negative feedback. This may provide clues to aid in the identification of this messenger, which to date remains elusive. It is certainly intriguing that reactive oxygen species (ROS) scavengers were sufficient to block inflammasome activation by a range of NLRP3 agonists [5], but

the causal relationship remains unclear as macrophages lacking functional phagosomal NADPH oxidase did not exhibit augmented NLRP3 activation [4] and ROS has also been shown to inhibit caspase-1 activity [6]. Certainly these pathways to NLRP3 inflammasome activation need not be mutually exclusive, and further work is required to delineate the relative contribution of each to antimicrobial defense.

The IPAF Inflammasome

The absence of IPAF in macrophages results in profound resistance to inflammasome activation catalyzed by infection with Gram-negative bacteria such as *Salmonella typhimurium*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, or *Shigella flexneri* [1, 7]. With the exception of *S. flexneri*, the universal feature of these pathogens is flagellin, which is typically translocated into the cytosol by a bacterial secretion system and subsequently detected by Ipaf leading to the assembly of the Ipaf-dependent inflammasome. In addition to the dependence of non-flagellated *Shigella* on Ipaf for caspase-1 activation, the ability of mutated *P. aeruginosa* (PAK Δ fliC), which does not have flagellin, to activate caspase-1 confirms the existence of a flagellin-independent pathway to Ipaf-dependent inflammasome activation [7]. Discrepancies between in vitro and in vivo response of Ipaf-deficient macrophages to *S. typhimurium* infection further defined the existence of parallel pathways to caspase-1 activation in vivo. Whereas Ipaf-deficient macrophages exhibit exquisitely attenuated caspase-1 activation and IL-1 β secretion in vitro [1], mice given this pathogen orally are not more susceptible to infection than controls [8]. This is in contrast to the enhanced susceptibility of caspase-1-deficient animals to *S. typhimurium* infection [8], thus confirming the importance of integrated, multifaceted regulation, both positive and negative, for effective inflammatory responses in vivo.

The NLRP1 and AIM2 Inflammasomes

To date, the only drivers of NLRP1 inflammasome activation identified is the *Bacillus anthracis* lethal toxin (LT) and muramyl dipeptide (MDP) [9]. Initial clues regarding the role of NLRP1 came from the observation that various in-bred mouse strains are differentially sensitive to LT, which was linked to polymorphism of the NLRP1 locus [10]. Similarly to NLRP1, AIM2 has a highly restricted spectrum of activating stimuli, being involved in the detection of dsDNA. The HIN domain in AIM2's C terminus interacts directly with dsDNA, facilitating recruitment and activation of caspase-1 in an ASC-dependent manner via its N-terminal pyrin domain (Fig. 1).

Despite key similarities among NLR members, it is evident that a level of inflammasome regulation is conferred by the ability of NLRs to specifically detect different substrates. Conceivably, the nature of inflammatory response requires that the sum total of inflammasome activation in the cell be maintained below a threshold to prevent unwanted damage to peripheral tissue, in addition to maintaining sensitivity to negative regulation. Furthermore, the decision whether to undergo apoptosis or produce cytokines appears to be inflammasome specific. Ipaf-dependent inflammasome activation of caspase-1 results predominantly in the rapid induction of pyroptosis, an inflammation-associated form of cell death, whereas NLRP3-dependent inflammasome activation results predominantly in processing of IL-1 β and IL-18. Again, this provides a division of labor across multiple cellular processes, presumably to prevent the failure to induce an inflammatory response to all known pathogens if any one process is deregulated. Another issue of critical importance is whether there is reciprocal regulation of different inflammasomes to confer substrate specificity, for example, does engagement of the NLRP3 inflammasome result in active neutralization of the IPAF-dependent inflammasome or vice versa? This may be conferred at the level of subcellular localization as inflammasome components are also known to shuttle between compartments or perhaps due to differential tissue expression patterns as has been demonstrated for NLRP1 and NLRP3 in human tissues [11].

Once a productive immune response has been mounted, the restraint of pro-inflammatory cytokine and chemokine production becomes the primary goal. Inflammasome activity can be suppressed by protein–protein interactions, including the association of the NACHT domain of the NLR protein with its LRR region prior to ligand recognition or sequestration of inflammasome components by CARD- and PYD-containing proteins. For example, the PYD-domain-containing Pyrin protein has a clear function as a negative regulator of inflammasome activation as individuals containing mutations of this protein have a disorder called familial Mediterranean fever (Table 2), and mice with targeted disruption of the C-terminal portion of PYRIN exhibit increased endotoxin sensitivity and caspase-1 activation [12]. Biochemically, this inhibition is based on the interaction of the SPRY domain of PYRIN with NLRP3 and caspase-1 leading to attenuation of IL-1 β secretion [13]. CARD-domain-containing proteins with negative regulatory capacities include CARD-only protein, ICEBERG, and inhibitor of CARD (INCA), and again function by disrupting protein–protein interactions required for inflammasome assembly. A thorough understanding of these negative regulatory mechanisms will provide invaluable guidance for the rational design of novel anti-inflammatory therapeutics.

Table 2 Human Diseases Associated with Deregulated Inflammasome Activity

Condition	Symptomatology	Causal mutation(s) affecting inflammasome
Cryopyrinopathies		
Familial hereditary periodic fever syndromes		
Muckle–Wells syndrome (MWS)	Fever, hives, sensorineural hearing loss and arthritis	Mutations in CIAS1 (gene encoding NLRP3) or the promoter region
Familial cold auto-inflammatory syndrome (FCAS)	Cold-induced fevers, skin rash	Mutations in CIAS1 (gene encoding NLRP3) or the promoter region
Neonatal onset multisystem inflammatory disease (NOMID)	Fever, hives, chronic aseptic meningitis	Mutations in CIAS1 (gene encoding NLRP3) or the promoter region
Familial Mediterranean fever (FMF)	Fever, abdominal or chest pain, arthritis	Mutations in MEFV (gene encoding pyrin)
Protein misfolding/aggregation diseases		
Alzheimer's disease	Cognitive impairment	None identified; environmental + disease susceptibility
Gout and pseudogout	Joint swelling and pain, limited movement, and red skin surrounding the affected joint	None identified; environmental + disease susceptibility
Autoimmune diseases		
Generalized vitiligo	Loss of pigmentation due to melanocyte destruction	Genetic variants of NLRP1
Type I diabetes	Fatigue, excessive thirst, weight loss	Genetic variants of NLRP1
Other		
Guadeloupe variant periodic fever syndrome	Cold-induced heterogeneous symptoms including fever, joint or muscle pain, sensorineural hearing loss, ulcers, and lymphadenopathy	Mutations in FCAS2 (gene encoding NLRP12)
Crohn's disease	Abdominal pain, fever, diarrhea	Genetic variants of ATG16L1; polymorphism of Nod2

Exploiting Knowledge of Inflammasome Activation for the Treatment of Auto-inflammatory Disorders

The host response to inflammatory stimuli is usually self-limiting to prevent damage to neighboring tissues, but in conditions such as familial periodic fever syndromes (FPFS) and Muckle–Wells syndrome (MWS), there is a persistent and rampant inflammatory response (Table 2). These disorders are routinely caused by gain-of-function germline mutations in the NLR signaling pathway, most notably NLRP3. Approximately 60 disease-associated mutations have been shown to cluster around the NACHT domain, suggesting that alteration of the binding properties results in spontaneous, deregulated assembly of the inflammasome and production of mature IL-1 β . Indeed, in the basal state, monocytes from MWS patients secrete more mature IL-1 β [14], and mice harboring mutations of NLRP3 equivalent to the human disease exhibit hyperactive inflammasome leading to unrestrained IL-1 β production [15].

Emergent data also identifies a potential link between unscheduled NLRP3 activation and the pathogenesis of Alzheimer's disease. Aggregates of extracellular β -amyloid are internalized by microglia leading to lysosomal damage, activation of the NLRP3 inflammasome, and IL-1 β production [16]. Genetic variants of the autophagy gene

ATG16L1 are associated with the inflammatory bowel disorder Crohn's disease, and disruption of this gene in mice leads to hyper-responsiveness to endotoxin-induced IL-1 β production [17] as well as perturbed homeostasis of Paneth cells of the intestine [18]. It remains to be determined whether the autophagy pathway functions at the level of inflammasome assembly and/or activation or whether its primary role is to regulate the nonclassical secretion of mature IL-1 β . Diseases associated with metabolic disturbance such as gout and pseudogout have also been linked to deregulated inflammasome activity [19]. In the case of type II diabetes, glucotoxicity and lipotoxicity induce oxidative stress, pro-inflammatory cytokine production, and systemic inflammation leading to β -islet cell destruction and insulin resistance [20]. Collectively, these observations underscore the potential benefit of developing therapies that neutralize the inflammasome.

Currently, therapeutics that neutralize downstream effectors of inflammasome activity such as IL-1 β show remarkable promise for the treatment of these disorders. Patients with FPFS and gout [19] respond to IL-1 receptor antagonists, including recombinant IL-1R (anakinra and rilonacept) and neutralizing human anti-IL-1 β antibody (canakinumab), and interestingly, even diseases with complex etiology such as type II diabetes have been effectively

treated with anakinra [20]. In addition, these are the only compounds currently approved by the Food and Drug Administration for the treatment of cryopyrin-associated periodic syndromes and rheumatoid arthritis [21]. These products downregulate the inflammatory response by “mopping up” inflammatory cytokines; however, although IL-1 β is certainly the most potent, it is not the sole cytokine produced by inflammasome activation. IL-18 is also produced in response to inflammasome activation [22] and influences inflammation in a different manner to IL-1 β , most prominently by inducing a T helper type 2 response [23]. This provides a link between the innate and adaptive immune response and, therefore, represents an attractive node for therapeutic targeting with compounds that neutralize components upstream of inflammasome activation. Conceivably, this approach could entail neutralization of the putative secondary messenger and/or prevention of ligand–receptor binding; however, this awaits experimental verification.

Translating knowledge of the molecular basis for a disease to rational drug design, in most cases, leads to improved therapeutic index and reduced side effects. Much is now known about what activates inflammasomes, how inflammasomes assemble and become activated, and what negative regulatory mechanisms exist to prevent the deleterious effects of unrestrained pro-inflammatory cytokine production. This provides a unique opportunity to develop targeted therapeutics that can be used to treat the variety of human disorders associated with deregulated inflammasome activity.

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