The Inflammasome in Lung Diseases.

Authors:

Gimena dos Santos¹, Mikhail Kutuzov¹ and Karen M. Ridge¹,².

¹Division of Pulmonary and Critical Care Medicine. Feinberg School of Medicine. Northwestern University. Chicago, IL. USA.

²Jesse Brown VA Medical Center. Chicago, IL. USA.

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Corresponding author: Karen M. Ridge, PhD.


Division of Pulmonary and Critical Care Medicine. Feinberg School of Medicine. Northwestern University. Chicago, IL. 60611. USA.

E-mail: kridge@northwestern.edu

Telephone: (312) 908-8163

Fax: (312) 503-0411

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Abstract

Inflammation, the process aimed at restoring homeostasis after an insult, can be more damaging than the insult itself if uncontrolled, excessive or prolonged. The inflammasome is an intracellular multimeric protein complex that regulates the maturation and release of proinflammatory cytokines of the IL-1 family in response to pathogens and endogenous danger signals. Growing evidence indicates that the inflammasome plays a key role in the pathogenesis of both acute and chronic respiratory diseases. The inflammasome can be activated by the pathogens that account for the most prevalent infectious diseases of the respiratory tract, such as Influenza A virus, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*. The inflammasome also plays a role in the chronic inflammation of the airways of patients with asthma and COPD, as well as in the initiation and progression of the inflammatory process seen in pulmonary fibrosis. The aim of this review is to summarize the most relevant points of the inflammasome activation in lung diseases.

Key words: innate immunity, interleukin-1β, respiratory diseases.
Introduction

Inflammation is an adaptive response to noxious stimuli (41). The innate immunity comprises a system of germline-encoded receptors that inspect both the intracellular and extracellular compartments for signs of infection and recognize highly conserved microbial motifs or ‘Pathogen Associated Molecular Patterns’ (PAMPs). These Pattern-Recognition Receptors (PRRs) are expressed by cells at the front line of host defense against infection, such as macrophages, monocytes, dendritic cells, and epithelial cells. PRRs are not clonally distributed; therefore, all cells expressing PRRs immediately identify PAMPs-expressing microbes as a potential threat (34). Membrane-bound toll-like receptors (TLRs) and C-type lectins (CTL) are the PRR that probe the extracellular milieu and the endosomal compartments for PAMPs, while the cytosol is constantly scanned by intracellular nucleic-acid sensors like AIM2 and RIG-like helicases. Activation of these receptors causes proinflammatory cytokine production and type I interferon-dependent antiviral responses, via the transcription factor NF-κB (53).

NOD-like receptors (NLRs) are a particular type of intracellular PRRs that recognize both PAMPs and host derived signals named DAMPs (Danger Associated Molecular Patterns). NLRs are composed of a conserved central domain that mediates nucleotide binding and oligomerization (NACHT, NOD or NBS domain), a C-terminal leucine-rich domain (LRR) that senses NLRs agonists and has an auto-inhibitory effect in their absence (14), and an N-terminal region required for protein-protein interaction. Human NLR gene family is composed of 22 members, and depending on their N-terminal domains they are classified into 4 subfamilies: NLRA, NLRB, NLRC and NLRP. NLRA contains an acidic transactivation domain; NLRB contains a baculovirus inhibitory repeat domain, NLRC a caspase recruitment domain (CARD) and NLRP a pyrin (PYD) domain. Activation of certain NLRs (NLRP1, NLRP3 and NLRC4)
leads to the assembly of the **inflammasome**, a high-molecular-weight platform for the activation of caspase-1 which is required for the proteolytic maturation and release of proinflammatory cytokines IL-1β and IL-18 (15, 28, 42, 51).

**Historical perspective**

The link between mutations in NLR genes and inflammatory diseases was established in 2001 by Hoffmann and colleagues (24) when they described mutations in NLRP3 in individuals affected by Muckle-Wells syndrome (MWS), a rare autoinflammatory disease characterized by recurrent episodes of fever and rash associated with ocular and articular manifestations (46). In 2002 Tschopp and colleagues (38) described for the first time an inducible high molecular weight complex containing NLRP3, an adaptor protein (ASC) and proinflammatory caspases which they called the inflammasome. Two years later they demonstrated that constitutive production of active IL-1β observed in MWS was the molecular basis of the NLRP3 inflammasome-dependent disorders (2). In 2004, Dixit and colleagues (36) demonstrated the requirement of the adaptor ASC within the inflammasome, since macrophages from ASC-/- mice were unable to produce mature IL-1β and IL-18 when challenged with ATP. Moreover, ASC-/- mice also showed defective caspase-1 dependent cell death, establishing a molecular link between inflammation and cell death pathways.
The NLRP3 inflammasome

The NLRP3 inflammasome is the best characterized (51) and participates in immune responses to infectious and non-infectious agents. It consists of the aforementioned NLRP3 receptor, the adaptor protein ASC and caspase-1 (Figure 1).

ASC (apoptosis-associated speck-like protein containing a CARD domain), also known as Pycard, is an adaptor protein that bridges NLRP3 and caspase-1. In resting monocytes and macrophages ASC is sequestered in the nucleus; inflammatory stimuli induce ASC redistribution to the cytosol (9) where it interacts with NLRP3 through its pyrin domain and with caspase-1 through its Caspase Activation and Recruitment Domain (CARD).

Caspases are cysteine proteases involved in either inflammatory or apoptotic pathways (57). Caspase-1, the most fully characterized of the proinflammatory caspases, is synthesized as an inactive zymogen. Upon appropriate stimulation, NLRP3 recruits ASC and pro-caspase 1 to form the inflammasome, where pro-caspase 1 is cleaved into a p10 and p35 fragment. The latter is subsequently processed into the p20 subunit and its CARD, and two molecules of p20 heterodimerize with two molecules of p10 to form the mature and active enzyme (38, 50). Active caspase 1 in turn, cleaves the precursors of two potent proinflammatory cytokines IL-1β and IL-18 in the cytoplasm, which are then released to the extracellular milieu in a yet undefined mechanism (35, 47).

NLRP3 inflammasome can be activated by multiple stimuli: whole pathogens (bacteria, viruses, fungi), PAMPs, DAMPs (extracellular ATP, monosodium urate crystals) and environmental irritants (silica, asbestos, UVB radiation). Two signals are thought to be required for activation: a ‘priming signal’ that induces both the transcription of pro-IL1β and pro-IL-18 and the expression
of NLRP3 after TLR stimulation (29), and a ‘second signal’ that activates the inflammasome. However, the molecular mechanism that triggers inflammasome assembly remains unclear (Figure 2). One of the proposed models of activation is P2X7 receptor-dependent pore formation by pannexin-1 hemichannel in response to extracellular ATP and potassium efflux, which allows extracellular PAMPs and/or DAMPs to access the cytosol and directly activate NLRP3 (10, 27). Another model is lysosomal rupture. After engulfment of particulate or crystalline agonists such as silica and asbestos, phagosomal destabilizes and releases its content in the cytosol, which is sensed by NLRP3 and causes inflammasome activation (25). Finally, reactive oxygen species (ROS) appear to be a crucial event upstream of inflammasome assembly (66). ROS are sensed by thioredoxin and thioredoxin-interacting protein (TXNIP) complex, causing dissociation of the complex and subsequent binding of TXNIP to NLRP3. This leads to recruitment of ASC and pro-caspase-1 by NLRP3 and assembly of the inflammasome (65). A recent study showed that ROS act as priming signals required for transcriptional upregulation of NLRP3 rather than its oligomerization (5).

Other inflammasomes

Other inflammasomes have also been characterized, such as NLRP1, NLRC4 and AIM2 inflammasomes (Figure 3, Table 1). The NLRP1 inflammasome, the first to be described, is activated by anthrax lethal toxin (7). Unlike most NLRs, NLRP1 activates caspase-1 directly and ASC is not required to produce mature IL-1β (20). The NLRC4 or IPAF inflammasome is activated by cytosolic flagellin or by the basal body rod component of the type 3 secretion.
system found in *Salmonella typhimurium, Shigella flexneri, Legionella pneumophila* and *Pseudomonas aeruginosa* (55). Differences from the NLRP3 and NLRP1 inflammasomes include the ability to activate caspase-1 without the adaptor ASC (56), and the constitutive expression of NLRC4 without a TLR-mediated priming signal (29). Finally AIM2, a PRR that senses cytosolic dsDNA (Figure 2), is also capable of forming inflammasomes. Since ligand requirements for AIM2 include dsDNA from virus, bacteria or the host itself, it may also contribute to autoimmune responses (49).

**Regulation of inflammasome activity**

Inflammasome activity requires precise regulation to avoid an excessive production of cytokines and it deleterious effects. Regulation takes place at transcriptional and post-transcriptional levels. For instance, NLRP3 is expressed at limited levels in macrophages and is highly inducible in response to proinflammatory stimuli such as LPS, cytokines or ROS (5). Moreover, differential splicing of ASC can generate protein variants with an inhibitory function instead of the classical adaptor molecule (8). Another level of regulation is the subcellular location of the inflammasome components; one example is the aforementioned redistribution of ASC from the nucleus to the cytoplasm in activated inflammatory cells (9). Additional regulation of the inflammasome activity can be achieved by secreted factors. In fact, type I interferons can suppress inflammasome activation and its subsequent production of IL-1β, which may contribute to the increased risk of secondary bacterial infections after influenza or other viral infections (23). Autophagy, the lysosomal-mediated process required to maintain cell homeostasis in response to
stress, also plays a role in the regulation of inflammasome activity. Actually, Saitoh and colleagues demonstrated that cells deficient in autophagy-specific proteins have an enhanced inflammasome activation in response to stimuli (52) which can be explained by the increase in cellular ROS levels caused by insufficient clearance of defective mitochondria (66). Another possible explanation to this phenomenon is the cytosolic translocation of mitochondrial DNA in dysfunctional mitochondria, which could not only engage AIM2 inflammasome but also signal downstream an AIM2-independent pathway (45).

The role of the inflammasome in lung diseases.

Lung Infections

Community-acquired pneumonia (CAP) is the most common cause of severe sepsis and the leading cause of death from infection in the United States (60). Since the mortality rate from CAP has not changed significantly in the past four decades (44), a thorough comprehension of its pathogenesis is mandatory to find both a suitable treatment and effective vaccines.

Many bacterial pathogens that can cause CAP have been shown to activate the NLRP3 inflammasome, the most common mechanism involving the secretion of pore-forming toxins. The virulence of Streptococcus pneumoniae, the leading cause of life-threatening infections such as CAP, meningitis, and sepsis, depends on the polysaccharide capsule and pore-forming toxins such as cytolysin pneumolysin (PLY) (61). The disruption of the plasma membrane caused by PLY induces potassium efflux, one of the proposed mechanisms of activation of the NLRP3
inflammasome. Listeriolysin O, a pore-forming toxin produced by *Listeria monocytogenes*, and the α-hemolysin toxin produced by *Staphylococcus aureus* activate the NLRP3-inflammasome in human and mouse monocytic cell a similar manner (13, 37).

Infection by certain viruses also results in inflammasome activation. Double-stranded DNA viruses can activate AIM2 inflammasome while both DNA and RNA viruses can trigger the assembly of NLRP3 inflammasome. Influenza A virus (IAV), a major cause of lung infections and mortality, is known to activate the NLRP3 inflammasome (4, 58, 62) but the mechanism is currently unclear. Recent reports indicate that IAV ion channel M2, which is involved both in fusion during viral entry and in synthesis of new virions, can trigger inflammasome assembly and activation (26, 48). Infection of mice with IAV induces secretion of ATP into the bronchoalveolar lavage fluid (1) and ATP released from influenza-infected dying cells may also trigger the inflammasome-dependent response (1).

Active tuberculosis is primarily a disease of the lung but it can progress to a generalized inflammatory disease. *Mycobacterium tuberculosis* (MTB) is a peculiar pathogen because it resides within a phagosome-like compartment of host macrophages during infection where it can suppress inflammasome activation (39). However, MTB has the ability to activate the NLRP3 inflammasome depending on the expression of a functional protein secretion system (ESX-1). (17) The pathways that lead to inflammasome activation in this pathogen are not completely understood, but the export of ESAT-6 (Early Secreted Antigenic Target, 6 kDa, a family of small proteins secreted by *Mycobacterium tuberculosis*) via the mentioned ESX-1 appears to be of critical importance. (43)
Airway Diseases

Although the incidence and severity of both asthma and Chronic Obstructive Pulmonary Disease (COPD) is increasing worldwide (32) there is currently no treatment to slow the progression of the latter and there is still a significant group of asthmatics resistant to available therapies. Hence, understanding the pathophysiology of these diseases to find effective therapies is of prime importance. Chronic inflammation of the airway is the common feature of both diseases and there is a growing body of evidence suggesting a role for the NLRP3 inflammasome in the pathogenesis of this inflammation.

Extracellular ATP is strongly and persistently upregulated in the airways of patients with COPD (31) and this is associated with a decline in lung function (12) and with an increase in airway infiltration by inflammatory cells. As we previously mentioned, extracellular ATP activates the NLRP3 inflammasome by engaging the purinergic receptor P2X7. This receptor is also upregulated in alveolar macrophages and blood neutrophils from patients with COPD (31) as well as in blood and airway neutrophils and alveolar macrophages in the mouse model of lung inflammation induced by cigarette smoke (CS) (33).

The role of the inflammasome components NLRP3 and ASC in CS-models has not been fully investigated. However, there is evidence of inflammasome activation since levels of caspase-1 are increased in lung tissue after CS challenge in mice. Caspase-1 levels are also higher in lung tissue from COPD patients and smokers compared to non-smoking donors (19). Selective inhibition of caspase-1 significantly decreased inflammation after CS challenge in animal models (11). This data supports a role for the inflammasome in the airway inflammation in COPD and asthma, although further studies are required.
The role of NLRP3 inflammasome in the development of allergic airway disease is currently controversial. As with COPD, ATP levels are also elevated in the airways of asthmatic patients and in animal models in response to allergen challenge (6). Moreover, IL-1β levels are higher in serum of asthmatic than non-asthmatic patients (59) and induced sputum contain higher levels of IL-1β in symptomatic than in non-symptomatic asthmatics, suggesting a putative role of the NLRP3 inflammasome in both chronic disease and acute exacerbations. However, in a recent report Allen et al (3) failed to see a difference in clinical outcome of four allergic asthma models in NLRP3-/- mice as compared to controls.

**Pulmonary Fibrosis**

The term ‘pulmonary fibrosis’ includes a broad range of lung disorders characterized by progressive and irreversible destruction of normal lung architecture by excessive accumulation of collagen and other extracellular matrix (ECM) components in basement membranes and interstitial tissues. ECM expansion impairs effective gas exchange and leads to death due to extreme respiratory failure (30). However, the pathophysiologic mechanisms underlying pulmonary fibrosis are not completely understood.

The importance of inflammation in initiation and progression of pulmonary fibrosis has been previously established (63). Silica, asbestos and bleomycin, among other irritants, can injure lung epithelial cells and activate the NLRP3 inflammasome in macrophages, leading to IL-1β secretion (18, 21, 25). IL-1β secretion is also increased in macrophages treated with pravastatin, as demonstrated by Xu and colleagues suggesting a role for statins in pulmonary fibrosis (64). IL-1β promotes production of TGF-β, the most potent and ubiquitous profibrotic cytokine (30) that
triggers activation, proliferation and transdifferentiation of epithelial cells and resident fibroblasts into collagen-producing myofibroblasts. IL-1β also promotes the secretion of neutrophils-attracting CXC chemokines and platelet-derived growth factor (PDGF), the former favoring the influx of neutrophils and exacerbating the damage to epithelial cells, and the latter further inducing fibrosis. Finally, both TGF-β and IL-1β can increase the expression of plasminogen activator inhibitor 1 (PAI-1) which inhibits ECM degradation, promotes recruitment of more inflammatory cells (67) and suppresses the release of antifibrogenic growth factors (30). The initial proinflammatory scenario of inflammasome activation can rapidly progress to a profibrotic one, leading to the chronic and devastating disease.

**Acute Respiratory Distress Syndrome (ARDS)**

Acute lung injury and its most severe form, ARDS, is a frequent complication of patients admitted to the intensive care unit and is associated with a mortality of approximately 25 % (54). ARDS can be triggered by different disorders such as pneumonia, sepsis, isquemia and trauma; however, there is neither an effective tool to predict patients’ susceptibility to ARDS nor an effective therapy. Pathophysiologically, the disease is characterized by dysregulated inflammation, with excessive permeability of epithelial and endothelial barriers leading to lung edema formation and severe hypoxemia (40). Inflammasome-regulated cytokines appear to play a major role in the development of ARDS, as demonstrated by Dolinay and colleagues (16), since increases in the levels of circulating IL-18 in critically ill patients correlate with disease severity and mortality, even after adjusting for important confounders such as Acute Physiology and Chronic Health Evaluation (APACHE) II score.
Conclusion

The lung is exposed to a variety of insults that can be detected by different PRRs, such as microbial molecules (LPS, flagellin), inhaled particles (asbestos, silica) as well as cell injury-associated endogenous molecules (ATP and K+). The activation of different PRRs and the resultant signaling pathways play a critical role in host protection and in the pathology of lung diseases. New reports (21) now implicate the inflammasome in the sensing of dangers/stress signal, leading to the increased levels of active IL-1β. Further, the role of IL-1β as a critical inflammatory mediator of acute inflammation and tissue remodeling has been well established (22). Over the last ten years, the discovery and characterization of the inflammasome has led to a comprehensive insight in the innate and adaptive immune responses to different lung insults. However, our knowledge of activation and regulation of the inflammasome is still incomplete. Further understanding of the inflammasome complex would be advantageous in the development of new treatments modalities in acute lung injury and chronic lung disease.
References


**Figure captions**

**Figure 1:** **NLRP3 inflammasome.** Upon proper stimulation (1) NLRP3 receptor recruits pro-caspase-1 through the adaptor protein ASC to form the inflammasome (2). Within the inflammasome pro-caspase-1 goes autocatalytic processing resulting in active caspase-1 (3), which in turn cleaves the precursors pro-IL-1β and IL-18 into the mature and active forms (4).

**Figure 2:** **Activation of the inflammasome.** Maturation and secretion of IL-1β requires two signals: the first signal (‘priming signal’) leads to the synthesis of pro-IL-1β, pro-IL-18 and other components of the inflammasome such as NLRP3; the second signal results in the assembly of the inflammasome, activation of caspase-1 and release of mature cytokines IL-1β and IL-18 to the extracellular milieu. Currently, the nature of the second signal is debated. The three proposed models of activation are depicted in this figure: 1) extracellular ATP activates P2X7 receptor and causes subsequent recruitment of pannexin-1 hemichannel to the plasma membrane, leading to K⁺ efflux; 2) lysosomal rupture after engulfment of crystalline or particulate agonists; and 3) ROS that both upregulates NLRP3 expression and the activation of the inflammasome.

**Figure 3:** **Human inflammasome-related proteins.** NLRPs contain an N-terminal PYD, a conserved NACHT and a C-terminal leucine-rich repeat domains. NLRP1 also contains a C-terminal CARD. NLRC4 contains an N-terminal CARD that interacts directly with caspase-1. AIM2, a DNA-interacting human interferon-inducible 200 protein (HIN200) with an N-terminal
PYD, can also form a caspase-1-activating inflammasome. With the exception of NLRP1 that can bind directly to pro-caspase 1 and does not require the adaptor protein ASC, inflammasomes are also composed of ASC and caspase-1.
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Agonist

1. NLRP3
2. ASC
3. Pro-Caspase 1
4. Active Caspase-1

Pro-IL-1β Pro-IL-18
IL-1β IL-18

Inflammasome