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NLRs and the danger of pollution and aging

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Abstract

Inflammatory interleukin 1 β production after silica and alum crystal or β -amyloid uptake occurs via a process involving lysosomal destabilization and release of cathepsin B that activates the NALP3 inflammasome.

Members of the mammalian nucleotide-binding domain, leucine-rich repeat-containing (NLR) family of proteins have emerged as key regulators of inflammation. Within this family much attention has focused on NLRP3 (cryopyrin, NALP3). Naturally occurring mutations in NLRP3 are linked to a trio of dominantly inherited auto-inflammatory disorders characterized by the excessive production of interleukin 1 β (IL-1 β)¹. This highly inflammatory cytokine is normally stored within cells as inactive pro-IL-1 β , which, upon stimulation of the cells, is rapidly cleaved enzymatically by activated caspase-1. NLRP3 and the adaptor protein ASC are core constituents of a caspase-1-activating complex called the ‘inflammasome’. Once activated, caspase-1 cleaves and activates pro-IL-1 β and pro-IL-18, which then elicit inflammation upon release from the cell². To date mainly microbial pathogen-associated molecules and toxins have been identified to stimulate activation of the NLRP3 inflammasome and IL-1 β secretion. However, a growing number of host-derived molecules that alert the immune system to cell injury and tissue damage have also been identified as key triggers of NLRP3 inflammasome activation. These molecules, termed damage-associated molecular patterns (DAMPs), include ATP and gout associated monosodium urate (MSU) crystals. Two reports in this issue of *Nature Immunology*, by Hornung *et al.* and Halle *et al.*, now expand the list of NLRP3 activators to include environmental and neurodegenerative stimuli^{3, 4}. Moreover, these reports identify lysosomal permeabilization and rupture, followed by the subsequent release of cathepsin B, as a common pathway that leads to IL-1 β release via the NLRP3 inflammasome.^{3, 4} (Fig.1)

Although many NLRP3 activators have been identified, it remains unclear how these diverse stimuli trigger NLRP3 activation. One model posits that NLRs, like some Toll-like receptors (TLRs) directly interact with pathogen associated molecular patterns (PAMPs); another model posits that these divergent stimuli are detected through indirect pathways. The latter mode of detection seems to be the case for a majority of nucleotide-binding site–leucine-rich repeat (NBS-LRR) proteins in plants, a related set of structurally conserved proteins required for pathogen detection, as few NBS-LRR proteins bind directly to pathogen derived molecules. Instead most NBS-LRRs are triggered by host proteins that have been ‘preferentially’ modified by pathogen-derived molecules. In this manner, NBS-LRRs act as ‘guard’ proteins capable of detecting a broad spectrum of stimuli by monitoring relatively few host targets.⁵ Based on the mechanisms found in plants, it is possible that the NLRP3 inflammasome is also activated through a common pathway that lies downstream of diverse stimuli. The two papers in this issue provide evidence to support that hypothesis.

In the first report, Hornung *et al* investigate the mechanisms governing silica induced inflammation. Long-term, typically occupational exposure to silicon dioxide (silica) particles results in silicosis, a progressively debilitating respiratory condition characterized by pulmonary inflammation and fibrosis. Following inhalation, silica crystals accumulate within the lung airways, whereupon they are engulfed by resident macrophages. This results in the production of several proinflammatory cytokines, including IL-1 β . Though first recognized by Bernardino Ramazzini in 1705, the molecular mechanisms governing silica-induced inflammation have yet to be fully elucidated.

Hornung *et al.* demonstrate that silica crystals are potent activators of inflammation *in vitro*, as stimulation of LPS-primed human peripheral blood mononuclear cells elicited a dose dependent release of IL-1 β . To investigate the *in vivo* relevance of these results, Hornung *et al.* trans-orally challenged mice with silica crystals. In contrast to wild-type mice, mice lacking the IL-1 receptor demonstrated dramatically decreased neutrophil recruitment to the lung, indicating that silica induced IL-1 β further perpetuates the development of inflammation *in vivo*. To determine which NLR family members are involved in the innate immune response to silica crystals, Hornung *et al.* stimulated macrophages isolated from NLRP3- and ASC-deficient mice. Their results are similar to two very recent reports in that both NLRP3 and ASC are required for caspase-1 activation and IL-1 β release in response to silica^{6, 7}. Hornung *et al.* further show that this same pathway is important in mediating caspase-1 activation and IL-1 β release induced by aluminium hydroxide salt (alum), a common adjuvant used in human vaccines. This finding agrees with recent findings from two other groups^{8, 9}. Furthermore, by utilizing cytochalasin D to disrupt actin filaments, Hornung *et al.* demonstrate that phagocytosis of silica, MSU, or alum crystals is required to activate the NLRP3 inflammasome, which also agrees with published observations^{3, 4}.

However, the report by Hornung *et al.*⁴ provides evidence at odds with previous publications of Dostert *et al.*⁶ and Cassel *et al.*⁷ on the requirement of reactive oxygen species (ROS) in crystal induced activation of the NLRP3 inflammasome. In their report, Dostert *et al.* proposed that ROS generated by NADPH oxidase upon crystal phagocytosis are responsible for activation of the NLRP3 inflammasome⁶. Using pharmacological inhibitors of NADPH oxidase, ROS detoxifiers, and shRNA to 'knockdown' the p22phox subunit of NADPH oxidase, Dostert *et al* show decreased caspase-1 activation and IL-1 β processing in cells exposed to asbestos, MSU or silica crystals⁶. Similar results based on pharmacological inhibition of NADPH oxidase were observed by Cassel *et al.*⁷. In contrast to those studies, Hornung *et al.* evaluate the contribution of ROS to NLRP3 activation by utilizing macrophages isolated from mice lacking the gp91phox subunit of NADPH-oxidase and find no reduction in caspase-1 activation or IL-1 β production after stimulation with either silica or MSU crystals. As different methodological approaches were used in these three studies, additional work will be required to resolve the discrepancy.

In the other report in this issue of *Nature Immunology*, Halle *et al* demonstrate that NLRP3 is also activated by fibrous particles of β -amyloid³. Insoluble β -amyloid aggregates have long been associated with Alzheimer's disease, a neurodegenerative disorder characterized by progressive memory loss, cognitive impairment, and dementia. Aggregates of misfolded β -amyloid protein arise from proteolytic processing of the amyloid precursor protein (APP) and are typically observed as extracellular 'senile plaques'. These plaques trigger the production of inflammatory cytokines, including IL-1 β , following phagocytosis by resident microglia and infiltrating macrophages. Halle *et al* demonstrate that fibrillar β -amyloid elicited caspase-1 cleavage and IL-1 β production in wild-type microglia and macrophages and that the NLRP3 inflammasome is required for this response³. Interestingly, the inflammatory cytokines TNF and nitric oxide (NO) were reduced in NLRP3- and caspase-1-deficient cells following β -amyloid stimulation, suggesting that IL-1 β release from wild-type cells acts in an autocrine

fashion to amplify inflammation through the induction of other proinflammatory mediators. In all cases, non-fibrillar β -amyloid failed to activate the NLRP3 inflammasome, indicating that the structural properties of β -amyloid influence its inflammatory potential. Relevance *in vivo* was demonstrated by the stereotactic injection of β -amyloid into the striatum of mice. Microglial accumulation and activation was reduced in ASC- and caspase-1-deficient animals, although the role of NLRP3 was not directly examined.

While the appearance of several recent reports on activation of the NALP3 inflammasome undoubtedly advances our understanding of the mechanisms underlying the inflammation associated with silicosis, alum adjuvant activity, and Alzheimer's disease, the two papers in this issue *Nature Immunology* provide an additional significant finding: activation of the NLRP3 inflammasome by all of these stimuli is mediated by lysosomal 'destabilization' or permeabilization. The authors in both studies conclude that NLRP3 activation by lysosomal destabilization requires phagocytosis of the crystals or β -amyloid fibrils because treatment with cytochalasin D prevented lysosomal damage and NLRP3-mediated IL-1 β release. Moreover, the studies show that permeabilization of lysosomes results in the release of the endosomal-lysosomal protease cathepsin B into the cytoplasm. Treatment with the cathepsin B specific inhibitor Ca-074-Me dramatically decreased the amount of IL-1 β elicited by both stimuli, suggesting that the cytoplasmic localization and proteolytic activity of cathepsin B plays a role in activating the NLRP3 inflammasome. As cathepsin B was previously shown to promote pyro necrosis, a necrotic-like form cell death initiated by NLRP3^{10, 11}, the new results here indicate that cathepsin B plays even a more intricate role in NLRP3 inflammasome activation than previously thought, and may represent a viable target for therapeutic intervention to ameliorate NLRP3-mediated inflammation.

In an additional key experiment Hornung *et al* demonstrate that lysosomal rupture alone is sufficient to activate the NLRP3 inflammasome⁴. In the absence of silica crystals or any other known NLRP3 stimuli, the authors show that osmotic or chemical permeabilization of lysosomes activates caspase-1 cleavage and IL-1 β production; this 'sterile' lysosomal rupture did not result in IL-1 β release in macrophage lacking NLRP3 and ASC. These results support the notion that silica crystals, rather than being detected directly by NLRP3, are likely being 'sensed' indirectly by NALP3 by 'monitoring' lysosomal integrity. These data, in combination with the work by Halle *et al*, illuminate a mechanism by which many diverse stimuli activate the NLRP3 inflammasome and elicit IL-1 β release. This mode of 'indirect' activation is similar to activation of many plant NBS-LRR proteins: one intracellular sensor responds to many cellular insults by detecting a common 'activating target' elicited 'downstream' of the molecules that similarly provoke the stimulus.

These reports raise several interesting questions. First, how does cathepsin B activate NLRP3? It is currently unknown if these proteins directly interact or how cathepsin B may regulate either the formation or activity of the inflammasome. However, given the requirement for cathepsin B proteolytic activity, it is likely that NLRP3 activation involves the processing of one or more inflammasome associated components. It is worth noting that several plant NBS-LRR proteins are activated following proteolytic cleavage of an associated host protein following pseudomonas infection.¹² A second question involves the mechanisms of lysosomal permeabilization. Another report demonstrated that the pore forming protein pannexin-1 can transport extracellular NLRP3 stimuli into the cytoplasm following ATP binding by P2X7 receptors.¹³ It is possible that ion translocation, particularly potassium efflux, through these same pannexin pores may initiate lysosomal permeabilization, thereby facilitating the delivery of cathepsin B into the cytoplasm. Finally, in the case of all NLRP3 activating stimuli, future studies will need to address the *in vivo* consequences of NLRP3-mediated inflammation on pathogenesis and whether therapeutic intervention to inhibit NLRP3 is ultimately beneficial to the host.

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Figure 1. Lysosomal permeabilization activates the NLRP3 inflammasome

Phagocytosis of silica crystals, aluminum salts, or β -amyloid fibrils results in lysosomal permeabilization (dashed red line). This causes the release of the lysosomal protease cathepsin B into the cytoplasm which contributes to NLRP3 activation through a yet unknown mechanism. Once activated, NLRP3 initiates cell death and formation of a caspase-1 activating complex termed an “inflammasome”. Processed caspase-1 then cleaves and activates pro-IL-1 β , which elicits inflammation upon release from the cell. PYR, pyrin domain; NBD, nucleotide binding domain; LRR, leucine-rich repeats; CARD, caspase recruit domain.