The Nalp3 inflammasome is essential for the development of silicosis

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Inhalation of crystalline silica and asbestos is known to cause the progressive pulmonary fibrotic disorders silicosis and asbestosis, respectively. Although alveolar macrophages are believed to initiate these inflammatory responses, the mechanism by which this occurs has been unclear. Here we show that the inflammatory response and subsequent development of pulmonary fibrosis after inhalation of silica is dependent on the Nalp3 inflammasome. Stimulation of macrophages with silica results in the activation of caspase-1 in a Nalp3-dependent manner. Macrophages deficient in components of the Nalp3 inflammasome were incapable of secreting the proinflammatory cytokines interleukin (IL)-1 β and IL-18 in response to silica. Similarly, asbestos was capable of activating caspase-1 in a Nalp3-dependent manner. Activation of the Nalp3 inflammasome by silica required both an efflux of intracellular potassium and the generation of reactive oxygen species. This study demonstrates a key role for the Nalp3 inflammasome in the pathogenesis of pneumoconiosis.

asbestosis | NLRP3 | caspase-1 | interleukin-1 β

Silica crystals are abundant and naturally occurring. Airborne silica particles are commonly encountered in numerous occupations, including mining, construction, manufacturing, and farming. The inhalation of these particles causes the development of silicosis, which is a chronic interstitial pulmonary disease characterized by the delayed development of fibrosis with progressive pulmonary dysfunction (1–3). Similarly, inhalation of asbestos particles causes the development of the associated fibrotic disease asbestosis (1–3). There is no effective therapy for silicosis or asbestosis, nor are the underlying immunologic mechanisms leading to disease clearly understood. Alveolar macrophages, and the IL-1 β produced by these cells, have been suggested to play a crucial role during the early inflammatory response after exposure to silica or asbestos. Alveolar macrophages from patients with asbestosis spontaneously secrete elevated amounts of IL-1 β compared with healthy controls (4, 5). Macrophages treated with silica have been shown to secrete IL-1 β , and IL-1 β -deficient mice are more resistant to developing pulmonary pathology in response to inhaled silica (6, 7). Additionally polymorphisms in the *IL-1RA* allele have been linked to human susceptibility to silicosis (8).

The mammalian NLR family is composed of over 20 members that contain a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding NACHT domain, and an N-terminal protein–protein-interaction domain composed of a CARD (caspase activation and recruitment domain) or pyrin domain (9, 10). A number of NLR family members have been shown to activate the cysteine protease caspase-1 within a multiprotein complex called the inflammasome (11). Caspase-1 is capable of initiating the cell death pathway known as pyroptosis as well as cleaving the precursor cytokines pro-IL-1 β , pro-IL-18, and pro-IL-33 into their mature forms, IL-1 β , IL-18, and IL-33, respectively. The NLR family member Nalp3 (also known as cryopyrin, CIAS1, and NLRP3) can activate caspase-1 in re-

sponse to a number of diverse stimuli, including ATP, monosodium urate crystals (MSU), and bacterial pore-forming toxins (12–14). Upon activation Nalp3 recruits the adaptor molecule ASC by a pyrin–pyrin-domain interaction; ASC then recruits caspase-1 by CARD–CARD interactions, thus forming the NALP3 inflammasome.

In this study we identify Nalp3 as being an important innate immune receptor involved in the recognition of silica and asbestos by macrophages. Stimulation of macrophages with either silica or asbestos resulted in the robust secretion of IL-1 β in a manner dependent on the Nalp3 inflammasome. We also demonstrate that silica-induced production of reactive oxygen species (ROS) is required for the activation of the Nalp3 inflammasome. Finally, we show that the chronic fibrosis seen in a murine model of silicosis *in vivo* is dependent upon the presence of ASC and Nalp3. These findings suggest that silica-induced production of IL-1 β through the Nalp3 inflammasome plays an important role in the initial inflammatory responses that lead to silicosis.

Results and Discussion

Silica Induces IL-1 β but Not TNF α or IL-6 Secretion *in Vitro*. The role of inflammation in the development of silicosis is well accepted. Surprisingly, crystalline silica (SiO₂) was found to be a poor inducer of macrophage TNF α and IL-6 (Fig. 1*A* and *B*) when compared with the potent inflammatory stimulus LPS. The expression of IL-1 β and IL-12p40 mRNA in macrophages after stimulation with either silica or LPS was examined by RT-PCR. LPS stimulation resulted in the expression of both IL-1 β and IL-12p40 mRNA, which was not detected in silica-stimulated macrophages (Fig. 1*C*). However, treatment of LPS-primed macrophages with silica resulted in the rapid secretion of IL-1 β in a dose-dependent manner (Fig. 1*D*). Treatment of LPS-primed macrophages with asbestos also resulted in secretion of IL-1 β in a dose-dependent manner (Fig. 1*E*). Silica, asbestos, or LPS-priming alone was not capable of inducing IL-1 β secretion (Fig. 1*D* and *E*), which is consistent with the actions of other stimuli such as MSU and ATP that require macrophage priming to activate caspase-1 (12–14). In contrast, titanium dioxide (TiO₂), which does not cause fibrotic disease upon inhalational exposure (15), did not induce the secretion of IL-1 β from

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The authors declare no conflict of interest.

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Fig. 1. Silica induces IL-1 β secretion from LPS-primed macrophages. (A–C) Macrophages from WT mice were stimulated with either 50 $\mu\text{g}/\text{cm}^2$ silica or 50 ng/ml LPS. Culture supernatants were collected at the indicated time after stimulation; TNF α (A) and IL-6 (B) release into culture supernatants was measured by ELISA. (C) IL-1 β and IL-12p40 expression in silica- or LPS-stimulated macrophages was determined by RT-PCR. (D–F) Macrophages from WT mice were either primed with 50 ng/ml LPS or left untreated. Macrophages were stimulated with the indicated dose of silica (D), asbestos (E), or titanium dioxide (F), and culture supernatants were collected 4 h later. IL-1 β release into culture supernatants was measured by ELISA. (G) Human alveolar macrophages were either primed with 50 ng/ml LPS or left untreated. Macrophages were stimulated with 50 $\mu\text{g}/\text{cm}^2$ silica or 40 $\mu\text{g}/\text{cm}^2$ asbestos for 4 h, and IL-1 β release into culture supernatants was measured by ELISA. Determinations were performed in triplicate and expressed as the mean $\pm$ SEM. Results are representative of two separate experiments.

LPS-primed macrophages (Fig. 1F). LPS-primed human alveolar macrophages were also capable of secreting IL-1 β in response to silica or asbestos (Fig. 1G), demonstrating that this effect was not restricted to murine macrophages. These data suggest that silica induced IL-1 β production is an early step in the inflammatory response to silica and likely drives the subsequent production of other inflammatory mediators. This makes IL-1 β an attractive target for potential therapeutic intervention. The specificity for silica to induce IL-1 β secretion and not TNF α and IL-6 is consistent with the clinical syndrome of silicosis, which is a chronic low-level inflammatory process that is manifest years after the initial exposure to silica.

Silica and Asbestos Induce IL-1 β Secretion in a Nalp3-Dependent Manner. Nalp3 along with the adaptor molecule ASC can activate caspase-1 in response to a variety of stimuli, including ATP,

nigericin, maitotoxin, MSU, and the pathogens *Listeria monocytogenes* and *Staphylococcus aureus* (12–14). We used macrophages deficient in specific components of the Nalp3 inflammasome to test whether they were required for silica-induced IL-1 β secretion. LPS-primed Nalp3-, ASC- and caspase-1-deficient macrophages all displayed a marked defect in their ability to secrete IL-1 β in response to silica compared with macrophages from WT mice (Fig. 2A). In contrast, macrophages deficient in the NLR molecule IPAF, which is important for macrophage caspase-1 activation in response to infection with type III and type IV secretion system carrying Gram-negative bacteria (16–18), had an intact response to silica and were capable of secreting IL-1 β (Fig. 2A). In addition, LPS-primed Nalp3-, ASC-, and caspase-1-deficient macrophages were also unable to secrete the other caspase-1-processed cytokine IL-18 in response to silica (Fig. 2B).

Caspase-1 activation involves autocatalytic processing of the 45-kDa pro-caspase-1 to generate two subunits, p20 and p10. Caspase-1 activation in silica-stimulated LPS-primed WT macrophages was detected by Western blotting by the appearance of the p10 cleavage product 2 h after stimulation with silica (Fig. 2C). We did not observe caspase-1 activation in response to silica in either Nalp3- or ASC-deficient LPS-primed macrophages (Fig. 2C). As expected, IPAF-deficient LPS-primed macrophages did not display any defect in caspase-1 activation in response to silica (Fig. 2C). These data identify the Nalp3 inflammasome to be required for caspase-1-mediated IL-1 β and IL-18 secretion in response to stimulation with silica.

The inhalation of asbestos leads to a similar fibrotic syndrome, asbestosis, characterized by chronic inflammation and insidious deterioration of lung function, which usually first manifests more than 15 years after the initial exposure to asbestos. Asbestos, but not titanium dioxide, stimulation of LPS-primed WT macrophages resulted in the secretion of IL-1 β in a Nalp3-dependent manner (Fig. 2D). This suggests a common mechanism for inflammation in response to the pro-fibrotic agents silica and asbestos requiring Nalp3, which is not shared by other, nonfibrotic, inhalant particles.

Silica-Induced Cytotoxicity Is Independent of Nalp3. It has been shown that treatment of macrophages with silica is cytotoxic (19). To determine whether silica-induced cytotoxicity was associated with Nalp3, we assessed the degree of cell death in WT and Nalp3-deficient macrophages after silica treatment. We confirmed the cytotoxic effect of silica and found it to be equivalent in macrophages from WT and Nalp3-deficient mice (Fig. 3A). These data demonstrate that silica-induced cytotoxicity is not dependent on Nalp3, and that IL-1 β release was not a consequence of cell lysis, because WT and Nalp3-deficient macrophages had similar amounts of cell death but only the former released IL-1 β .

Silica-Induced IL-1 β Secretion Requires Crystal Internalization and Potassium Efflux. To understand how silica might activate the Nalp3 inflammasome, we tested whether the endocytic ability of macrophages was required for silica-stimulated IL-1 β production. Inhibiting actin polymerization with either cytochalasin B or cytochalasin D inhibited IL-1 β production by silica in LPS-primed macrophages (Fig. 3B). Neither cytochalasin B nor cytochalasin D reduced IL-1 β production in response to stimulation with ATP, which uses the P2X7 receptor to activate the Nalp3 inflammasome, confirming that macrophages were still viable and capable of secreting Nalp3-dependent IL-1 β (Fig. 3B). These data suggest that, unlike ATP, endocytosis of silica by macrophages is needed to activate the Nalp3 inflammasome in response to silica for the resultant processing and secretion of proinflammatory cytokines. Endocytosis of particulates may be occurring through the scavenger receptors SR-A and MARCO,

Fig. 2. Silica- and asbestos-induced IL-1 β secretion is dependent on Nalp3. LPS-primed macrophages from WT, caspase-1-, ASC-, Nalp3-, or IPAF-deficient mice were stimulated with 50 $\mu\text{g}/\text{cm}^2$ silica. Culture supernatants were collected at 4 h or at the indicated time after stimulation (A–C) and IL-1 β and IL-18 release into culture supernatants was measured by ELISA (A and B). Determinations were performed in triplicate and expressed as the mean $\pm$ SEM. Results are representative of three (A) and two (B) separate experiments. (C) Lysates from LPS-primed WT, ASC-, NALP3-, or IPAF-deficient macrophages stimulated with 50 $\mu\text{g}/\text{cm}^2$ silica for the indicated times were immunoblotted with antibodies against the p10 subunit of caspase-1. (D) LPS-primed macrophages from WT and NALP3-deficient mice were stimulated with either 40 $\mu\text{g}/\text{cm}^2$ asbestos or 50 $\mu\text{g}/\text{cm}^2$ titanium dioxide. Culture supernatants were collected 6 h later and IL-1 β release into culture supernatants was measured by ELISA. Determinations were performed in triplicate and expressed as the mean $\pm$ SEM. Results are representative of three separate experiments. *, Nondetectable.

which have been implicated in playing an important role in the inflammatory response to silica (20). Further studies will be required to determine whether these receptors are also required for silica-induced Nalp3 inflammasome activation.

A number of diverse stimuli can activate the Nalp3 inflammasome. However, a common step shared by these stimuli to induce Nalp3-dependent caspase-1 activation is the efflux of cellular potassium (21). Preventing the potassium efflux by increasing extracellular potassium inhibits caspase-1 activation in response to stimuli that activate Nalp3 but not stimuli that activate caspase-1 through IPAF (21). Increased extracellular potassium significantly inhibited silica-induced IL-1 β production from macrophages (Fig. 3C). As expected, increased extracellular potassium also inhibited ATP-mediated IL-1 β production, which is also dependent on Nalp3. Silica therefore is a previously unrecognized Nalp3 stimulus, which like other Nalp3 stimuli requires a cellular potassium efflux to activate caspase-1.

Silica-Induced ROS Are Required for Nalp3 Inflammasome Activation.

There are two main sources of cellular ROS. The first is the phagocyte NADPH oxidase, which generates superoxide anions as part of the antimicrobial response to ingested microbes (22). In the resting phagocyte, components of the NADPH oxidase are spatially segregated, with flavocytochrome b_{558} in the membrane and $p47^{\text{phox}}$, $p67^{\text{phox}}$, and Rac in the cytoplasm. Upon stimulation, the cytosolic components translocate to the membrane and assemble into a functioning enzyme complex, shuttling electrons from cytoplasmic NADPH to molecular oxygen to generate extracellular or phagosomal superoxide anion. Mitochondria have also long been known to generate reactive oxidants and are a major source of cellular ROS (22). The mitochondrial respiratory chain is made up of four electron-transporting complexes and a proton-translocating complex, with the majority of intracellular ROS produced during mitochondrial respiration by complex I. ROS have also been shown to play important signaling functions within the cell either for homeostatic functions or to signal stress. This is particularly the case for the mitochondria, which can either confine its production of

ROS locally or flood the cell with oxidants, causing damage to itself and the cell in the process.

The Nalp3 inflammasome activator ATP has been shown to be capable of inducing the production of ROS, which are important for caspase-1 activation (23). As silica can also induce ROS production from macrophages (19) we examined whether this was required for caspase-1 mediated IL-1 β secretion. Using HRP-dependent luminol-enhanced chemiluminescence to detect both intra- and extracellular ROS, we confirmed that silica induced ROS production from macrophages (Fig. 3D) and that the response was blocked by preincubation of macrophages with the inhibitor of NADPH oxidase diphenyleneiodonium chloride (DPI) (data not shown). DPI also markedly inhibited silica-induced IL-1 β secretion and caspase-1 activation (Fig. 3F and G), thereby suggesting that ROS signaling was required for silica-induced Nalp3 inflammasome activation. DPI did not inhibit silica-induced caspase-3 activation in LPS-primed macrophages (Fig. 3G). Nalp3-deficient macrophages were capable of producing ROS in response to silica (Fig. 3E), indicating that ROS signaling is likely occurring upstream of Nalp3. Although these data demonstrate a role for ROS in silica-induced IL-1 β secretion, they do not distinguish between the two most likely sources for ROS in stimulated cells, because DPI can also exert inhibitory effects on mitochondrial ROS production in addition to NADPH oxidase. However, taken together these studies clearly define a novel signaling pathway used to activate the Nalp3 inflammasome.

ASC- and Nalp3-Deficient Mice Have Decreased Pulmonary Pathology in Response to Inhaled Silica.

To confirm the physiologic relevance of our *in vitro* data, we assessed the role of the Nalp3 inflammasome in a murine model of silicosis. We treated WT, ASC-/-, and Nalp3-/- mice with intranasal instillation of silica and assessed pulmonary inflammation and fibrosis after 3 months. WT mice had profound cellular inflammatory changes and granulomas as seen on H&E staining; in contrast there was a reduction in inflammation and granuloma formation in both ASC- and Nalp3-deficient mice (Fig. 4A). The reduction in

Fig. 4. Decreased inflammation and collagen deposition in ASC^{-/-} and Nalp3^{-/-} mice after intranasal instillation of silica. WT, ASC^{-/-}, and Nalp3^{-/-} mice received either intranasal PBS or silica. Three months after the initial challenge, lungs were stained with H&E to examine inflammation (A) or trichrome stain to examine collagen deposition (B). H&E-stained sections were scored in a blinded fashion for cellular tissue infiltration (*, $P = 0.039$; **, $P = 0.0062$ compared with WT by one-tailed unpaired Student's t test). Images were obtained at $\times 10$ (A) and $\times 20$ (B) magnification and are representative of two mice per group for PBS and five mice per group for silica.

consequence of silica-induced IL-1 β production and not a direct result of silica's action upon the macrophage. Recent studies have implicated caspase-1 in modulating unconventional protein secretion and in particular that of fibroblast growth factor-2 (FGF-2) (26). This has intriguing implications for additional roles of caspase-1 in disease pathogenesis. The central role of Nalp3 in recognition of silica and activation of caspase-1 makes it an attractive target for potential therapeutic intervention.

Materials and Methods

Mice. The generation of NALP3^{-/-}, ASC^{-/-}, caspase-1^{-/-}, and IPAF-deficient mice has been described previously (12, 27, 28). Caspase-1^{-/-}, ASC^{-/-}, and NALP3-deficient mice were backcrossed onto the C57BL/6 genetic background for at least nine generations. IPAF-deficient mice were backcrossed onto the C57BL/6 genetic background for at least six generations. Age- and sex-matched C57BL/6 mice purchased from the National Cancer Institute were used as WT controls. All protocols used in this study were approved by the Institutional Animal Care and Use Committees at the University of Iowa and Yale University.

Macrophages. Thioglycollate-elicited peritoneal macrophages were generated by injecting 1 ml of 3% thioglycollate solution into the peritoneal cavity of mice. Three to 4 days later macrophages were collected by peritoneal lavage and plated in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin G, and 100 μ g/ml streptomycin. Unless indicated, mac-

rophages were activated by stimulating with 50 ng/ml LPS from *Escherichia coli* serotype O111:B4 (InvivoGen) for 4–12 h before stimulation with silica or asbestos.

Human alveolar macrophages were obtained from normal volunteers by bronchoalveolar lavage as described in ref. 29, and the procedure was approved by the Committee for Investigations Involving Human Subjects at the University of Iowa.

In Vitro Stimulation of Macrophages. Macrophages that were either unstimulated or had been activated with 50 ng/ml LPS for 4–12 h were stimulated with either 50 μ g/cm² silica (Min-U-Sil-5; Pennsylvania Glass Sand), 40 μ g/cm² crocidolite asbestos (North American Insulation Manufacturers Association Fiber Repository), or 50 μ g/cm² titanium dioxide (Sigma) unless otherwise indicated. Four hours, or at the indicated time, after stimulation supernatants were collected. Culture supernatants were then assayed for IL-1 β and TNF α and IL-6. Antibody pairs for the IL-1 β ELISAs were from R&D Systems. Antibody pairs for the TNF α and IL-6 ELISAs were from eBiosciences. Macrophage cell death was determined 4 h after stimulation with silica by using a cytotoxicity (LDH release) detection kit (Promega). The inhibitors cytochalasin B (10 μ M), cytochalasin D (20 μ M), and diphenyleneiodonium chloride (DPI) (2 μ M and 10 μ M) were added 10 min before the addition of silica.

RT-PCR. RNA was isolated from macrophages by using the Qiagen RNeasy Mini kit and following the manufacturer's instructions. cDNA was synthesized by using SuperScript II reverse transcriptase (Invitrogen). The primers used for RT-PCR amplification were as follows: HPRT, 5'-GTGGATACAGGCCAGACTT-TGTT and 3'-GAGGGTAGGCTGGCTATAGGCT; IL-12p40, 5'-ATGCCATGTGG-GAGCTGGAGAAAG and 3'-GTGGAGCAGCAGATGTGAGTGCT; IL-1 β , 5'-GACAGTGATGAGAATGACCTGTC and 3'-CCTGACCACTGTTGTTCC.

Inhibition of Potassium Efflux. To inhibit potassium efflux, macrophages were primed with 50 ng/ml LPS for 12 h and then medium was replaced with serum-free buffer with 150 mM KCl (10 mM Hepes, 5 mM NaH₂PO₄, 150 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% BSA, pH 7.4). In parallel, a buffer with 150 mM NaCl was used (10 mM Hepes, 150 mM NaCl, 5 mM KH₂PO₄, 1 mM MgCl₂, 1 mM CaCl₂, 1% BSA, pH 7.4).

ROS Measurement. Production of ROS was measured by the peroxidase-dependent luminol-amplified chemiluminescence technique, as previously described (30). Briefly, chemiluminescence was monitored with a Lumistar Galaxy (BMG Labtech) luminometer. Macrophages were plated on white polystyrene microtiter plates (Optiplate, Perkin-Elmer) in Hanks balanced salt solution containing 1 mM CaCl₂, 1.5 mM MgCl₂, 10 mM glucose, and 1% human serum albumin. Cells were incubated at 37°C in the presence of 50 μ M luminol and 4 units of horseradish peroxidase. Stimuli were added to the respective wells and chemiluminescence was followed continuously for 60 min. Assays were run in duplicate.

Western Blotting. Electrophoresis of proteins was performed by using the NuPAGE system (Invitrogen) according to the manufacturer's protocol. Briefly, after stimulation with silica, macrophages and supernatants were lysed in lysis buffer [50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and a protease inhibitor mixture (Roche)] and stored at -80°C until analyzed. Proteins were separated on a NuPAGE gel and transferred to a PVDF membrane by electroblotting. To detect caspase-1 a rabbit polyclonal anti-mouse caspase-1 p10 antibody (Santa Cruz Biotechnology) was used. Caspase-3 and actin were detected by using antibodies from Cell Signaling and Calbiochem, respectively.

In Vivo Silicosis Model. Six to 8-week-old C57BL/6, ASC^{-/-}, and Nalp3^{-/-} mice were anesthetized with metophane before intranasal instillation of either 50 μ l of sterile PBS or 50 μ l of a PBS suspension of silica (20 mg/ml). Mice received instillations of silica on days 0 and 14. Three months after the first instillation of silica mice were killed and lungs were harvested. Lungs were fixed and embedded in paraffin. Sections were stained with either trichrome or hematoxylin and eosin (H&E). H&E stains were used for analysis of airway inflammation and pathological changes. Inflammation was scored on a scale of 1–3 arbitrary units by a pathologist blinded to the experimental protocol, taking into account both the degree of inflammation and its distribution. Lymphoid and macrophage infiltration severity were scored separately and the scores were pooled. For example, none to rare lymphoid or macrophages were given a score of 1, small to moderate-sized perivascular lymphoid aggregates or activated/foamy macrophages scattered within alveoli/interstitium were given a score of 2, and large perivascular lymphoid aggregates or activated/foamy macrophages filling alveoli/interstitium

were given a score of 3. The percentage of lung affected was given a score of 0–3 with 0% getting a score of 0, 1–5% getting a score of 1, 6–40% getting a score of 2, and >40% getting a score of 3.

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- Mossman BT, Churg A (1998) Mechanisms in the pathogenesis of asbestosis and silicosis. *Am J Respir Crit Care Med* 157:1666–1680.
- Otsuki T, et al. (2007) Immunological effects of silica and asbestos. *Cell Mol Immunol* 4:261–268.
- Wagner GR (1997) Asbestosis and silicosis. *Lancet* 349:1311–1315.
- Kline JN, Schwartz DA, Monick MM, Floerchinger CS, Hunninghake GH (1993) Relative release of interleukin-1 β and interleukin-1 receptor antagonist by alveolar macrophages. A study in asbestos-induced lung disease, sarcoidosis, and idiopathic pulmonary fibrosis. *Chest* 104:47–53.
- Zhang Y, Lee TC, Guillemin B, Yu MC, Rom WN (1993) Enhanced interleukin-1 β and tumor necrosis factor- α release and mRNA expression in macrophages from idiopathic pulmonary fibrosis or following asbestos exposure. *J Immunol* 150:4188–4196.
- Sarih M, Souvannavong V, Brown SC, Adam A (1993) Silica induces apoptosis in macrophages and the release of interleukin-1 α and interleukin-1 β . *J Leukocyte Biol* 54:407–413.
- Srivastava KD, et al. (2002) Crucial role of interleukin-1 β and nitric oxide synthase in silica-induced inflammation and apoptosis in mice. *Am J Respir Crit Care Med* 165:527–533.
- Yucesoy B, et al. (2001) Polymorphisms of the IL-1 gene complex in coal miners with silicosis. *Am J Ind Med* 39:286–291.
- Mariathasan S, Monack DM (2007) Inflammasome adaptors and sensors: Intracellular regulators of infection and inflammation. *Nat Rev Immunol* 7:31–40.
- Pétrilli V, Dostert C, Muruve DA, Tschopp J (2007) The inflammasome: A danger sensing complex triggering innate immunity. *Curr Opin Immunol* 19:615–622.
- Martinon F, Burns K, Tschopp J (2002) The inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL- β . *Mol Cell* 10:417–426.
- Sutterwala FS, et al. (2006) Critical role for NALP3/CIA1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 24:317–327.
- Mariathasan S, et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228–232.
- Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237–241.
- Fryzek JP, et al. (2003) A cohort mortality study among titanium dioxide manufacturing workers in the United States. *J Occup Environ Med* 45:400–409.
- Mariathasan S, et al. (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213–218.
- Zamboni DS, et al. (2006) The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of *Legionella pneumophila* infection. *Nat Immunol* 7:318–325.
- Sutterwala FS, et al. (2007) Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. *J Exp Med* 204:3235–3245.
- Hamilton RF, Jr, Thakur SA, Holian A (2008) Silica binding and toxicity in alveolar macrophages. *Free Radic Biol Med* 44:1246–1258.
- Hamilton RF, Jr., Thakur SA, Mayfair JK, Holian A (2006) MARCO mediates silica uptake and toxicity in alveolar macrophages from C57BL/6 mice. *J Biol Chem* 281:34218–34226.
- Pétrilli V, et al. (2007) Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14:1583–1589.
- Terada LS (2006) Specificity in reactive oxidant signaling: Think globally, act locally. *J Cell Biol* 174:615–623.
- Cruz CM, et al. (2007) ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 282:2871–2879.
- Gasse P, et al. (2007) IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. *J Clin Invest* 117:3786–3799.
- Piguet PF, Vesin C, Grau GE, Thompson RC (1993) Interleukin 1 receptor antagonist (IL-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. *Cytokine* 5:57–61.
- Keller M, Rüegg A, Werner S, Beer HD (2008) Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 132:818–831.
- Lara-Tejero, et al. (2006) Role of the caspase-1 inflammasome in *Salmonella typhimurium* pathogenesis. *J Exp Med* 203:1407–1412.
- Kuida K, et al. (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1 β converting enzyme. *Science* 267:2000–2003.
- Monick MM, Carter AB, Hunninghake GW (1999) Human alveolar macrophages are markedly deficient in REF-1 and AP-1 DNA binding activity. *J Biol Chem* 274:18075–18080.
- Bánfi B, et al. (2004) NOX3, a superoxide-generating NADPH oxidase of the inner ear. *J Biol Chem* 279:46065–46072.