ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation

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Deposition of uric acid crystals in joints causes the acute and chronic inflammatory disease known as gout and prolonged airway exposure to silica crystals leads to the development of silicosis, an irreversible fibrotic pulmonary disease. Aluminum salt (Alum) crystals are frequently used as vaccine adjuvant. The mechanisms by which crystals activate innate immunity through the Nlrp3 inflammasome are not well understood. Here, we show that uric acid, silica and Alum crystals trigger the extracellular delivery of endogenous ATP, which just precedes the secretion of mature interleukin-1 β (IL-1 β) by macrophages, both events depending on purinergic receptors and connexin/pannexin channels. Interestingly, not only ATP but also ADP and UTP are involved in IL-1 β production upon these Nlrp3 inflammasome activators through multiple purinergic receptor signaling. These findings support a pivotal role for nucleotides as danger signals and provide a new molecular mechanism to explain how chemically and structurally diverse stimuli can activate the Nlrp3 inflammasome.

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Endogenous or environmental particulate dangers trigger innate immunity through assembly of the Nlrp3 inflammasome, an intracellular molecular platform which under activation allows the cleavage of the potent pro-inflammatory cytokine interleukin-1 β (IL-1 β). Numerous studies showed that a first signal triggers synthesis of pro-IL-1 β and Nlrp3 by transcriptional induction, whereas a second stimulus leads to inflammasome oligomerization, caspase-1 auto-activation, caspase-1-dependent cleavage and release of the biologically active, mature IL-1 β . This second signal may be triggered by a group of chemically and biologically unrelated molecules such as pathogen-associated molecular patterns (PAMPs), toxins or danger-associated molecular patterns (DAMPs). DAMPs originate from environmental pollutants such as silica and asbestos,^{1,2} from vaccines such as aluminum salt (Alum) adjuvant³ or from endogenous metabolic stresses such as high glucose,⁴ cholesterol,⁵ β -amyloid protein,⁶ biglycan,⁷ ATP⁸ and monosodium urate crystals (MSU).^{3,9} Several major upstream mechanisms are currently proposed for Nlrp3 inflammasome activation such as membrane disruption (for bacterial toxins and soluble ATP) or internalization of particulate activators by phagocytosis.¹⁰ Extracellular ATP (eATP) or bacterial toxins lead to K⁺ efflux and pannexin-1 (Panx-1) pore formation, both required for Nlrp3 activation.¹¹ Phagocytosis of particles including silica, Alum, fibrillar amyloid- β or MSU was shown to result in

lysosomal destabilization/permeabilization with release of the endosomal-lysosomal protease cathepsin B into the cytoplasm^{2,6} and/or to mediate reactive oxygen species (ROS) driven activation.

We hypothesized that eATP release from stimulated cells and further purinergic signaling may be a corner stone of Nlrp3 inflammasome activation pathway. ATP signaling is emerging as an important mechanism to control various cell functions.¹² Cellular stimulation triggers ATP release and subsequently activation of purinergic receptors (P2X or P2Y receptors) at the cell surface (autocrine activation) and/or in adjacent cells (paracrine activation), thereby regulating or modulating cellular functions in particular immunity. Essentially all cells are able to release nucleotides nevertheless the mechanisms of nucleotide release in epithelia, endothelial cells or other non-excitable cells are poorly understood.^{12,13} After release, eATP interacts with specific purinergic receptors or is degraded *via* different ecto-ATPases to di- and monophosphates and then to adenosine, ATP or its metabolites being able to signal through different purinergic receptors (P2X, P2Y or adenosine P1 receptors).¹⁴ In pathological conditions, high level of ATP is released from necrotic cells and acts as a pro-inflammatory danger signal, activating the Nlrp3 inflammasome through binding to the ionotropic receptor P2X7.¹⁵

Here, for the first time we provide evidence that uric acid, silica and aluminum particles induce the active release of

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Abbreviations: ATP, adenosine triphosphate; IL-1 β , interleukin-1 beta; NLR, Nod-like receptor; MSU, monosodium urate crystal; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; ROS, reactive oxygen species

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intracellular ATP to the outside of human macrophages, depending in some extent to functional P2X7 receptor and connexin/pannexin channels. Moreover, we show a strong correlation between ATP release and secretion of mature IL-1 β after stimulation of primed human macrophages. In addition, crystals exposure to primed murine macrophages leads to maturation of IL-1 β that highly depends on purinergic receptor signaling with the involvement of multiple autocrine purinergic receptor loops.

We identify a new mechanism for crystals-mediated Nlrp3 inflammasome activation and mature IL-1 β release through multiple purinergic receptors and connexin/pannexin pore activation. We provide a link between ATP- and crystals-mediated inflammasome activation demonstrating that crystals act through ATP release to induce inflammasome activation and propose a new hypothesis allowing understanding how such a variety of activators can activate Nlrp3 inflammasome.

Results

MSU, silica or Alum crystals trigger active ATP release and IL-1 β secretion in human macrophages. Recently, we showed that stimulation of lung epithelial cells with bleomycin, a cytotoxic agent that causes DNA damage, leads to a rapid release of ATP partly dependent on functional P2X7 receptor and Panx-1 pore formation.¹⁶ Since activated or stressed cells were shown to release ATP, we investigate whether crystals or particles known as activators of the Nlrp3 inflammasome may act through ATP leakage to trigger IL-1 β maturation.^{13,17} We observed that MSU or silica crystals cause active ATP release by pulmonary epithelial cell lines, which depends on purinergic receptors and

pannexin/connexin channels but not mature IL-1 β production (data not shown). To determine whether particulate Nlrp3 inflammasome activators are able to trigger the release of endogenous ATP by the macrophage lineage, the human monocyte/macrophage cell line THP-1 was stimulated with MSU crystals during various times. Luciferase ATP assays revealed that MSU crystals trigger the release of ATP by human macrophages, which peaks at 2 h (Figure 1a). Since the extracellular accumulation of ATP reflects the balance between ATP release by cells and ATP hydrolysis by ectonucleotidases,¹⁸ we used the ecto-ATPase inhibitor ARL67156 to limit ATP catabolism. In the presence of ARL67156, the levels of ATP detected in the different supernatants increased up to sixfold (Figure 1b). From the same macrophage culture and at a given end time point, we co-analyzed ATP release and mature IL-1 β in the presence of ARL67156 and observed that ATP release is strongly correlated with IL-1 β secretion (Figure 1c). Hence, ATP release induced by MSU or Alum is transient and precedes sustained IL-1 β production by the same macrophages. After silica stimulation, responses were delayed and we did not observe the peak of ATP release even after 6 h. In contrast allopurinol crystals, known to fail in activating the Nlrp3 inflammasome,⁹ induced neither ATP release nor IL-1 β secretion (Figure 1c). Importantly, cell death measured by MTT was minimal, suggesting that ATP release is not the consequence of any macrophage death (Figure 1d). These data suggest that IL-1 β secretion is a consequence of ATP leakage induced by particulate Nlrp3 activators.

Phagocytosis or cystein proteases inhibition blocks both ATP release and IL-1 β production in human macrophages. Particle phagocytosis is known to be

Figure 1 MSU, silica or Alum crystals trigger active release of ATP by human macrophages. PMA-primed THP-1 cells incubated with MSU crystals release ATP in the supernatant that peaks after 2 h and rapidly return to baseline (a). Higher concentrations were obtained using the ecto-ATPase inhibitor ARL67146 (ARL) to preserve ATP (b). In the presence of ARL67146, there is a strong correlation between MSU, silica and Alum-induced ATP release and IL-1 β production, whereas allopurinol crystals do not induce ATP release or IL-1 β secretion (c). MTT assay revealed no significant change in cell viability at studied time points (d). Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D.

essential for inflammasome activation and mature IL-1 β production.² However, cytoskeleton is not necessary for IL-1 β secretion.³ Here, we show that ATP leakage is completely impaired by cytochalasin D, which inhibits actin polymerization, demonstrating that ATP release is a physiologic process needing particle phagocytosis by human macrophages (Figure 2a). Moreover, we confirmed that cytochalasin D abrogates IL-1 β production (Figure 2b). Importantly, cysteine proteases, for example, cathepsin B and L, were shown to be involved in Nlrp3 activation in response to particles.^{2,5} We show here that E-64d, a broad inhibitor of cysteine proteases is able to dramatically reduce ATP release and IL-1 β secretion (Figure 2b) whereas the control acidic protease inhibitor pepstatin A had no effect (Figures 2a and b). In addition, cathepsin B specific inhibitor Ca074-Me dose dependently reduced eATP and IL-1 β production induced by silica, suggesting a specific role for cathepsin B (Figure 2c). Altogether, these data suggest that particle internalization/lysosome destabilization with lysosomal cathepsin leakage precedes ATP release and IL-1 β production by human macrophages.

Purinergic signaling and pannexin/connexin hemichannels are required for ATP release and IL-1 β secretion in human macrophages. We then investigated the mechanisms of particle-induced ATP release leading to IL-1 β secretion. Since P2X7 receptor and Panx-1 hemichannel were involved in ATP release from different cell types after stress or activation, we next investigated their role in a particle-induced context.¹⁹ Three potential mechanisms for regulated nucleotide release after cell stimulation,

exocytosis, blebbing or passage *via* a plasma membrane channel may be considered.¹⁴ We focused on channel(s) that have been shown to mediate nucleotide release and among them the connexin and pannexin family channels.²⁰ Connexins were first shown to form gap junctions between cells, mediating intercellular communication, but emerging literature reports direct hemichannel activity,²¹ hence able to act similarly as pannexin hemichannels which upon activation mediate molecule and ion transfers between intracellular and extracellular compartments.^{20,22} Using pharmacological inhibitors, we show that the connexin/pannexin channel blocker carbenoxolone (CBX) and the connexin channel blocker flufenamic acid (FFA) reduced significantly both ATP and IL-1 β release, indicating that connexin and/or pannexin hemichannels are involved in ATP release and IL-1 β production (Figures 3a and b). Moreover, specific inhibition of the P2X7 receptor by A740003²³ at 10 μ M led to partial inhibition of ATP release and IL-1 β production by Alum but not by silica, whereas higher concentrations were efficient in both ATP and IL-1 β releases (Figures 3a and b). In conclusion, particles induce P2X7 receptor-dependent ATP release through connexin and/or pannexin channels leading to mature IL-1 β secretion in the same extent.

IL-1 β production by murine macrophages depends on cysteine proteases, purinergic receptors and pannexin/connexin hemichannels. Conversely to THP-1 human monocyte/macrophage cell line, we could not measure significant ATP increase in the supernatant of stimulated murine bone marrow-derived macrophages (BMDM) or dendritic cells (BMDC) even adding the ecto-ATPase

Figure 2 Phagocytosis or cysteine proteases inhibitors block both IL-1 β production and ATP release by human macrophages. PMA-primed THP1 cells were incubated with silica or Alum salt crystals for 3 h. Phagocytosis inhibitor cytochalasin D (Cyt D) and cysteine proteases inhibitor E-64d but not acidic protease inhibitor pepstatin A (Peps A) were able to strikingly decrease both silica- or Alum-induced ATP release (a) and IL-1 β production (b) in PMA-primed THP-1 cells. Cathepsin B specific inhibitor Ca074-Me dose dependently reduced eATP and IL-1 β production induced by silica (c). Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D. (***) $P < 0.001$; ns, non-significant)

Figure 3 P2X7 receptor or pannexin/connexin hemichannel involvement in ATP release and IL-1 β secretion in human macrophages. PMA-primed THP-1 cells were incubated with silica or Alum salt crystals. Connexin/pannexin channel blocker carbenoxolone (CBX) and connexin channel blocker flufenamic acid (FFA) significantly reduced both ATP and IL-1 β release induced by silica and Alum (a and b). Specific inhibition of the P2X7 receptor with 10 μ M A740003 leads to partial inhibition of ATP release without inhibition of IL-1 β production, whereas higher concentrations are efficient in both ATP release and IL-1 β production but might be non-specific (a and b). Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D. (** $P < 0.01$; *** $P < 0.001$; ns, non-significant)

inhibitor ARL67156. This may be due to fastest ATP degradation by these cells and/or to limitation of our measurement assay. Using cytochalasin D, we first confirmed that MSU, silica or Alum salt crystal-induced IL-1 β secretion by murine macrophages required phagocytosis (Supplementary Figures 1a and b) whereas allopurinol crystals did not induce IL-1 β (Supplementary Figure 1c). Conversely, ATP- or nigericin-induced IL-1 β secretion was not affected by cytochalasin D confirming cytochalasin D abrogates particle phagocytosis but not P2X7 receptor and/or pannexin channel activities (Supplementary Figure 1d). Since a role for cathepsin B in crystal-induced inflammasome activation is still controversial, we analyzed the effect of protease inhibitors. The cysteine cathepsin and calpain inhibitors (E64d, Z-FA-FMK), the cysteine cathepsin B inhibitor (Ca074-Me) but not the cysteine cathepsin L inhibitor or the aspartic acid protease cathepsin D inhibitor (pepstatin A) dose dependently decreased IL-1 β upon silica and Alum crystals (Supplementary Figures 1a and b). In addition, macrophages from cathepsin B-deficient mice stimulated with MSU, silica or Alum crystals presented a significant decrease of IL-1 β (Supplementary Figure 1e). These data indicate that mature IL-1 β secretion depends at least partially on the cysteine protease cathepsin B but not on the cysteine protease cathepsin L and the aspartic acid protease cathepsin D. Moreover, the P2X7 receptor specific inhibitor A740003, the broad-spectrum P2 receptor inhibitor

oATP or the phospholipase C inhibitor U73122 blocking signaling through the P2Y1-like subfamily (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) strikingly decreased or suppressed IL-1 β production upon MSU, silica or Alum crystals in murine BMDM (Figure 4a) and BMDC (not shown) in a dose-dependent manner. CBX and FFA were also efficient in inhibiting IL-1 β secretion upon silica or Alum in BMDM (Figure 4b). The use of oATP, A740003 or CBX blocked crystal-induced IL-1 β but not TNF- α production by BMDM (Supplementary Figure 2). These data demonstrate a specific effect of purinergic signaling and connexin/pannexin inhibition in Nlrp3 inflammasome activation pathway without toxic effect. Western blotting analysis confirmed that inhibition of purinergic signaling or connexin activity decreased amount of secreted 17 kDa mature IL-1 β produced by BMDM upon silica or Alum particles stimulation (Figure 4c). Altogether, purinergic receptors and connexin/pannexin channels are required for IL-1 β production by the three tested Nlrp3 inflammasome activators.

Single deletion in P2X7, P2X4 or P2Y2 receptor does not affect IL-1 β production by murine macrophages.

In an attempt to better delineate particle-mediated IL-1 β production, we performed experiments with macrophages from Nlrp3 inflammasome components or for ATP purinergic receptors deficient mice. Consistent with published reports,^{1,2,9} IL-1 β but not TNF- α secretion upon MSU,

Figure 4 IL-1 β production by murine macrophages upon MSU, silica or Alum depends on purinergic signaling and pannexin/connexin hemichannels. IL-1 β production by LPS-primed BMDM stimulated with silica or Alum is dose dependently decreased using P2X7 inhibitor A740003 and more potently with the broad-spectrum purinergic inhibitor oATP or with the phospholipase C inhibitor U73122 blocking several P2YRs (a). The same inhibitory effect is observed with the connexin/pannexin inhibitor CBX and with the connexin channel blocker FFA (b). Western blot analysis of LPS-primed BMDM cell extracts (XT) and supernatants (SN) confirmed that oATP (500 μ M) or CBX (50 μ M) significantly decreased 17 kDa mature IL-1 β production in response to silica or Alum (c). Molecular weight markers are shown at the right (c). Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D.

silica or Alum, was Nlrp3 inflammasome dependent (Supplementary Figures 3a and b), whereas deficiency for Nlrp4 in macrophages (Supplementary Figure 3c) or dendritic cells (Supplementary Figure 3d) had no effect. In contrast, macrophages from P2X7 receptor-deficient mice stimulated

with MSU, silica or Alum did not present a significant decrease in IL-1 β production (Figure 5a). As expected, P2X7 receptor but not P2X4 receptor, was fully required for Nlrp3 activation in response to high ATP concentrations (Figure 5b). In addition, single deletion of the ATP ionotropic

Figure 5 Single P2R deficiency does not lead to reduced IL-1 β production. BMDM deficient for P2X7 or P2X4 receptors were LPS-primed for 3 h and stimulated with MSU, silica or alum for 6 h. IL-1 β production is not significantly decreased (a). In contrast, high exogenous ATP (5 mM; 45 min)-induced IL-1 β production is fully dependent on P2X7 but not on P2X4 receptors (b). P2Y2 receptor-deficient BMDM (c) or BMDC (d) presented no significant decrease in IL-1 β production. Data are representative of three independent experiments and are expressed as mean values $\pm$ S.D.

P2X4 receptor (Figure 5a) known to act together with P2X7 receptor⁷ or of the ATP/UTP metabotropic P2Y2 receptor involved in cell chemotaxis in response to ATP leakage,²⁴ did not lead to significant impairment in IL-1 β production by BMDM (Figure 5c) or BMDC (Figure 5d). Discrepancies between strong effect of biochemical inhibitors and single gene deficiency may reflect redundancy between purinergic receptors or multiple purinergic receptors signaling.

ATP, ADP and UTP nucleotides are involved in particle-mediated inflammasome activation. In order to confirm the role of ATP and purinergic signaling in microparticle-induced IL-1 β secretion, we stimulated murine macrophages in the presence of apyrase, an ATP consuming enzyme. Figure 6a shows apyrase (grade VII that hydrolyzes ATP and ADP) abrogated silica-induced IL-1 β secretion similarly to ATP-induced IL-1 β whereas apyrase alone had no effect on IL-1 β secretion and cell viability (not shown). These results demonstrate the role of ATP and/or ADP in particle-mediated IL-1 β secretion. To further study the involvement of multiple purinergic receptors signaling in sensing Nlrp3 inflammasome particulate activators, we stimulated macrophages with particles in the presence of different nucleotides or purinergic receptor agonists. Addition of 500 μ M ATP, UTP or ADP to LPS-primed murine macrophages did not lead alone to IL-1 β secretion but exacerbated MSU, silica or Alum-induced production of IL-1 β (Figure 6b). Consistently, stable ATP γ S or ADP β S agonists were more potent than ATP or ADP to enhance particles-induced IL-1 β production (Figure 6b). Interestingly, addition of equivalent concentrations of MeATP (P2X agonist) or UTP (P2Y agonist) were not able to induce IL-1 β but potentiated the response to MSU or silica crystals (Figure 6c). We obtained similar results using human macrophages and smaller concentration of ATP, ATP γ S,

ADP or ADP β S (1, 10, 50, 100 or 200 μ M) which dose dependently increased IL-1 β production induced by sub-optimal quantities of silica or Alum whereas they had not effect alone (Figure 6d). Moreover, a small effect of UTP was observed (Figure 6d). The same results with lower concentrations of ATP, ATP γ S, ADP or ADP β S were obtained with murine BMDM macrophages (not shown). In addition, ADP, a specific ligand for P2Y receptors, dose dependently increased the murine macrophage response to MSU or silica further, suggesting the involvement of ADP purinergic receptors (Figure 6e). In contrast, the downstream nucleotide catabolism product adenosine inhibited MSU and silica-induced production of IL-1 β by murine dendritic cells (Figure 6f). These data demonstrate that not only ATP but also its metabolic derivative ADP or UTP play an active role in inflammasome activation in response to microparticles. In conclusion, purinergic signaling by both P2X and P2Y receptors in the context of particle-induced cell response is necessary for efficient IL-1 β production by the Nlrp3 inflammasome.

Discussion

Here, we provide a link between ATP- and crystal-mediated inflammasome activation, demonstrating that crystals act through ATP release to induce IL-1 β maturation. Large amount of eATP (5 mM) was identified as a powerful second signal inducing pro-IL-1 β processing into mature IL-1 β in a two-step model for IL-1 β secretion by murine²⁵ and human macrophages⁹ through P2X7 receptor signaling.²⁶ In contrast, primary human monocytes were shown to require only one signal since exogenous LPS was sufficient to induce secretion of mature IL-1 β , although exogenous ATP strongly accelerates IL-1 β processing and secretion.^{27,28} In addition in

Figure 6 Involvement of ATP, ADP and UTP nucleotides in crystal-induced inflammasome activation. IL-1 β secretion by LPS-primed murine BMDM stimulated for 6 h with silica crystals (250 μ g/ml) or ATP (5 mM) was dose dependently abrogated in the presence of apyrase (**a**). LPS-primed murine BMDM stimulated with 500 μ M of ATP, ADP or stable derivatives, respectively, ATP γ S and ADP β S, do not lead alone to production of IL-1 β . However, they strongly enhance IL-1 β production induced by particles (**b**). Similarly, 600 μ M of P2X receptor agonist MeATP or P2YRs agonist UTP are able to increase the IL-1 β production from 25 to 50% (**c**). The same synergistic effects of ATP, ATP γ S, ADP and ADP β S are observed in PMA-primed THP1 stimulated with alum (125 μ g/ml) or silica (250 μ g/ml) dose dependently (1, 10, 50, 100 or 200 μ M) (**d**). UTP also slightly increased IL-1 β production (**d**). ADP up to 5 mM is not sufficient alone for IL-1 β maturation, but it highly exacerbates MSU or silica-induced IL-1 β production by LPS-primed BMDM (**e**). By contrast, adenosine dampens MSU or silica-induced IL-1 β production by BMDC (**f**). Data are representative of two to three independent experiments and are expressed as mean values $\pm$ S.D.

human monocytes several PAMPs and one DAMP were shown to trigger both the first and the second signals with extracellular release of endogenous ATP acting on the P2X7 receptor in an autocrine loop, triggering mature IL-1 β

secretion in a one-step model of inflammasome activation.²⁹ Here, we demonstrate that in the presence of the first signal, MSU, silica or Alum salt crystals trigger the second one leading to mature IL-1 β production by human macrophages

through ATP release and subsequent purinergic signaling. Importantly, allopurinol crystals known to fail in Nlrp3 inflammasome activation⁹ are unable to trigger ATP release.

We show that oATP recognized as a broad inhibitor of P2X and P2Y receptor was able to prevent crystal-induced maturation of IL-1 β by murine or human macrophages, highlighting the importance of the P2 purinergic receptor family. Moreover, crystal-induced secretion of mature IL-1 β was abrogated by ATP/ADP consuming enzyme apyrase confirming the role of ATP and purinergic signaling in inflammasome activation. Nevertheless, P2X7 receptor deficiency in murine macrophages does not change the cell capacity to produce IL-1 β albeit high concentrations of the P2X7 receptor inhibitor A740003 were able to severely impair the cytokine production, suggesting the involvement of other P2 receptors. Several works have reported that blocking or deleting P2X7 receptor did not affect MSU,⁹ silica¹⁵ or Alum salt³ crystals-induced IL-1 β production, supporting a role for other purinergic receptors in non-pathologic conditions. In the opposite, Alzheimer disease-related amyloid- β protein aggregates were shown to promote IL-1 β release through P2X7 receptor-mediated ATP release.³⁰ Similarly single deficiency in P2X4 or P2Y2 receptors had no major effects on mature IL-1 β production. Even if further experiments need to be performed to better decipher the role of each receptor, our data suggest the involvement of multiple purinergic signaling.

Nlrp3 inflammasome activation by large amount of ATP (5 mM, 30 min) in the presence of LPS constitutes an *in vitro* model as prolonged stimulation results in membrane disruption and cell death underlying the pathological impact of high ATP levels. Importantly, we demonstrate that mild doses of ATP, and unexpectedly also of ADP and UTP, albeit having no effect by themselves, synergize with particles for highest IL-1 β production. These data strongly suggest that the two purinergic receptor families P2X and P2Y are involved since both ADP and UTP acting only on P2Y, seem to play a role. As mild doses of ATP, ADP or UTP were unable alone to promote mature IL-1 β production, another signal triggered by crystal stimulation may be required, for example, a P2X7 receptor-independent potassium efflux. All particles affecting inflammasome activity were shown to be fully dependent on potassium efflux, suggesting a role for ionotropic P2X receptors or other potassium channels. It was shown before that not only ATP but other nucleotides such as ADP, UTP or UDP are released in the extracellular space from mechanically stressed cells, in particular endothelial and epithelial cells.^{13,31} These compounds may act on different purinergic receptors to generate a fine-tuned response.

In contrast to human macrophages, we were unable to detect eATP after murine macrophages stimulation by crystals. This is likely due to a higher level of ecto-ATPase activity by murine cells since we observed that exogenous ATP added to murine macrophages rapidly became undetectable in contrast to the same amount of exogenous ATP added to human macrophages. These observations suggest a local increase of ADP/AMP/adenosine and possible action on IL-1 β production pathway. It is worth to note that the derivative compound adenosine has been shown to act as an anti-inflammatory mediator and could hence counter-balance ATP/ADP activity.³²

Using pharmacological inhibitors, we found that MSU, silica or Alum crystal-induced mature IL-1 β secretion by murine or human macrophages is dependent on connexin/pannexin channels. Panx-1 hemichannel has been involved in Nlrp3 inflammasome activation in response to high ATP, nigericin, maitotoxin and bacterial components.^{33–35} Nevertheless, a recent work using Panx-1-deficient mice observed no impact on mature IL-1 β production upon ATP, MSU or silica particles.³⁶ Compensation between pannexins and/or connexins able to form hemichannels may exist in classical deficient mice and conditional Panx-1 mice would be useful. The use of pharmacologic inhibitors indicates that ATP is released through pannexin/connexin hemichannels as shown elsewhere.^{36,37} We also show that ATP release by human macrophages depends on purinergic signaling and in particular on P2X7 receptor, suggesting the existence of an amplification loop allowing ATP-dependent ATP release. Importantly using cytochalasin D, we show that ATP release requires particle phagocytosis as mature IL-1 β production. Importantly, ATP- or nigericin-induced IL-1 β secretion was not affected in the presence of cytochalasin D, confirming that cytochalasin D acts on particle phagocytosis but not P2X7 receptor and/or pannexin channel activities. Our results do not rule out previous finding showing that phagolysosomal acidification and disruption with cathepsin B release are required for Nlrp3 inflammasome activation.^{1,2} We confirm the involvement of cysteine cathepsins in the production of mature IL-1 β but for the first time we demonstrate that these proteases including cathepsin B play a role in ATP release, suggesting they may be involved in channel maturation and/or opening processes upstream of Nlrp3 inflammasome assembly. In an attempt to summarize our data, we propose the model presented in Figure 7.

We show that phagocytosis of particulate activators induces membrane permeabilization through membrane channel opening with sequential ATP release and purinergic signaling leading to inflammasome activation. This model provides a link between particle internalization- and membrane permeabilization-mediated Nlrp3 inflammasome activation since particulate molecules and eATP are still considered as two completely independent ways to activate the Nlrp3 inflammasome. We show here that ATP release and purinergic signaling is a step of crystal-induced inflammasome stimulation. This is of clear account in regards to extensive work showing that eATP could act as a danger signals released in numerous situations.

Materials and Methods

Reagents. MSU (average length 14.02 μ M $\pm$ 7.72) and allopurinol crystals (prepared as described³⁸) silica (Dr. F Huaux, Bruxelles, Belgium) and Alum (Imject Alum, Thermo Scientific, Rockford, IL, USA) were used at 125–500 μ g/ml as mentioned. Particles were sonicated 30 min prior use.

A740003 is a gift from Dr. F Rassendren (Montpellier, France). ATP, ATP γ S, ADP, ADP β S, MeATP, UTP, nigericin, A438079, oATP, CBX, FFA, bafilomycin A1, pepstatin A, ARL67156 and apyrase grade VII were from Sigma (St. Quentin Fallavier, France), Ca074-Me, E64d and cytochalasin D from Calbiochem Merck (Fontenay sous bois, France).

Mouse bone marrow-derived cells culture and stimulation.

Deficient mice and methods used to prepare primary BMDM and BMDC are described in Supplementary Information. BMDM and BMDC were plated in 96-well microculture plates (2×10^5 cells/well) and primed 3 h with 100 ng/ml ultrapure

Figure 7 Schematic diagram illustrating the specific cascade and signaling pathway in LPS-primed macrophages stimulated with MSU, silica or Alum salt crystals. LPS priming induces transcription of pro-IL-1 β gene and other genes in the nucleus upon activation of the transcription factor NF κ B and subsequent production of pro-IL-1 β protein in the cytosol (1). Particle internalization (2), fusion to lysosome (3) and further phagolysosome destabilization may lead to cathepsin leakage (4), which precedes pannexin/connexin (Pannx/Conx) hemichannel and purinergic signaling dependent intracellular ATP release (5). Extracellular ATP may act through P2X7 receptor to amplify ATP release in a P2X7 receptor dependent way (6). ATP or its derived degradation products such as ADP, generated by ecto-endonucleases, act through an autocrine loop on other purinergic receptor P2X and/or P2Y receptors (7) leading to Nlrp3 receptor activation (8). This allows inflammasome complex formation and maturation of pro-IL-1 β to IL-1 β production (9) and IL-1 β secretion (10)

LPS (*Escherichia coli*, serotype O111:B4, Invivogen, Toulouse, France) then washed and stimulated by particles and listed compounds for 6 h or 30–45 min with ATP (5 mM). Supernatants were collected and IL-1 β or TNF- α cytokines were assayed by ELISA assay.

Human monocytes/macrophages THP1 cells culture and stimulation. THP1 cells were cultured in RPMI, 10% FCS (Hyclone, Cramlington, UK), penicillin (100 U/ml, Invitrogen, Illkirch, France) and streptomycin (100 μ g/ml, Invitrogen). For experiments, THP-1 cells were differentiated 3 h with 0.5 μ M phorbol 12-myristate 13-acetate (Sigma Aldrich, Saint Louis, MO, USA), washed and overnight plated (2×10^5 cells/well). Cells were stimulated for indicated time, supernatant collected for concomitant ATP measurement and/or further IL-1 β quantification.

ATP measurement. eATP in cell culture supernatant was quantified using ATP Lite One-Step kit (Perkin-Elmer, Waltham, MA, USA). Quick centrifugation of the plates was performed to avoid any cell debris contamination. Luminescence was measured with a luminometer (Mithras, Berthold Technologies, Bad Wildbad, Germany) and ATP concentration determined using standard curve allowed samples quantification.

Cytokine measurement by ELISA. IL-1 β and TNF- α levels in cell supernatant were determined using ELISA assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Immunoblotting. Supernatants were collected and stored for further analysis. Cells were washed with cold PBS and scraped in lysis buffer solution (150 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, 0.2% SDS and 1% Nonidet P-40) supplemented with a protease inhibitor cocktail (1%) and pepabloc (0.1 mg/ml) (Roche Applied Science, Meylan, France). Lysis extracts were sonicated and after centrifugation, supernatants were collected and protein content measured (DC protein assay, Bio-Rad, Munich, Germany). Proteins were denatured by boiling (95 $^{\circ}$ C, 5 min), separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with primary goat anti-IL-1 β antibody (Sigma Aldrich) and proteins detected with appropriate secondary rabbit polyclonal antibody (Sigma Aldrich) conjugated to horseradish peroxidase followed by enhanced chemiluminescence (ECL; Fisher, Illkirch, France).

Statistics. Statistical evaluation of differences between the experimental groups was determined by Mann–Whitney test using Prism software (La Jolla, CA, USA). *P*-values of <0.05 were considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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