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To cite this version:

Ludivine Baron, Aurélie Gombault, Manoussa Fanny, Bérengère Villeret, Florence Savigny, et al.. The NLRP3 inflammasome is activated by nanoparticles through ATP, ADP and adenosine.. Cell Death and Disease, 2015, 6 (2), pp.e1629. 10.1038/cddis.2014.576. hal-01128180

HAL Id: hal-01128180
https://univ-rennes.hal.science/hal-01128180
Submitted on 10 Jun 2015

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The NLRP3 inflammasome is activated by nanoparticles through ATP, ADP and adenosine

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The NLR pyrin domain containing 3 (NLRP3) inflammasome is a major component of the innate immune system, but its mechanism of activation by a wide range of molecules remains largely unknown. Widely used nano-sized inorganic metal oxides such as silica dioxide (nano-SiO2) and titanium dioxide (nano-TiO2) activate the NLRP3 inflammasome in macrophages similarly to silica or asbestos micro-sized particles. By investigating towards the molecular mechanisms of inflammasome activation in response to nanoparticles, we show here that active adenosine triphosphate (ATP) release and subsequent ATP, adenosine diphosphate (ADP) and adenosine receptor signalling are required for inflammasome activation. Nano-SiO2 or nano-TiO2 caused a significant increase in P2Y1, P2Y2, A2A and/or A2B receptor expression, whereas the P2X7 receptor was downregulated. Interestingly, IL-1β secretion in response to nanoparticles is increased by enhanced ATP and ADP hydrolysis, whereas it is decreased by adenosine degradation or selective A2A or A2B receptor inhibition. Downstream of these receptors, our results show that nanoparticles activate the NLRP3 inflammasome via activation of PLC-InsP3 and/or inhibition of adenylyl cyclase (ADCY)-cAMP pathways. Finally, a high dose of adenosine triggers inflammasome activation and IL-1β secretion through adenosine cellular uptake by nucleotide transporters and by its subsequent transformation in ATP by adenosine kinase. In summary, we show for the first time that extracellular adenosine activates the NLRP3 inflammasome by two ways: by interacting with adenosine receptors at nanomolar/micromolar concentrations and through cellular uptake by equilibrative nucleoside transporters at millimolar concentrations. These findings provide new molecular insights on the mechanisms of NLRP3 inflammasome activation and new therapeutic strategies to control inflammation.

Cell Death and Disease (2015) 6, e1629; doi:10.1038/cddis.2014.576; published online 5 February 2015

The inflammasome is a major factor of the innate immune system acting as a multiprotein platform to activate caspase-1. We showed recently that nanoparticles of TiO2 (nano-TiO2) and SiO2 (nano-SiO2) are sensed by the NLRP3 inflammasome to induce the release of mature IL-1β, as observed previously with the environmental irritants asbestos or silica. Despite the identification and characterisation of numerous sterile or microbial activators, the precise mechanisms mediating NLRP3 inflammasome activation remain to be determined. Here, we investigated whether ATP release and purinergic signalling through ATP, ADP and adenosine may be involved in inflammasome activation by nanoparticles. Intracellular ATP is released after cellular stress and/or activation, and purinergic signalling has been shown to modulate inflammation and immunity. In the extracellular space, ATP is rapidly hydrolysed in a stepwise manner to ADP, AMP (adenosine monophosphate) and adenosine by ectoenzymes. Adenosine is then irreversibly hydrolysed to inosine by adenosine deaminase (ADA). Extracellular ATP (eATP) signals through both ATP-gated ion channels P2X and G protein-coupled receptor (GPCR) P2Y membrane receptors, whereas ADP signals through P2Y receptors and adenosine through P1 receptors (or A receptors). P2Y receptors and A receptors may be coupled to the Gq protein, which activates phospholipase C-beta (PLC-β), to the stimulatory G (Gs) protein, which stimulates adenylyl cyclase inducing an increase in cyclic AMP (cAMP) levels, or to the inhibitory (Gi) protein, which inhibits adenylyl cyclase. Extracellular adenosine level is the result of adenosine production from extracellular ATP and ADP, its degradation into inosine and its reuptake by cells. Both ATP and adenosine can be transported outside of the cell via diffusion or active transport, whereas only adenosine can enter the cells through adenosine transporters. Most cells possess equilibrative and concentrative nucleoside transporters, whereas only adenosine can enter the cells through adenosine transporters.
concentrative adenosine transporters (respectively, ENTs and CNTs), which allow adenosine to quickly cross the plasma membrane. Intracellular adenosine is converted to ATP via phosphorylation steps mediated by adenosine kinase (AK) and AMP kinase (AMPK). The basal physiological level of extracellular adenosine has been estimated to be in the range of 30–200 nM. ATP-derived adenosine and its subsequent signalling through P1 receptors have beneficial roles in acute disease states. However, during tissue injury, elevated adenosine levels participate in the progression to chronic diseases by promoting aberrant wound healing leading to fibrosis in different organs including the lungs, liver, skin and kidney. In these conditions the blockade of adenosine signalling is beneficial. In murine models, ADA-knockout mice present high persistent adenosine levels, which lead to airspace enlargement and fibrosis, cardinal signs of COPD and IPF. Here we investigate in more detail the critical contribution of purinergic signalling in driving NLRP3 inflammasome activation in response to nanoparticles pointing out the effect of ATP, ADP, as well as adenosine and its receptors. We also identify ATP-derived adenosine as a potential activator of the inflammasome.

**Results**

Nano-SiO2 or nano-TiO2 particles trigger active ATP release and IL-1β secretion through purinergic signalling and pannexin/connexin hemichannel activity. We recently showed that nano-SiO2 and nano-TiO2, but not nano-ZnO, activate the NLRP3 inflammasome in human and murine macrophages. Here we studied whether active ATP release, purinergic signalling and connexin/pannexin channel activity are involved in inflammasome activation by nano-SiO2 and nano-TiO2. Using the ecto-ATPase inhibitor ARL67156 to limit ATP catabolism, we observed that nano-SiO2 (Figure 1a) or nano-TiO2 (Figure 1b), but not nano-ZnO (Figure 1c), causes an active release of endogenous ATP in primed THP1 macrophages, which peaks at 3–4 h and just precedes mature IL-1β secretion. Importantly, nano-TiO2, nano-SiO2 or nano-ZnO did not induce necrosis or apoptosis even after 6 h of stimulation (Figures 1d and e). We confirmed the importance of the inflammasome in IL-1β production in response to nanoparticles using THP1 cells stably expressing short hairpin ribonucleic acid (shRNA) against components of the inflammasome, the NLRP3 protein itself or the adaptor protein apoptosis-associated speck-like protein containing a CARD domain (ASC) (Figure 1f). By investigating the mechanisms of nanoparticle-induced ATP release leading to IL-1β secretion, we observed that specific inhibition of the P2X7 receptor (P2X7R) by A740003 at 10 μM led to partial inhibition of ATP release and IL-1β secretion by nano-SiO2 and nano-TiO2 (Figures 1g and h). Among several potential mechanisms of nucleotide release, we focused on the connexin and pannexin families, which are able to form hemichannels.20,21 The connexin/pannexin channel blockers carbenoxolone (Cbx) and flufenamic acid (FFA) significantly reduced both ATP and IL-1β releases (Figures 1g and h). Although unable to induce IL-1β by themselves, the addition of the nucleotides ATP or ADP or their stable derivatives ATPγS or ADPβS greatly increased IL-1β production by THP1 cells in response to nanoparticles (Figure 1i). Unlike what we observed with THP1 human monocyte/macrophage cell line, we were unable to measure significant ATP increase in the supernatant of stimulated murine bone-marrow-derived macrophages (BMDMs). This might probably be owing to the fastest ATP degradation by these cells as proposed.

However, the use of two different P2R antagonists, suramin and periodate-oxidised ATP (oATP), dose-dependently led to the reduction of IL-1β production induced by nano-SiO2 or nano-TiO2 (Figure 2a). Cbx and FFA also induced the reduction of IL-1β release (Figure 2b). Western blotting analysis confirmed that nano-SiO2 or nano-TiO2 triggers the cleavage of pro-IL-1β into the mature 17 kDa IL-1β form and its secretion in primed BMDMs. The addition of oATP, A740003, Cbx or FFA strongly reduced the secretion of mature IL-1β (Figure 2c). Similarly, the cleavage of procaspase-1 into the secreted mature p10 subunit was reduced in the presence of oATP, Cbx or FFA (Figure 2d), confirming that NLRP3 inflammasome activation depends on purinergic signalling and connexin/pannexin channels.

**Nanoparticles induce IL-1β secretion through metabotropic P2Y receptor signalling.** To identify more precisely the purinergic receptors involved, we performed quantitative mRNA expression analysis of P2 purinergic receptors. P2Y2 receptor (for ATP/UTP) mRNA level was increased after nano-SiO2 or nano-TiO2 particle stimulation, whereas P2Y1 receptor (ADP) mRNA level was increased only after nano-SiO2 stimulation (Figure 3a). In contrast, mRNA levels of P2Y7 (ATP), P2Y4 (UTP), P2Y6 (UDP) or P2Y12 (ADP) receptors were slightly reduced after nano-SiO2 stimulation (Figure 3a) and also P2Y12 receptor after nano-TiO2 stimulation. Deficiency in the ATP ionotropic P2X7 or P2X4 did not lead to significant impairment in IL-1β production by BMDMs upon nanoparticle stimulation (Figure 3b). Deficiency in the ATP/UTP metabotropic P2Y2 receptor, notably involved in cell chemotaxis in response to ATP leakage, promoted a slight decrease in IL-1β production (Figure 3c). In addition, we found that P2Y1 receptor antagonist MRS2500 (Figure 3d) decreased nano-SiO2-, but not nano-TiO2-, induced mature IL-1β secretion, whereas P2Y6 receptor (UDP) antagonist MRS2578 (Figure 3e) and P2Y12 receptor (ADP) antagonist MRS2395 (Figure 3f) had no effect on nanoparticle-induced IL-1β secretion. Altogether, these results suggest that P2Y1 (ADP) and P2Y2 (ATP/UTP) receptors are involved in the activation of the NLRP3 inflammasome by nano-SiO2.

**Nanoparticles induce mature IL-1β secretion through adenosine and P1 receptors signalling.** We stimulated murine macrophages in the presence of the ATP-consuming enzyme apyrase grade VII, which hydrolyses ATP and ADP into AMP. Apyrase did not abrogate IL-1β secretion induced by nano-TiO2 or nano-SiO2 but, on the contrary, slightly increased it (Figure 4a). Then, in the presence of the adenosine deaminase (ADA), IL-1β secretion by nano-SiO2 or nano-TiO2 was greatly reduced (Figure 4b). Similarly, we stimulated THP1 cells with nano-SiO2 in the presence of...
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Figure 1  Nano-SiO2 or nano-TiO2 particles trigger active ATP release and IL-1β secretion through purinergic signalling and pannexin/connexin hemichannel activity. Nano-SiO2 (a) or nano-TiO2 (b) triggered active release of ATP in the supernatant by PMA-primed THP1 that peaks between 3 and 4 h. This ATP release was correlated with a secretion of IL-1β (a,b). Nano-ZnO did not induce ATP release or IL-1β secretion (c). Apoptotic (PI−/annV+) and necrotic (PI+/annV−) cell death of primed THP1 was monitored using the Annexin/PI staining (d,e). ARL67156 (50 μM) was added to the supernatant during stimulation to limit ATP catabolism (a–c). IL-1β secretion by nano-SiO2 or nano-TiO2 was attenuated in THP1 cells stably expressing shRNA directed against ASC (sh ASC) or NLRP3 (sh NLRP3) in comparison with THP1 transfected with lamin-specific shRNA (sh CTL) (f). Nano-ZnO did not induce IL-1β secretion after 4 h of stimulation (f). Specific inhibition of P2X7R by A740003 partially decreased ATP release and IL-1β secretion by PMA-primed THP1 after 4 h nanoparticle stimulation (g,h). Connexin/pannexin channel blocker carbenoxolone (Cbx) and connexin channel blocker flufenamid acid (FFA) reduced both ATP release and IL-1β secretion upon nano-SiO2 or nano-TiO2 (g,h). PMA-primed THP1 stimulated for 4 h with 200 μM ATP, ADP or their stable derivatives ADP/S or ADP/S greatly increased IL-1β production in response to nanoparticles, whereas these nucleotides had no effect alone (i). Nanoparticles are at the concentration of 250 μg/ml (a–h) or 125 μg/ml (i). Data are representative of 2–4 independent experiments. Data are mean ± S.D. of triplicates, compared between untreated and nanoparticle-stimulated THP1; *** and ααα, P ≤ 0.001 for THP1, βββ, P ≤ 0.001 for THP1 sh CTL, γγγ, P ≤ 0.001 for THP1 sh ASC, δδδ, P ≤ 0.001 for THP1 sh NLRP3 (f). Data are mean ± S.D. of triplicates, compared between nanoparticle-stimulated THP1 and nanoparticles plus inhibitor or agonist; *** and ααα, P ≤ 0.001 for nano-SiO2 and nano-TiO2 stimulated THP1, respectively (g–i).
Figure 2  Nano-SiO$_2$- or nano-TiO$_2$-induced IL-1$\beta$ in mouse macrophages is dependent on purinergic signalling. IL-1$\beta$ production by LPS-primed BMDMs stimulated for 6 h with nano-SiO$_2$ or nano-TiO$_2$ was dose dependently decreased by P2R antagonists suramin (200, 400 or 600 $\mu$M) and oATP (100, 200 or 400 $\mu$M) (a). Cbx (50 or 100 $\mu$M) and FFA (25, 100 or 250 $\mu$M) significantly reduced IL-1$\beta$ release by murine macrophages (b). Western blotting analysis of LPS-primed BMDM supernatants (SN) confirmed that FFA (100 $\mu$M), Cbx (50 $\mu$M) oATP (200 $\mu$M) or A740003 (100 $\mu$M) strongly reduced the secretion of the mature 17kD IL-1$\beta$ form mIL-1$\beta$ in response to nano-SiO$_2$ or nano-TiO$_2$ (c). Similarly FFA, Cbx or oATP significantly reduced autoproteolytic cleavage of the pro-caspase-1 into the secreted p10 subunit (d). Stimulation of BMDMs with ATP (5 mM, 45 min) induced mature IL-1$\beta$ (c) and caspase-1 (d) releases in the supernatant, whereas nano-ZnO did not induce these cleavages (c,d). BMDM extracts (XT) prepared from the same experiments were blotted with anti-$\beta$-actin, anti-pro-IL-1$\beta$ and anti-pro-caspase-1 for control (c,d). Molecular-weight markers are shown at the right (c,d). BMDMs were stimulated with 250 $\mu$g/ml nanoparticles during 6 h. Data are representative of three independent experiments. Data are mean $\pm$ S.D. of triplicates, compared between nanoparticle-stimulated THP1 and nanoparticles plus inhibitor; *** and $\alpha\alpha\alpha$, $P \leq 0.001$ for nano-SiO$_2$ and nano-TiO$_2$ stimulated THP1, respectively (a,b)
apyrase or ADA. Measurement of eATP levels showed that, even when ATP was degraded by apyrase, IL-1β secretion was still observed and even slightly increased (Figure 4c). When ADA was added to the nanoparticles, we noted a potent decrease in IL-1β and ATP levels probably owing to a shift in the balance of the ATP/ADP towards adenosine (Figure 4d). Next, the addition of the non-degradable pan-adenosine receptor agonist 5′-N-Ethylcarboxamidoadenosine (NECA; 0.3–30 μM) significantly increased nano-SiO₂-but not nano-TiO₂-induced IL-1β secretion (Figure 4e). In contrast, adenosine had no effect at these concentrations, but only increase IL-1β at higher concentrations.
(100–300 mM) probably because adenosine is rapidly degraded into inosine by ADA (not shown). In addition, IL-1β induced by nano-SiO₂ but not by nano-TiO₂ was slightly decreased in the presence of the CD73 inhibitor AMP-CP, suggesting that adenosine is more important for nano-SiO₂-induced IL-1β (Figure 4f). These results indicate that adenosine is more important for nano-SiO₂-induced IL-1β (Figure 4f).

**Figure 4** Nanoparticles induce mature IL-1β secretion through adenosine and P1 receptor signalling. LPS-primed murine BMDMs were stimulated for 6 h with nano-SiO₂ or nano-TiO₂ in the presence of increasing concentrations of apyrase grade VII (0.3, 1, 3 or 10 U/ml) (a). Nano-SiO₂- and nano-TiO₂-induced IL-1β were slightly decreased and then rapidly increased (a). LPS-primed BMDMs were also stimulated for 6 h with nano-SiO₂ or nano-TiO₂ in the presence of increasing doses of ADA (0.3, 1, 3 and 10 U/ml) (b). Apyrase grade VII or ADA alone had no effect on IL-1β secretion by murine macrophages (a,b). PMA-primed THP1 were stimulated for 6 h with nano-SiO₂ (250 μg/ml) in the presence of different concentrations of apyrase VII (c) or ADA (d). Nano-SiO₂-induced eATP decreased by increasing apyrase VII, whereas IL-1β remained elevated (c). ADA dose dependently decreased both nano-SiO₂-induced IL-1β and eATP secretions (d). Apyrase VII or ADA alone had no effect on IL-1β and eATP secretions by human macrophages (c,d). LPS-primed murine BMDMs were stimulated for 6 h with nano-SiO₂ or nano-TiO₂ in the presence of increasing concentrations of NECA (e) or AMP-CP (f). IL-1β secretion induced by nanoparticles was increased in the presence of NECA (e) and remained stable in the presence of AMP-CP (f). Data are representative of three independent experiments (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ns: not statistically different).
Adenosine induced NLRP3 inflammasome activation: P1 purinergic receptors mRNA expression showed that both A2A and A2B mRNAs are increased in the presence of nano-SiO₂ or nano-TiO₂, whereas A3 and A1 mRNA expression levels did not significantly change (Figure 5a). Moreover, we showed that the specific A2A (SCH58261), A2B (MRS1754) or the specific A3 (MRS1523) inhibitors decreased IL-1β secretion after nano-SiO₂ or nano-TiO₂ stimulation (Figures 5b–d). In contrast, the specific antagonist of A1 receptor (DPCPX) had no effect on IL-1β secretion (Figure 5e). These results identified adenosine as a crucial mediator of IL-1β secretion through the high-affinity A2A receptor and the low-affinity A2B and A3 receptors in response to nanoparticle activation in murine macrophages.

Nanoparticles trigger NLRP3 inflammasome through the activation of PLC-InsP3 and inhibition of ADCY-cAMP pathways. We investigated pathways leading to inflammasome downstream of purinergic receptors. Both P2Y and P1 receptors belong to the GPCR family acting through numerous signalling cascades and have been linked to inflammation.24 P2Y1, P2Y2, A3 and A2B receptors involved in nanoparticle-mediated inflammasome activation can be coupled to the heterotrimeric G proteins of the Go family that activate phospholipase C-β (PLC-β). We show that the inhibitor of PLC-β, U73122, blocked nanoparticle-induced IL-1β secretion (Figure 6a). PLC-β is able to hydrolyse phosphatidylinositol-4, 5-bisphosphate into diacylglycerol (DAG), activating the protein kinase C and the production of the inositol trisphosphate (InsP3), which in turn causes an increase in cytosolic Ca²⁺ by binding to InsP3 receptors located in the endoplasmic reticulum. As intracellular Ca²⁺ (iCa²⁺) increase was shown to directly activate the NLRP3 inflammasome,25 we analysed the effect of 2-APB, a molecule chelating and hence blocking the increase of iCa²⁺. We observed that 2-APB strongly inhibited nanoparticle-induced IL-1β secretion (Figure 6b). Moreover, adenosine receptors can also be coupled to the Gs family activating ADCY or the Gi/o family inhibiting ADCY with subsequent augmentation or reduction of cyclic AMP (cAMP). As cAMP was shown to bind and suppress NLRP3 inflammasome activation directly,26 we examined the involvement of ADCY in nanoparticle-induced IL-1β secretion. The addition of the ADCY activator forskolin dose dependently inhibited nanoparticle-induced IL-1β secretion (Figure 6c), whereas the addition of the ADCY inhibitor SQ22536 had no effect (Figure 6d). Collectively, these results indicate that nanoparticles trigger the NLRP3 inflammasome plemson through both activation of PLC-InsP3 and inhibition of ADCY-cAMP pathways.

Adenosine induces IL-1β secretion and ATP release in THP1 human macrophages. We observed that high concentrations of adenosine (100 μM), which does not correspond to adenosine receptor affinities, enhanced ATP release and IL-1β secretion on nanoparticles in THP1 macrophages (Figure 7a). Moreover, adenosine at 5 mM was alone able to trigger ATP release and IL-1β secretion, whereas 100 μM adenosine had no effect (Figure 7a). IL-1β secretion induced by nanoparticles plus adenosine (5 mM) or adenosine alone in the presence of the specific caspase-1 inhibitor Z-YVAD-fmk was greatly reduced (Figure 7c). Moreover, IL-1β secretion in response to a high dose of adenosine was not induced in THP1-expressing shNLRP3 or shASC (Figure 7d). Intracellular and extracellular adenosine levels are regulated by equilibrative nucleoside transporters (ENTs) present at the cell membrane.7 Cellular adenosine uptake may lead to intracellular metabolism of adenosine in ATP by adenosine kinase and subsequent release of ATP and IL-1β secretion.6 To test these possibilities, we stimulated THP1 macrophages with increasing doses of adenosine, NECA, the non-degradable analogue of adenosine or inosine, the metabolite of adenosine degradation by ADA. We observed that even if high doses of adenosine triggered ATP release and IL-1β secretion, the same doses of NECA had no effect, demonstrating that metabolism of adenosine is necessary for these responses (Figure 7e). One possibility for adenosine to be metabolised is its hydrolysis in inosine by ADA. Nevertheless, high doses of inosine were unable to promote eATP and IL-1β release (Figure 7e). Millimolar doses of adenosine, NECA or inosine did not induce cell death (Figure 7e). The other possibility is an adenosine reuptake through ENTs and intracellular metabolism of adenosine in ATP by adenosine kinase. To test this hypothesis, we measured eATP and IL-1β induced by millimolar concentrations of adenosine in the presence of 5-iodotubercidin, a pharmacological inhibitor of both adenosine kinase and ENTs, and showed that eATP and IL-1β release was reduced (Figure 7f). We used NBMPR, a pharmacological inhibitor of ENTs that is specific for ENT1 (Ki = 0.4 nM) and ENT2 (Ki = 2.8 μM) at mM and μM concentrations, respectively. We observed that NBMPR inhibited eATP and IL-1β only at μM doses (Figure 7g). The mRNA expression of ENT2 was significantly increased by millimolar concentrations of adenosine (Figure 7h). Millimolar concentrations of adenosine significantly increased NLRP3 mRNA expression, supporting the role of adenosine in NLRP3 inflammasome activation (Figure 7i). In addition, intracellular ATP contents were increased after addition of extracellular adenosine at high doses (Figure 7j). Altogether, these results indicate that extracellular adenosine when present at a high concentration is recaptured by macrophages through ENT2 transporters and metabolised in ATP by adenosine kinase leading to ATP release and NLRP3 inflammasome activation.

Early nanoparticle-induced pulmonary inflammation depends on adenosine. Airway exposure to ultrafine particles is associated with strong infiltration of neutrophils in the airways in humans and mice.1,26 We instilled mice with nano-TiO₂ or nano-SiO₂ and visualised the presence of nanoparticle aggregates in lung parenchyma at 24 h (Figure 8a). Similarly to nano-TiO₂,1 nano-SiO₂ elicited a considerable neutrophil influx in the BALF at 6 h (Figure 8b), which correlated with the production of the neutrophil chemoattractant KC (Figure 8c) and the metalloproteinase-9 (MMP-9) present in neutrophil β2 gelatinase granules.
(Figure 8d). Moreover, myeloperoxidase (MPO) present in neutrophils α azurophilic granules (Figure 8e) and IL-1β levels (Figure 8f) were also increased in lung homogenates. As we observed that nano-SiO2 instillation induced a transient increase of ATP content locally and that ATP is rapidly degraded, adenosine can act as a danger signal involved in lung inflammation. We performed local adenosine depletion experiments in mice by using ADA, which catalyses

![Graph showing expression ratios of GOI/RNA 18S for A1, A2A, A2B, and A3 receptors.]  

**Figure 5**  A2A, A2B, and A3 receptors were involved in NLRP3 inflammasome activation. Quantitative PCR analysis of P1 receptor expression in LPS-primed BMDMs stimulated for 4 h with nano-SiO2 or nano-TiO2. A2A and A2B mRNA levels were greatly increased by nanoparticles, whereas A3 mRNA was slightly increased only by nano-TiO2, and A1 expression remained unchanged (a). The specific A2A (SCH58261), A2B (MRS1754) and A3 (MRS1523) inhibitors dose dependently decreased IL-1β production by LPS-primed BMDMs, whereas specific A1 inhibitor (DPCPX) had no inhibitory effect on IL-1β secretion (b–e). Inhibitor concentrations were 0.1, 0.3, 1, 3 and 10 μM for DPCPX and SCH58261, and 0.3, 1, 3, 10 and 30 μM for MRS1754 and MRS1523; inhibitors alone did not induce IL-1β production after 6 h (b–e). Nanoparticles were used at 200–250 and 300 μg/ml for nano-SiO2 and nano-TiO2, respectively. Data are representatives of 2–4 independent experiments (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ns: not statistically different)
the conversion of adenosine into inosine. We observed that local treatment with ADA reduced nano-SiO₂-induced acute inflammation, resulting in markedly reduced neutrophils (Figure 8b), KC (Figure 8c), MMP-9 (Figure 8d) contents in BALF and attenuated MPO (Figure 8e) and IL-1β levels (Figure 8f) in the lung. These data indicate that adenosine generated in vivo after nanoparticle-induced lung injury has an early pro-inflammatory role, as observed in vitro for macrophages.

Discussion

Despite extensive studies, the mechanisms of NLRP3 inflammasome activation are not well understood. Here we demonstrate that SiO₂ and TiO₂ nanoparticles promote the secretion of mature IL-1β by macrophages through the active release of ATP in the extracellular space. Interestingly, ATP but also its degrading products ADP and adenosine are important signalling molecules that allow NLRP3 inflammasome activation and mature IL-1β secretion in macrophages.

We showed that nano-SiO₂ and nano-TiO₂ but not nano-ZnO induced the active release of ATP through connexin and/or pannexin hemichannels leading to IL-1β secretion by macrophages. ATP release and IL-1β secretion depend on purinergic signalling and in particular on the P2X7R for ATP, contrarily to IL-1β secretion. The addition of nucleotides such as ATP or ADP, or their stable derivatives ATPγS or ADPβS, greatly increased IL-1β production by macrophages, indicating that ATP and ADP are involved in nanoparticle-mediated NLRP3 inflammasome activation. Importantly, nano-SiO₂ and/or nano-TiO₂ increased the mRNA expression of P2Y1 and/or P2Y2, whereas P2Y4, P2Y6, P2Y12 and/or P2X7 receptor mRNAs were downregulated in primed murine macrophages. When CD39, which degrades extracellular ATP into ADP and AMP, was inhibited in THP1 cells using ARL67156, eATP and IL-1β levels were increased. One can hypothesise that it favours IL-1β secretion through the ATP-specific P2Y2 receptor. On the contrary, in the presence of adenosine deaminase (ADA), IL-1β was greatly but not totally reduced, pointing out an additional important role for adenosine as a major ATP-derived signalling nucleoside promoting IL-1β secretion after nano-SiO₂ or nano-SiO₂ macrophage activation. Importantly, nano-SiO₂ and/or nano-TiO₂ increased mRNA expression levels of A2A, A2B and slightly of A3 receptors, but not of A1 receptors. In addition, specific A2A and A2B inhibitors decreased IL-1β secretion, confirming that adenosine is a crucial mediator of IL-1β secretion essentially through the high-affinity A2A and the low-affinity A2B adenosine receptors in response to nanoparticle activation in macrophages. In THP1 cells, adenosine degradation by ADA leads to a decrease of eATP, potentially due to the deficit of conversion of ADP/ATP from adenosine because of the increased degradation of adenosine to inosine. This reduction...
in ATP, ADP and adenosine allowed greatly reducing IL-1β secretion probably through signalling by P2Y2, P2Y1, A2a, A2b and A3 receptors. Downstream of purinergic receptors, which are coupled to G proteins (GPCR), we propose that nanoparticles trigger maturation of IL-1β through activation of PLC-β/InosP3 and inhibition of ADCY-cAMP pathways. This suggests that intracellular Ca²⁺ increase and cAMP decrease are second signals required for NLRP3 inflammasome activation. As ATP or ADP, participates in inflammasome activation via multiple receptor signalling pathways.

**Figure 7** Adenosine induces IL-1β secretion and ATP release in THP1 human macrophages. PMA-primed THP1 cells were stimulated during 6 h with 250 μg/ml nano-SiO₂, 500 μg/ml nano-TiO₂ and/or high concentrations of adenosine (Ado), and eATP and IL-1β releases were measured. Adenosine potentiated eATP release and IL-1β secretion upon nanoparticle stimulation without inducing change in cell viability (a). High doses of adenosine alone induced IL-1β secretion by PMA-primed THP1 in a dose-dependent manner (b). The caspase-1-specific inhibitor Z-YVAD-fmk (5 μM) remarkably reduced adenosine- and/or nanoparticle-induced IL-1β secretion; Z-YVAD-fmk alone had no effect (c). Adenosine-dependent induction of IL-1β secretion was reduced in THP1 sh NLRP3 or THP1 sh ASC but not in unmodified THP1 or THP1 sh CTL (d). PMA-primed THP1 cells were stimulated with increasing doses of adenosine, the non-metabolisable analogue of adenosine NECA or inosine (Ino), the product of adenosine hydrolysis by ADA, and eATP and IL-1β releases were measured at 6 h (e,f). Quantitative PCR analysis of ENT1, ENT2 and NLRP3 expression in PMA-primed THP1 stimulated for 6 h with increasing concentrations of Ado was performed (g,h). Intracellular ATP contents were measured 6 h after stimulation with high concentrations of adenosine (i). Data are representative of 2–3 independent experiments (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ns: not statistically different).
As extracellular ATP is degraded in adenosine within minutes, it is more likely that adenosine participates in early steps of NLRP3 inflammation activation rather than in sustained inflammasome activation, as suggested recently. Extracellular adenosine is finely regulated by adenosine degradation and cellular uptake. Nevertheless, adenosine accumulation can lead to chronic inflammation and diseases. Surprisingly, using millimolar concentrations (1–5 mM), we observed that exogenous adenosine alone was able to induce NLRP3 inflammasome activation.

Figure 7 (Continued)
Adenosine receptors which affinities to adenosine range between 1 nM to 20 μM are probably desensitized and not involved in response to adenosine 1–5 mM. Indeed, this adenosine receptor-independent effect was not mediated by millimolar doses of the non-degradable adenosine analogue, NECA, indicating that the inflammasome activation depends on adenosine metabolism and/or transport. Here, we show for the first time that exogenous adenosine at millimolar concentrations promoted NLRP3 expression and inflammasome activation and mature IL-1β secretion through cellular uptake and transformation into ATP by macrophages, leading to the increase of intracellular ATP content, subsequent ATP release and IL-1β secretion. Moreover, the functional activity of the nucleotide transporter ENT2 present at the cell membrane and of the intracellular adenosine kinase, which transforms intracellular adenosine into ATP, was required. Indeed, millimolar concentrations of adenosine were shown to efficiently increase intracellular ATP contents in primary lymphocytes and multiple cancer cell lines. In an attempt to summarise our data, we propose a model presented in Figure 9. As we showed very recently that the NLRP3 inflammasome is released as a particulate danger signal and phagocytosed by surrounding macrophages, one can imagine that adenosine uptake by these neighbouring cells may amplify the inflammatory response. Our in vitro results provide a new mechanism by which adenosine accumulation in vivo may sustain inflammasome activation, leading to chronic inflammatory diseases. Adenosine degradation in inosine by ADA could be a good strategy to attenuate adenosine-mediated inflammation via specific receptor signaling and to avoid adenosine accumulation and cellular uptake. To evaluate the role of adenosine in pulmonary inflammation and the potential anti-inflammatory effect of degradation of adenosine by ADA, we exposed mice to nano-SiO2 in the presence of ADA. Our results indicate that adenosine produced locally after nanoparticle exposure presents pro-inflammatory effects and that irreversible degradation of adenosine to inosine by ADA treatment reduced early

Figure 8 Pulmonary inflammation upon instillation of nanoparticles is partially dependent on adenosine production. C57BL/6 mice were instilled with saline solution or nano-TiO2 (5 mg/kg). Lung histology was performed 24 h after instillation to visualise aggregates of nanoparticles and inflammation in tissue (haematoxylin and eosin staining; original magnification, ×1000; micrographs are representatives of 5 mice per group) (a). Simultaneously to the instillation of vehicle or nano-SiO2, mice were treated or not intraperitoneally with adenosine deaminase (ADA, 5 U/mouse). Inflammation parameters were investigated at 6 h. Neutrophil counts (b) KC (c) and MMP-9 (d) contents were measured in the BALF. Myeloperoxidase (MPO) level (e) and IL-1β secretion (f) in lung homogenates were measured. Data are representative of three independent experiments. (*P≤0.05, **P≤0.01, ***P≤0.001)
pulmonary inflammation. Several studies indicated that extra-cellular adenosine can rise from baseline to high local concentrations in chronic diseases, adenosine being pro-inflammatory.\(^8,10\)–\(^15\) Our study supports the idea that adenosine can act as a pro-inflammatory mediator in certain circumstances. Moreover, we demonstrate that depending on its microenvironment concentration adenosine acts through different mechanisms, in particular adenosine-receptor signalling or adenosine cellular reuptake. Altogether, this indicates that signalling through adenosine is finely tuned and may be involved in both pro-inflammatory of anti-inflammatory processes. Nanoparticles are known to exacerbate respiratory diseases such as asthma and COPD.\(^31\) Their toxicity or inflammatory effects depend on nanoparticle shape and size and the amount of metal ion released.\(^32\) Cobalt-chromium nanoparticles were shown to induce human fibroblast damages without crossing the plasma membrane, through transmission of ATP via hemichannels and pannexin channels and intercellular signalling.\(^33\) Nevertheless, the role of adenosine was never described in nanoparticle-mediated damage or inflammation.

In conclusion, after tissue injury, adenosine may activate the inflammasome through membrane receptor signalling. In case of chronic inflammation, adenosine may accumulate and act via its cellular uptake and conversion into intracellular ATP, allowing amplifying and/or prolonging inflammasome activation. This may explain why sustained high adenosine levels are pro-inflammatory. Our findings may provide new therapeutic approaches to control chronic inflammation by inhibiting nucleotide receptors, nucleoside transporters and/or adenosine kinase activation.

### Methods

#### Reagents.

Nano-SiO\(_2\) and nano-TiO\(_2\) were purchased from IoLiTec (Heilbronn, Germany), and nano-ZnO is a gift from Dr. Amir Yazdi (Lausanne, Switzerland). Nanoparticles were sonicated for 30 min and used at a concentration of 125–500 \(\mu\)g/ml in vitro at a concentration of 5 mg/kg in vivo, as mentioned. A74003 is a gift from Dr. F. Rassendren (Montpellier, France). Adenosine deaminase (ADA) (AS168), ADP, ADP/\(\beta\)S, adenosine (Ado), apyrase grade VII (A6535), ARL67156, ATP, ATP/\(\beta\)S, ATP, carbeneoxolone (Cbx), DPCPX, flufenamic acid (FFA), inosine (Ino), MRS1523, MRS1754, MRS2395, MRS2578, periodate-oxidised ATP (oATP), phorbol 12-myristate 13-acetate (PMA), SCH58261 and U73122 were from Sigma (St. Quentin Fallavier, France); suramin was from VWR (Fontenay-sous-bois, France); LPS (lipopolysaccharide from Escherichia coli, serotype 055:B5) was from Invivogen (Toulouse, France); 2-APB, 5-iodotubercidin, Forskolin, MRS2650, NBMPR and Z-YVAD-fmk were from Tocris (Bristol, UK) and NECA and SQ22536 were from Merck Millipore (Nottingham, UK).

#### Nano-SiO\(_2\) and nano-TiO\(_2\) in saline (5 mg/kg) or vehicle alone was administered by intranasal instillation under light ketamine (Imalgène 1000, 1.25 mg/ml) and xylazine (Rompun 0.1%) anaesthesia. Bronchoalveolar lavage (BAL) and lung tissue were assayed after 6 h. The lungs were homogenised in a solution containing 10 mM potassium phosphate and 0.1 mM EDTA (Sigma), centrifuged at 10 000 r.p.m. for 10 min and the supernatants were stored at \(-20^\circ\)C for further analysis. BAL was performed as previously described.\(^34\) Differential cell counts were performed by counting an average of 250 cells on cytopsin preparations (Shandon CytoSpin 3, Thermo Scientific, Illkirch, Germany), and nano-ZnO is a gift from Dr. Amir Yazdi (Lausanne, Switzerland). Nanoparticles were sonicated for 30 min and used at a concentration of 125–500 \(\mu\)g/ml in vitro at a concentration of 5 mg/kg in vivo, as mentioned. A74003 is a gift from Dr. F. Rassendren (Montpellier, France). Adenosine deaminase (ADA) (AS168), ADP, ADP/\(\beta\)S, adenosine (Ado), apyrase grade VII (A6535), ARL67156, ATP/\(\beta\)S, ATP, carbeneoxolone (Cbx), DPCPX, flufenamic acid (FFA), inosine (Ino), MRS1523, MRS1754, MRS2395, MRS2578, periodate-oxidised ATP (oATP), phorbol 12-myristate 13-acetate (PMA), SCH58261 and U73122 were from Sigma (St. Quentin Fallavier, France); suramin was from VWR (Fontenay-sous-bois, France); LPS (lipopolysaccharide from Escherichia coli, serotype 055:B5) was from Invivogen (Toulouse, France); 2-APB, 5-iodotubercidin, Forskolin, MRS2650, NBMPR and Z-YVAD-fmk were from Tocris (Bristol, UK) and NECA and SQ22536 were from Merck Millipore (Nottingham, UK).

#### Mice.

C57BL/6 wild-type mice were bred in our animal facility (CNRS, Orleans). The animals used were eight to ten weeks old, and they were kept in isolated and ventilated cages. All animal experiments complied with the French Government's ethical and animal experiment regulations.

#### Lung inflammation model.

Nano-SiO\(_2\) or nano-TiO\(_2\) in saline (5 mg/kg) or vehicle alone was administered by intranasal instillation under light ketamine (Imalgène 1000, 1.25 mg/ml) and xylazine (Rompun 0.1%) anaesthesia. Bronchoalveolar lavage (BAL) and lung tissue were assayed after 6 h. The lungs were homogenised in a solution containing 10 mM potassium phosphate and 0.1 mM EDTA (Sigma), centrifuged at 10 000 r.p.m. for 10 min and the supernatants were stored at \(-20^\circ\)C for further analysis. BAL was performed as previously described.\(^34\) Differential cell counts were performed by counting an average of 250 cells on cytopsin preparations (Shandon CytoSpin 3, Thermo Scientific, Illkirch, Germany), and nano-ZnO is a gift from Dr. Amir Yazdi (Lausanne, Switzerland). Nanoparticles were sonicated for 30 min and used at a concentration of 125–500 \(\mu\)g/ml in vitro at a concentration of 5 mg/kg in vivo, as mentioned. A74003 is a gift from Dr. F. Rassendren (Montpellier, France). Adenosine deaminase (ADA) (AS168), ADP, ADP/\(\beta\)S, adenosine (Ado), apyrase grade VII (A6535), ARL67156, ATP/\(\beta\)S, ATP, carbeneoxolone (Cbx), DPCPX, flufenamic acid (FFA), inosine (Ino), MRS1523, MRS1754, MRS2395, MRS2578, periodate-oxidised ATP (oATP), phorbol 12-myristate 13-acetate (PMA), SCH58261 and U73122 were from Sigma (St. Quentin Fallavier, France); suramin was from VWR (Fontenay-sous-bois, France); LPS (lipopolysaccharide from Escherichia coli, serotype 055:B5) was from Invivogen (Toulouse, France); 2-APB, 5-iodotubercidin, Forskolin, MRS2650, NBMPR and Z-YVAD-fmk were from Tocris (Bristol, UK) and NECA and SQ22536 were from Merck Millipore (Nottingham, UK).
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France) after May-Grünwald-Giemsa staining (Difl Quick, Medion Diagnostics, Dübendorf, Switzerland) according to the manufacturer’s instructions. After BAL and lung perfusion, the large lobe was fixed and 3-μm sections were stained as described previously.35

ELISA. IL-1β, KC, MMP-9 and MPO levels were determined using ELISA assay kits (Mouse or human DuoSet, R&D system, Minneapolis, MN, USA) according to the manufacturer’s instructions.

THP1 culture and stimulation. Monocyte/macrophage THP1 cells are a gift from Dr. Amir Yazdi (Lausanne, Switzerland) and cultured in RPMI Medium 1640 (Gibco, Illkirch, France) with 10% fetal calf serum (Hyclone, Cramlington, UK) and penicillin/streptomycin (100 U/ml, Invitrogen). For experiments, THP1 were differentiated for 3 h with 0.5 μM PMA, washed and plated overnight (2·10⁵ cells/well). Cells were stimulated for indicated times, the supernatant was collected and protein content was measured (DC protein assay, Bio-Rad, Munich, Germany). Proteins were denatured by boiling (95 °C, 5 min), separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with a primary goat anti-IL-1β antibody (Sigma Aldrich) or rabbit anti-caspase-1 p10 (Santa Cruz Biotechnology) and proteins were detected with appropriate secondary antibody followed by enhanced chemiluminescence (ECL, Fisher, Illkirch, France).

Sh RNA THP1. THP1 stably expressing short hairpin RNA (shRNA) against lamin (‘sh CTL’), ASC or NLRP3 are kind gifts from Dr. Fabio Martinon (Lausanne, Switzerland) and were obtained as previously described (Pétrilli et al., 2007). shRNA THP1 cells were cultured in RPMI Medium 1640 (Gibco) with 10% fetal calf serum (Hyclone) and 4 μg/ml puromycin (Gibco). Cell priming and stimulation are the same as previously described for untransfected THP1 cells.

ATP measurement. Extracellular ATP in cell-free medium supernatant was quantified using ATP Life one step kit (Perkin Elmer, Courtaboeuf, France) according to the manufacturer’s instructions, and the luminescence produced was measured (Mithras, Mikrowin 2000 software, Berthold Technologies, Thoiry, France).

BMDM culture and stimulation. Primary BMDMs were obtained from femoral bone marrow as described.36 In brief, cells from femurs of C57BL/6 mice were isolated and cultured at 10⁵ cells/ml for 7 days in Dulbecco’s minimal essential medium (DMEM, Sigma) supplemented with 20% horse serum as a source of M-CSF. Three days after washing and culturing in fresh medium, the cell preparation contained a homogeneous population of macrophages. THP1 cells were cultured in RPMI Medium 1640 (Gibco, Illkirch, France) with 10% fetal calf serum (Hyclone, Cramlington, UK) and penicillin/streptomycin (100 U/ml, Invitrogen). For experiments, THP1 were differentiated for 3 h with 0.5 μM PMA, washed and plated overnight (2·10⁵ cells/well). Cells were stimulated for indicated times, the supernatant was collected and transferred to nitrocellulose membranes. The membranes were immunoblotted with a primary goat anti-IL-1β antibody (Sigma Aldrich) or rabbit anti-caspase-1 p10 (Santa Cruz Biotechnology) and proteins were detected with appropriate secondary antibody followed by enhanced chemiluminescence (ECL, Fisher, Illkirch, France).

Statistical analysis. Statistical evaluation of differences between the experimental groups was determined by ANOVA followed by Bonferroni’s test using the Prism 5.0 software (GraphPad). P-values <0.05 were considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements. We thank Fabio Martinon (Eptalines, Switzerland) for kind gifts of THP1 stably expressing short-hairpin RNA (shRNA), and Valérie Queyniaux and François Érard (Orleans, France) for scientific discussions. Grant support by the «Fonds de Dotation pour la Recherche en Santé Respiratoire», the «Agence Nationale de la Recherche» and «Conseil Général du Loiré».

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