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Abbreviation list

AA: antibiotic antimycotic
AP-1 : activator protein-1
APC : antigen presenting cells
APDC : ammonium pyrrolidinedithiocarbamate
AQP : aquaporin
ASC : apoptosis-associated speck-like protein containing CARD
BAL : broncho-alveolar lavage
BALF : broncho-alveolar lavage fluid
BHA : butylated hydroxyanisole
BMDC : bone-marrow-derived dendritic cells
BMDM : bone-marrow-derived macrophages
CA-074-Me : CA-074 methyl ester
CNT : carbon-nanotube
CXCL1 : chemokine (C-X-C motif) ligand 1
CXCL2 : chemokine (C-X-C motif) ligand 2
CXCR2 : chemokine (C-X-C motif) receptor 2
DAMP : damage-associated molecular pattern
DPI : diphenyleneiodonium
ER : endoplasmic reticulum
GM-CSF : granulocyte-macrophage colony-stimulating factor
HMGB1 : high mobility group box 1
HSP : heat shock protein
IL-x : interleukin-x
IL-1R : interleukin-1 receptor
IL-1RAcP : interleukin-1 receptor accessory protein
IL-1Ra : interleukin-1 receptor antagonist
Leu-Leu-OMe : L-leucyl-L-leucine methyl ester
M-CSF : macrophage colony-stimulating factor
MSU : monosodium urate
MWCNT : multi-walled carbon-nanotube

MyD88 : myeloid differentiation primary response (88)

NFκB : nuclear factor-κ B

NLR : nod-like receptor

NLRP3 : NOD-like receptor protein 3

P2X7R : P2X purinoceptor 7

PAMP : pathogen-associated molecular pattern

PM2.5 or PM10 : particles with diameters that are less than or equal to 2.5 or 10 microns in size respectively

RAGE : receptor for advanced glycation endproducts

ROS : reactive oxygen species

RVD : regulatory volume decrease

SYK : spleen tyrosine kinase

SWCNT : single-walled carbon-nanotube

TACE : TNF-α converting enzyme

TAK1 : TGF-β-activated kinase 1

TLR : toll-like receptor

TNF-α : tumor necrosis factor α

TRP : transient receptor potential cation channel

TRPM2 : transient receptor potential cation channel, subfamily M member 2

TRX : thioredoxin

TXNIP : thioredoxin-interacting protein

I. Introduction

This review report will be submitted to the journal of "Particle and Fiber Toxicology".

Abstract

The innate immune system is the first line of defense against inhaled particles. Macrophages serve important roles in particle clearance and orchestrating inflammation. Following recognition and internalization by macrophages, particles are endocytosed in vesicular phagolysosomes. Intracellular phagosomal leakage, redox unbalance and ionic movements induced by toxic particles result in inflammasome complex engagement, caspase-1 activation, biologically-active IL-1β release and finally inflammatory cell death termed pyroptosis. The overall effect is to drive a local inflammatory environment evolving, when particles persist in the lung, to deleterious chronic inflammation characterized by the sustained production of highly potent pro-inflammatory mediators and neutrophil accumulation. In this introduction, we summarize the emerging pathways and signals involved in the expression, maturation and secretion of IL-1β during these responses to particles. We also draw attention on particle characteristics promoting inflammasome activation, in particular for nanoparticles which possess a strong capacity to induce IL-1β processing.

Interleukin-1β (IL-1β) is a highly active cytokine playing an essential role in inflammation and tissue damage. An abundant literature has precisely depicted IL-1β regulations and functions during infectious and inflammatory diseases. Various IL-1β-targeting agents such as IL-1 receptor antagonist, soluble decoy receptor and neutralizing monoclonal anti-IL-1β antibody have been used with success in several inflammatory diseases such as rheumatisms, diabetes and cancers in humans (1,2). The different components of the IL-1β signaling pathway have been largely elucidated and comprise the IL-1 type 1 receptor (IL-1RI), its co-receptor (IL-1RAcP) and the adaptor molecule (MyD88). Research on IL-1β has recently been revitalized by the discovery of the inflammasome machinery (constituted of the caspase-1 enzyme, ASC adaptor and NLRP sensor) which allows the release of mature IL-1β from the immature and inactive IL-1β pro-form (3). It is also well-established that active IL-1β serves as a primary initiating signal to coordinate the mobilization of immune cells to the damaged area caused by inflammation. The recruitment and traffic of neutrophils to the injured tissue is mediated by an IL-1β-dependent production of neutrophil-attracting chemokines such as CXCL1 and CXCL2 produced by resident cells such as fibroblasts and epithelial cells. IL-1β can also directly orchestrate the expansion and activation of macrophages by promoting the production of macrophage-related cytokines such as M-CSF and GM-CSF (4). Intense research on the IL-1β/inflammasome axis has resulted in a new concept of cell death pathway dependent on caspase-1 and known as pyroptosis. Pyroptosis induces cell swelling and plasma membrane rupture with release of cytoplasmic content leading to inflammatory reaction (5). These mechanism and outcome of pyroptosis are distinctly different from the aspects of apoptosis, which, in contrast, prevents inflammation.

Interestingly, early research on IL-1β revealed that monocytes and macrophages exposed to silica or asbestos particles strongly release IL-1β. A major role of IL-1β in asbestosis or silicosis was proposed more than 30 years ago (6-8). These seminal studies in lung toxicology showed that IL-1β produced by particle-exposed macrophages induces, in concert with TNF-α, the production of chemokines by epithelial cells and mediates particle-induced inflammatory lung responses (9-11). The *in vivo* role of IL-1β and its activation in the development of neutrophilic inflammation induced by particle has been extensively investigated and demonstrated by inhibiting the IL-1 signaling pathway (12,13) i.e. by IL-1Ra (IL-1 receptor antagonist) administration (14), deleting IL-1

receptor (13) or when inflammasome components were blocked ; i.e. by using ASC-, NLRP3-, caspase-1-deficient mice (13,15,16). The requirement of the IL-1β/inflammasome in lung inflammation has been extended to nanoparticles. Indeed, IL-1β cleavage and secretion after MWCNT (multi-walled carbon-nanotube), nickel oxide or air ambient nanoparticle exposure has been correlated to lung pathology induced by these particles (17-19). IL-1R-deficient mice developed less lung inflammation in response to titanium nanoparticles and MWCNT compared with wild-type mice (20-22).

Particle-induced inflammasome activation is most of the time described in macrophages, dendritic cells or monocytes. However, structural cells of various body compartments significantly participate to the initial inflammatory response after particle exposure by secreting mature IL-1β and amplify inflammation at the site of damage (21,23,24). For instance, activation of inflammasome takes also place in airway epithelial cells in response to particles (19,25-28). Carbon black nanoparticles induced IL-1β release by primary rat epithelial lung cells, cardiomyocytes and cardiofibroblasts, which then amplifies the subsequent pro-inflammatory cytokines production (24,27). Mesothelial cells also responded to asbestos by activating inflammasome (14,29,30).

The observation that diverse particles are able to activate caspase-1 and the inflammasome allowed considering pyroptosis as a new cell death pathway in particle toxicology. Indeed, cell death induced by nanometric carbon black was mainly dependent on caspase-1 in RAW264.7 macrophages (31). MWCNT also induced caspase-1 dependent cell death in primary human bronchial epithelial cells (26). However, other studies demonstrated that alum and silica particle-induced cell death was independent of caspase-1 in macrophages (32,33).

This introduction summarizes the current knowledge on the main cell signals responsible of mature IL-1β release after particle exposure. The first part delineates the intracellular events induced by particles (called signal 2) that result in NLRP3 inflammasome assembly. We particularly describe the physicochemical features of the particles which determine IL-1β processing. We finally recapitulate the possible endogenous mediators that prime inactive pro-form of IL-1β (pro-IL-1β) expression and function as signal 1 during the early response to particles.

Signals and mechanisms implicated in particle-induced IL-1β/inflammasome activation

The NLRP3 inflammasome is a multimeric complex mainly constituted by the sensor protein NLRP3, the adapter protein Apoptosis-associated Speck-like protein containing CARD (ASC), and the cysteine protease caspase-1. Detection of the stimuli triggers a conformational change of NLRP3 that leads to its oligomerization, recruitment of ASC through homotypic protein–protein interactions of the pyrin domains and subsequent recruitment and auto-activation of caspase-1 (34). NLPR3 inflammasome activation was first demonstrated by using monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) crystals (13). Subsequently, a plethora of studies confirmed that diverse inflammatory particles are able to activate the NLRP3-inflammasome machinery. However, it appeared obvious that the NLRP3-protein complex cannot be directly triggered by such a variety of particles. Intensive investigations exploring indirect cellular events (also called signal 2) have led to propose at least three main processes accounting for inflammasome activation in response to particles: (1) lysosomal damage and subsequent lysosome content release, (2) intracellular redox unbalance, organelle damage and ROS detoxifying protein modification and (3) intracellular ionic concentration and localization modifications (Figure 1).

a. Critical function of lysosomal impairment

Endocytosis

The importance of endocytosis for particle-induced inflammasome activity has been first demonstrated by using the actin polymerization inhibitors cytochalasin or lactrunculin in mononuclear and dendritic cells exposed to silica particles (12,35-37). The essential role of actin-mediated endocytosis was further robustly established in response to various particles such as aluminum salt-constituted (35,38-40), titanium dioxide (TiO_2) (41) or polymeric particles (40,42,43) and asbestos or CNT fiber-shaped particles (44,45). However, the simple diffusion across cell membrane of nanomaterials can be sufficient to activate inflammasome in keratinocytes (21). Additionally, cellular entry of particle is not an obligatory step for the processing and the secretion of IL-1β after particle exposure. Indeed, a direct contact between particles and the cell membrane can be sufficient for

activating the inflammasome complex in macrophages or dendritic cells. Macrophages significantly released IL-1β even if they were exposed to non-phagocytized polymethylmethacrylate microspheres or MSU crystals (46,47). In comparison with internalized particles, cell membrane-associated silica nanoparticles highly induced IL-1β release by macrophages (48). Cell contact of non-phagocytable polystyrene beads (40) or surface-glued alum crystals also resulted in IL-1β secretion by dendritic cells without internalization (49). Finally, lipid raft formation at cell membrane surface also leads to IL-1β secretion in response to titanium, alum and polymeric particles (41,42,46,49). Thus, it appears that particle recognition and/or endocytosis are competent to cause inflammasome and IL-1β processing.

Damage of lysosome

Lysosomal rupture induced by soluble lysosome-destabilizing agents such as L-Leucyl-L-leucine methyl ester (Leu-Leu-OMe) is sufficient for inflammasome activation (12). A clear correlation has also been found between the lysosomolytic ability of micro- and nanoparticles and inflammasome activation potency. Only silica particles accountable for a robust lysosomal destabilization induced IL-1β secretion (36,50). Implication of lysosomal leakage in inflammasome activation is now demonstrated in response to diverse silica particles in macrophages (36,37,48,51) or dendritic cells (40).

Particles are endocytosed in vesicular phagosome which then undergoes fusion with lysosomes, forming a phagolysosome. The fusion of particle-containing vesicles with lysosomes leads to acidic pH and ROS production in an attempt to digest particles. These two biological processes can be implicated in lysosomal destabilization and inflammasome activation. Indeed, inhibition of endosomal acidification by bafilomycin A1 successfully reduced lysosomal leakage and the subsequent IL-1β release in macrophages or dendritic cells exposed to silica, titanium, alum or polymeric particles (12,36,37,40,41,51). Also, pretreatment of silica-exposed macrophages with the ROS scavenger butylated hydroxyanisole (BHA) strongly reduced endosomal rupture and subsequent IL-1β release (37).

Cytosolic leakage of cathepsin B after lysosomal damage causes NLRP3 activation. The CA-074-Me cathepsin B inhibitor strongly affects IL-1β maturation induced by several

fibrous and non-fibrous particles such as silica (12,35,37,51,52), titanium (21,41,52,53), aluminum (35,38,40) or polymeric particles (40,42), asbestos or CNT (54,55). However, the role of cathepsin B in particle-induced inflammasome activation is still a matter of debate. Indeed, cathepsin B-deficient bone marrow-derived macrophages (BMDMs) did not secrete less IL-1β and did not cleave caspase-1 less than WT cells in response to alum or silica (44,47). Similarly, cathepsin B deficiency did not reduce IL-1β release induced by liposomes in BMDM cultures whereas a cathepsin B inhibitor did (56). This discrepancy could be explained by the lack of specificity of cathepsin inhibitors used or by the redundancy between cathepsins. How cathepsin release could be implicated in caspase-1 and inflammasome activation is also not clear. Cathepsin B could directly process pro-caspase-1 (57) but the general intracellular protein degradation by cathepsin B that in turn induces caspase-1 activation represents a more probable mechanism. In particular, cathepsin B leakage could also be involved in mitochondrial damage (58) or potassium efflux (39), two processes that are mandatory in inflammasome activation (see below).

Finally, the lysosome contains high concentrations of ions and their release upon lysosomal membrane damage may also explain inflammasome activation. In particular, lysosomal damage and leakage can lead to deleterious cytosolic calcium concentrations and inflammasome mobilization (59). In conclusion, particle-induced lysosomal damages induce multiple cellular perturbations that combine to maximize inflammasome assembly in response to particles.

Autophagy

Lysosomal functions are not limited to endocytosed material digestion but also concern endogenous material elimination by a process called autophagy (60). Autophagy is necessary to degrade and recycle damaged proteins and organelles, such as mitochondria. Inhibition of autophagy/mitophagy induces accumulation of ROS-generating damaged mitochondria and potentiates IL-1β processing in response to MSU crystals and soluble nigericin (61). Particles impair normal autophagolysosome formation and induce inflammasome activation by the following events (60). First, accumulation of non-destructible particles in phagolysosome leads to lysosome overload and prevents their

fusion with the autophagosome (62). Secondly, once in the cytosol, particles can bind to actin and impair cytoskeleton remodeling that is necessary for maturation and function of the autophagosome (63). Finally, particles present in the cytosol can diffuse through the membrane and be incorporated in autophagic vesicles leading to their destabilization. For instance, nanoparticles can be incorporated to autophagic vesicles when ubiquitinated or associated with ubiquitinated proteins (64,65). Altogether, these recent observations support that particles impair proper autophagic process, favoring damaged mitochondria accumulation and inflammasome processing.

b. Ionic modifications

Potassium

Decreased intracellular potassium concentration is mandatory for inflammasome activation. Reduction of intracellular potassium level induces a conformational change of NLRP3 allowing its activation (66). Additionally, potassium efflux could cause disruption of mitochondrial membrane potential (67) or ROS production (68). Potassium efflux has been observed in response to silica exposure before IL-1β release and its inhibition reduced IL-1β and caspase-1 activation in response to silica, alum, silver or polymeric particles, asbestos or CNT in macrophages or dendritic cells (15,16,39,40,43,45,52,56,69,70). How particle exposure leads to potassium efflux is still unknown. It has been suggested that plasma membrane damages or distortions caused by particle contact with cell surface may explain cell potassium leakage. Activation of the P2X7R cation-channel in response to ATP binding has also been implicated in particle-induced potassium efflux and inflammasome activation. Riteau and colleagues demonstrated that following silica or alum phagocytosis and lysosomal leakage, cellular ATP gained extracellular environment where it can bind to P2X7R and activate the inflammasome (71). IL-1β release in response to latex beads was also reduced in presence of apyrase (ATP diphosphohydrolase) or in P2X7R-deficient macrophages (43). However, the implication of ATP and P2X7R in potassium efflux in the context of toxic particles remains controversial since silica-induced IL-1β release by macrophages was not reduced by apyrase nor deficiency in P2X7R in other studies (70,72,73). Thus, the exact

mechanism by which potassium is release by particle-exposed cells still needs to be determined.

Calcium

Modification of free cytosolic calcium concentration has been strongly implicated in inflammasome activation in response to soluble activators (e.g. ATP) (59,74). Few studies have investigated calcium modification in cells exposed to particles and the role of this ion in inflammasome activation remains uncertain. It has been shown that alum crystals induce calcium mobilization from the endoplasmic reticulum that is required for NLRP3 inflammasome activation in BMDM cells (59). Extracellular calcium influx also affects intracellular calcium balance. Exposure to silica and alum increased free cytosolic calcium concentration by an extracellular entry through ROS-activated TRPM2 channel. Reduction of this influx by lowering extracellular calcium or suppressing TRPM2 channels leads to a partial decrease of IL-1β secretion (56,59). It is important to note that calcium is implicated in multiple cellular functions and could thus impact particle-induced inflammasome activation process at different levels. Indeed, actin polymerization and organelle trafficking necessary for phagolysosomal maturation are dependent of intracellular calcium movements. Thus, increased concentration of calcium could impact particle uptake and subsequent lysosomal damage. Potassium efflux necessary for inflammasome assembly could also be triggered by the activation of calcium-dependent potassium channels when cytosolic calcium concentrations are increased (75). Finally, high levels of cellular calcium can also induce mitochondrial dysfunction or trigger activation of TGF-β-activated kinase 1 (TAK1), both associated with inflammasome activation (59,66). In conclusion, it is probable that alteration of intracellular calcium homeostasis is involved in particle-induced inflammasome activation. However, the elucidation of the mechanism leading to this ionic dysregulation needs futures investigations in cells exposed to particles.

c. Oxidative stress

Increased cellular ROS production has been observed in response to most inflammasome activators. Interestingly, silica-induced ROS production was detected even in NLRP3-deficient macrophages, indicating that ROS production is upstream of inflammasome

assembly (16). The use of ROS scavengers such as N-acetylcysteine or ebselen, a glutathione peroxidase mimic, efficiently reduced IL-1β release and caspase-1 activation in response to particles such as silica, alum or asbestos in dendritic or mesothelial cells (29,52) and the deficiency in the ROS detoxifying protein thioredoxin (TRX) increased IL-1β maturation induced by silica and asbestos in macrophage cell lines (15). These data convincingly demonstrate that ROS formation is a crucial event in inflammasome processing in response to particles.

In addition to ROS produced intrinsically by the particles themselves, the NADPH oxidase pathway and the damaged mitochondria also lead to intracellular ROS production. Upon particle phagocytosis, phagosome-associated NADPH oxidase produces ROS that could be released in the cytosol upon lysosomal leakage. Inhibition of NADPH oxidase by using ROS inhibitors such as diphenyleneiodonium (DPI), ammonium pyrrolidinedithiocarbamate (APDC) or apocynin reduced IL-1β secretion or caspase-1 activation in response to silica, asbestos, CNT or titanium particles (15,16,37,41,44,54,56,76). The use of mice deficient in essential components of the membrane-associated phagocyte NADPH oxidase was however confusing. Cells lacking the $p22^{phox}$ expression had reduced inflammasome activation in response to asbestos whereas deficiency in $gp91^{phox}$ did not modify silica-induced inflammasome activation (12,15,44). Interestingly, mitochondrial ROS production during inflammasome activation has also been demonstrated after silica and alum treatment in macrophages (38,76). Altogether, these studies suggest that the enzymatic and cellular pathways leading to ROS-induced inflammasome activation are diverse, probably in function of the physicochemical properties of the particles.

How ROS activate NLRP3 is still debated but it is postulated that proteins modified by oxidative stress directly bind NLRP3. The complex formed by the ROS detoxifying protein thioredoxin (TRX) and thioredoxin-interacting protein (TXNIP) has also been proposed to link ROS and NLRP3 activation. Under normal conditions, TXNIP is associated with TRX. However, the presence of free radicals oxidizes TRX that cannot bind TXNIP anymore. TXNIP then interacts with and activates NLRP3. TXNIP deficiency in APC (Antigen Presenting Cell) reduced caspase-1 activation and IL-1β release induced by silica, asbestos and alum (15,29,61). The absence of TXNIP has also

been shown to prevent IL-1β release in a model of MSU-induced peritonitis (61). However, two studies failed to confirm requirement of TXNIP for inflammasome activation in response to silica and micrometric latex beads in BMDM (43,77). Finally, there is evidence that the sources of ROS are multiple and interconnected. Indeed, ROS released by particles or phagolysosomes directly or indirectly activate mitochondria to produce ROS. This amplification loop of free radical generation may explain why anti-oxidant cell defenses are supplanted after particle exposure and that the subsequent oxidative stress generated in cells activates inflammasome machinery.

d. Mitochondrial damage

Mitochondrial damage has been proposed as an important event in NLRP3 inflammasome activation in response to soluble activators (61,76) and has been associated with particle-induced inflammasome activation (43,48,69,78). Inhibition of damaged mitochondria clearance in BMDM exposed to latex beads leads to increased IL-1β release, probably due to uncontrolled ROS release (43). Under resting conditions NLRP3 localizes to endoplasmic reticulum (ER) structures in THP-1 macrophages but upon exposure to inflammasome-activating crystal such as alum, NLRP3/ER complexes and ASC are relocalized to mitochondria. The authors proposed that mitochondria recruit inflammasome components and favour their interactions. Additionally, voltage-dependent anion-selective channel protein 1 (VDAC1), a channel present at mitochondrial membrane and controlling calcium transfer from ER, was implicated in caspase-1 activation and IL-1β release in response to silica and alum, possibly via ROS production (61). Cardiolipin, a mitochondrial-specific phospholipid, bound and activated NLRP3 in macrophages exposed to silica and its depletion reduced IL-1β release (76). It is interesting to note that cathepsin, ROS and calcium release after lysosomal leakage participate to the mitochondrial damage induced by particles (58,79). Additionally, particles present in the cytosol after diffusion or lysosomal escape may directly affect normal mitochondria function which may result in inflammasome activation (69).

e. New mechanisms of particles-induced inflammasome activation

Macrophage swelling and subsequent regulatory volume decrease have been associated with NLRP3 inflammasome activation and IL-1β release in response to different stimuli

(52,66,80). Interestingly, cell volume modifications have been reported in the past in response to particle endocytosis (31,54,81). Recently, we demonstrated in murine macrophages that water movements through aquaporin (AQP), in particular AQP1, are necessary for inflammasome activation in response to micro- and nanoparticles. AQP was implicated in swelling and shrinkage of the cell to restore its homeostatic volume (82). Several mechanisms could explain the role of AQP in inflammasome activation. AQP mediates cytoskeleton rearrangement (83) necessary for particle endocytosis, intracellular vesicular trafficking and inflammasome components localization with filamentous actin (84-86). The reduction of AQP-controlling water flux and volume alterations probably affects potassium and calcium movements which are necessary for particle-induced inflammasome activation. For instance, AQP could be necessary for calcium channel TRP activation (87,88).

The ubiquitination process allows addressing protein to the proteasome for their elimination, and regulates inflammasome activity by targeting the degradation of inflammasome components by autophagy (89). For instance, NLRP3 ubiquitination reduced inflammasome activation in response to various activators such as silica crystals (90). On the other hand, it has been shown that the linear ubiquitination of ASC is necessary for silica-induced inflammasome activation in BMDM cells (91). Ubiquitination thus may repress or promote the particle-induced inflammasome machinery in function of the ubiquitinated protein and ubiquitination process considered.

Various kinases have been implicated in the pathway leading to IL-1β secretion after particle exposure (27,52,92,93). In particular, Spleen tyrosine kinase (SYK), a kinase regulating endocytosis and actin remodeling processes, has been involved in inflammasome activation in response to polymeric particles, silica, alum, asbestos and carbon nanotubes (35,46,49,54). In dendritic cells, contact between cell membrane and uric crystals results in membrane lipid alteration that induces activation of SYK and inflammasome activation (46,49). TAK1, a kinase involved in TLR signaling and activated by intracellular Ca^{2+} variations, has also been involved in inflammasome activation in response to ATP and osmotic stress (66,94). Interestingly, this kinase has also been involved in inflammasome activation consecutive to lysosomal rupture induced by Leu-Leu-OMe or uric crystals (95).

Recently, two different teams demonstrated that inflammasome activation leads to the release of ASC and NLRP3 that form functional oligomeric inflammasome particles. These complexes can be subsequently phagocytized by surrounding macrophages and trigger lysosomal damage and inflammasome activation. Additionally, ASC-NLRP3 complexes also form functional inflammasome in bystander macrophages after being internalized (96,97).

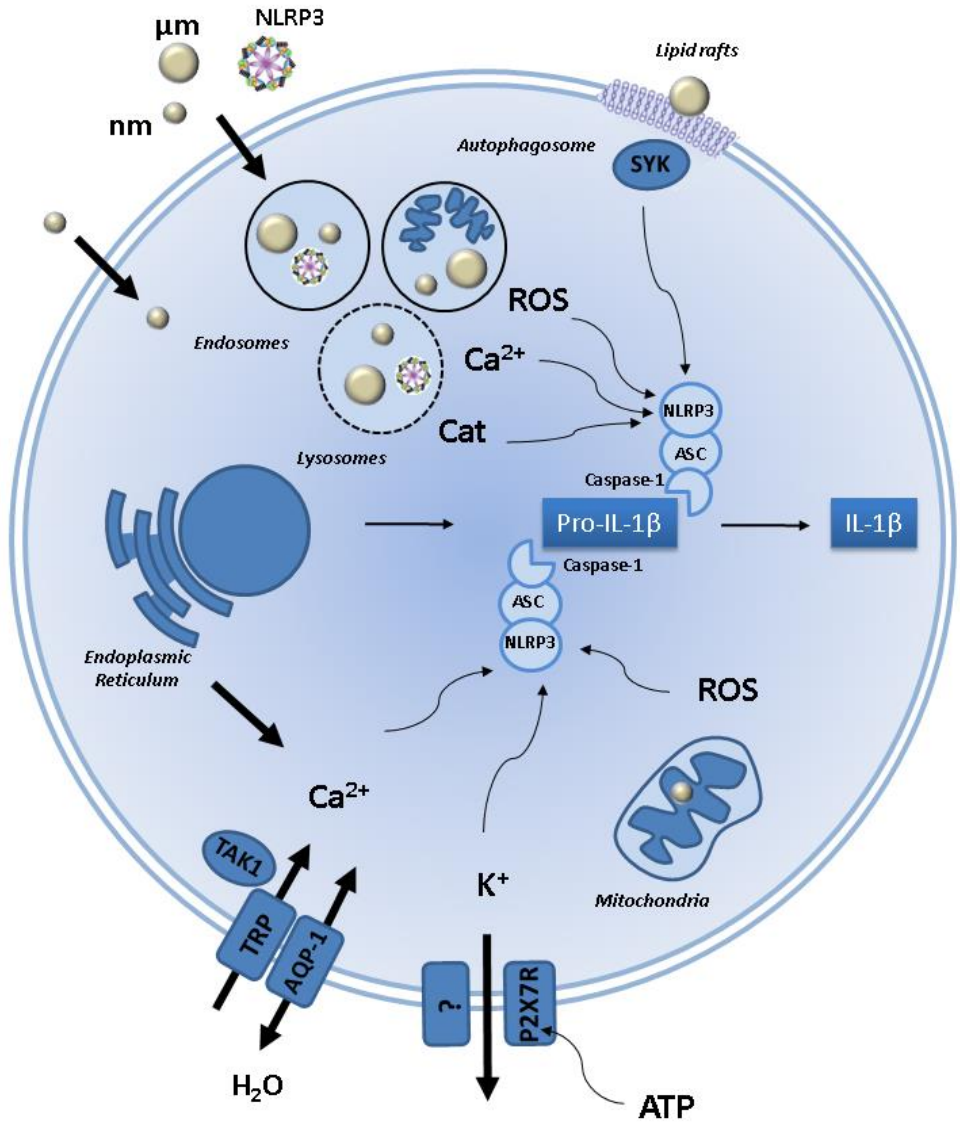


Figure 1 : **Cellular signals responsible of particle-induced inflammasome activation**

Inflammasome engagement after particle exposure results from various intracellular events (called signal 2) that are non-mutually exclusive. When endocytosed, nano- and micrometric particles or

exogenous NLRP3 complexes induce lysosomal destabilization and interfere with the autophagy/mitophagy resulting in the release of ROS, cathepsins or calcium in the cytosol. These vesicular leaking molecules promote inflammasome activation. Oxidative stress and active cathepsins modify undetermined proteic structures which are critical for NLRP3 inflammasome assembly. High calcium concentrations due to lysosomal and endoplasmic reticulum release or extracellular influx via TRP calcium-channels affect mitochondria which release high amount of ROS. TAK1, a kinase activated by increased intracellular calcium, is also implicated in inflammasome activation. Finally, depletion in intracellular potassium is mandatory for inflammasome activation. ATP release upon cell membrane damage could permeate P2X7R channels to potassium. Interestingly, particle endocytosis is not always required and contact between cell membrane and particles resulting in the formation of lipid raft is sufficient to trigger inflammasome engagement through SYK activation. In the case of nanoparticles, their small size allows them to cross biological membranes. Nanoparticles reach the cytosol even in absence of active endocytotic process and damage organelles such as mitochondria. Water movements via AQP, in particular AQP1, are necessary for inflammasome activation (see study 2). Water channels are involved in inflammasome by regulating cytoskeleton rearrangement, ionic movements and TRP activation.

Physicochemical characteristics determining inflammasome activation by particles

Contrary to water soluble agents, the toxicity of particles cannot only be determined by chemical composition and molecular structure. Various characteristics such as size, surface curvature and area or shape strongly affect particle internalization, intracellular localization and cellular damage. It is thus not surprising that all these parameters also determine the capacity of particles to induce IL-1β processing. A summary of studies considering particle characteristics, amplitude and mechanisms of inflammasome activation is provided in table 1.

a. Size

BMDM exposed to amorphous silica nanoparticles with size ranging from 30 nm to 10 µm released more IL-1β in response to the smallest particles (30 - 1000 nm > 3 µm > 10 µm). Lysosomal damage induced by the smallest particles, and not defect in internalization or actin polymerization, explained these differences (36). Another study confirmed that nanometric amorphous silica particles induced more IL-1β release than their submicrometric counterparts (50 nm vs 500 nm) in macrophages (51). BMDM and primary glial cells exposed to latex beads released more IL-1β in response to 20 nm than to 1 µm size particles. In this study, inflammasome activation was attributed to lysosomal destabilization and cathepsin B release for 20 nm particles and to ROS production and mitochondrial damage for 1 µm particles. Additionally, the inflammasome activation by the 20 nm particles was also associated with their capacity to induce cellular damage, ATP release (43). In dendritic cells, IL-1β release after polystyrene particle exposure was higher in response to 430 nm and 1 µm than to the particles of 10 µm or 32 µm. In this model, small polystyrene particles were significantly more efficiently internalized in comparison with bigger particles (40). Silver nanoparticles of 5 nm, 28 nm and 100 nm were all internalized in monocytes but only 5 nm and 28 nm induced vesicular damage with ROS production and IL-1β release (69). Altogether, these data suggest that in phagocytic cells, smallest particles are more potent to activate inflammasome, when the comparison is made on a mass dose basis. It is also important to note that the small size of nanoparticles allows them to reach intracellular compartments such as mitochondria that are not accessible to micrometric particles (98) or to bind proteins such as actin (63). The

limited activity of micrometric particles appears associated to reduced endocytosis and lysosomal damage. There are, however, some reported exceptions: THP-1 failed to release IL-1β in response to amorphous silica particles below 1 µm (37) and spherical polystyrene particles of 7-8 µm induced inflammasome activation in macrophages whereas 0.5 and 1 µm did not (42). Long fiber-shaped carbon, TiO_2 or CeO_2 nanoparticles induced more IL-1β release than shorter ones in macrophages (54,99-101). Importantly, the impact of particle size differs according to the cell type considered and their endocytic capacities. In non-phagocyting cells such as keratinocytes, only nanoparticles but not micrometric particles are internalized and induce inflammasome activity (21,61).

Aggregation or agglomeration state of particles (mainly nanoparticles) should also be important for inflammasome activation because it can modify particle diameter, curvature and density and thus subsequent endocytosis. Surface area available for particle reactivity can also be modified upon aggregation/agglomeration. Unfortunately, no study formally and directly assessed the impact of aggregation on IL-1β production. However, aluminum nanoplates and polyhedron of different aggregate sizes (hydrodynamic diameter of 93 nm and 333 nm, respectively) but with similar primary size, specific surface area and surface reactivity induced similar inflammasome activation suggesting minor impact of aggregation (38). Our group also demonstrated that aggregation in the nanorange did not modify the cytotoxic effect of silica nanoparticles in macrophages (102).

b. Surface area

On a mass basis, nanoparticles were frequently claimed to be more cytotoxic, however, once normalized by particle number or surface area, this difference was blunted (103,104). This issue has also been addressed in the frame of inflammasome activation. Expressed in mass concentration, 50 nm amorphous silica particles induced more IL-1β release than 500 nm in macrophages but when dose was expressed in total surface area this trend was reversed (51). Similarly, differences in IL-1β released by BMDM in response to amorphous silica particles ranging from 30 nm to 1000 nm, were dampened upon normalization by particle number and almost abolished once expressed in total surface area. However, in this study, particles over 1 µm of diameter were less internalized and macrophages released lower amounts of IL-1β after surface area

normalization (36). These data suggest that surface area dose determines inflammasome activity of internalized particles.

Reactional groups present at particle surface can also affect the ability of particles to induce inflammasome activation. It is already known that silanol groups present on silica are well associated with the particle toxic potential (105,106). Interestingly, reduction of silanol surface density by heating was associated with a decrease of IL-1β secretion by silica-exposed THP-1 cells. Subsequent rehydration restored initial silanol concentration on particles together with IL-1β processing capacity, demonstrating their implication in inflammasome activation. However, in the same study, particles with similar size, surface area and silanol characteristics but with different synthesis process presented different levels of inflammasome activation capacity, probably related to their ability to produce free radicals under acellular conditions (48). Hydroxyl content of particle surface also affected the secretion of IL-1β in response to aluminum nanoparticles in THP-1 cultures. Indeed, particle presenting higher amounts of surface hydroxyl induced more inflammasome activation. This effect was attributed by the authors to an increased capacity of hydroxyl-rich particles to induce ROS production and lysosomal damage (38). Metal contaminants can also exacerbate IL-1β processing by catalyzing surface radical production by particles. Chelation of iron reduced IL-1β maturation after asbestos exposure probably via reduced oxidative stress (15). Oxidative dissolution of silver nanoparticles upon lysosomal acidification lead to Ag ion release, ROS byproducts, mitochondrial ROS generation and ultimately inflammasome activation in THP-1 cells (107,108). Nickel contamination of carbon nanotubes was also implicated in CNT-induced inflammasome activation in mouse primary alveolar macrophages (17).

Importantly, functionalization of amorphous silica particles with various surface groups (-COOH, -NH_2, -SO_3H and -CHO) reduced particle-induced IL-1β release by THP-1 cells. These functionalizations reduced endosomal rupture, cathepsin B leakage and ROS production by limiting the access of surface silanol groups without affecting particle uptake (37). By clearing nickel contaminant without affecting particle uptake, carboxylic functionalization of MWCNT completely inhibited IL-1β release by macrophages (55,99). Carboxylation of titanium nanobelts also reduced IL-1β release by mouse alveolar macrophages and THP-1 cells, probably by reducing lysosomal damage (20).

Particle surface charge is another parameter important for inflammasome activation. Only after amino-functionalization, polystyrene beads induced lysosomal damage, cathepsin B release, mitochondrial ROS production and finally IL-1β release. According to the authors, amino-functionalization induced lysosomal destabilization consistent with the proton sponge theory. In this theory, to compensate protons that are trapped on amine present at the surface of the particle, proton pump activity is increased. For each proton that enters the lysosome, one Cl^- ion and one water molecule enter the lysosome, leading to lysosomal swelling and finally to destabilization (78).

Altogether, these studies emphasized the association between particle surface reactivity and their ability to induce lysosomal membrane destabilization and inflammasome activation. This effect on lysosomal membrane results from the surface characteristics inherent to particle chemical composition or contamination. Consequently, treatments altering particle surface reactivity by eliminating reactive groups or contaminants can be useful to reduce particle inflammogenicity.

c. Shape

By affecting internalization and lysosomal stability, the shape of particles is another important parameter which determines the activity of particles on the inflammasome machinery. In particular the high length/width ratio appears important in inflammasome activation. Inert in THP-1 cells, CeO_2 nanocubes or nanorods with low aspect ratio activate the inflammasome when their length and aspect ratio increase. Indeed, high length/width aspect ratio particles were able to destabilize lysosomal membrane leading to cathepsin B release and subsequent inflammasome activation (101). Long TiO_2 nanobelts induced more inflammasome assembly than short nanobelts and nanospheres in alveolar macrophages. This activity was also linked to lysosomal destabilization and cathepsin B release (100). Similarly, spiculated TiO_2 particles induced stronger IL-1β release by macrophages than spherical nanoparticles with similar size (41). Long well-dispersed carbon nanotubes as well as needle-like calcined fullerene nanowhiskers (HTCFNW) activate more intensively inflammasome than their shorter counterpart (109). Similarly, needle-like carbon nanotubes are more active than spherical carbon black nanoparticles and shorter nanotubes (54). Among spherical and rod-shaped gold nanoparticles in the same size range (20 and 40 nm diameter sphere and 10 nm witdh/40

nm lenght rods), only rods were able to induce IL-1β release, even if all were endocytosed and that both 20 nm spheres and rods escaped lysosomes (110). Curvature is also an important particle characteristic for inflammasome activation. Spherical polymeric particles composed of budding with combination of high positive and negative surface curvature released more IL-1β than smooth particles of the same size (7-8 μm). This effect was correlated with the level of internalized or associated budding particles (42). Thus the shape of particles is a major parameter determining inflammasome activation. Also, particles with aspect ratio close to one are particularly less efficient to induce inflammasome assembly than the others.

Table 1

Size					
Chemical structure	Primary size (nm)	Hydrodynamic diameter (nm)	Inflammasome activation	Identified mechanisms	Reference
Amorphous silica	20	295	++++	Lysosomal acidification and cathepsin B activity	(51)
	67	91	+++	Lysosomal acidification and cathepsin B activity	
	369	531	++	N.a.	
	500 - 10000	342 et 5560	+	Lysosomal acidification and cathepsin B activity	
	30	/	++++	Actin-mediated endocytosis and lysosomal acidification	(36)
	100	/	+++	Actin-mediated endocytosis and lysosomal acidification	
	300	/	+++	Actin-mediated endocytosis and lysosomal acidification	
	1000	/	+++	Actin-mediated endocytosis and lysosomal acidification	
	3000	/	++	Actin-mediated endocytosis and	

				lysosomal acidification	
	10000	/	+/-	Lysosomal acidification	
Carbon black	53,7	235	++	N.a.	(111)
	525	636	+	N.a.	
	14	/	++	N.a.	(112)
	260	/	-	N.a.	
Silver	5	/	++	Potassium efflux and oxydative stress	(69)
	28	/	+	Potassium efflux and oxydative stress	
	100	/	-	N.a.	
Polystyrene	1000	/	++++	N.a.	(40)
	430	/	+++	Actin-mediated endocytosis, lysosomal acidification cathepsin B activity and potassium efflux	
	10 000	/	++	N.a.	
	32 000	/	+	N.a.	

Surface					
Chemical structure	Surface characteristics	Radicals produced at cell surface	Inflammasome activation	Identified mechanisms	Reference
Amorphous silica	Midly -	+	++	Actin-mediated endocytosis, lysosomal acidification and cathepsin B activity, oxidative stress	(37)

	- (-COOH)	/	+	Actin-mediated endocytosis, lysosomal acidification and cathepsin B activity, oxidative stress	
	+ (-NH_2)	/	-	N. r.	
	Silanol +++	+++	+++	N.a.	(48)
	Silanol +++	++	++	Independent of entry and cathepsin B release	
	Silanol ++	+	+	N.a.	
	Silanol +	+	-	N.r.	
Polystyrene	+ (-NH_2)	/	+	Oxidative stress	(78)
	- (-COOH)	/	-	N.r.	
	/	/	-	N.r.	
Aluminum oxyhydroxyde	OH- +++++	++++	++++	Actin-mediated endocytosis, lysosomal acidification and cathepsin B activity, oxidative stress	(38)
	OH- ++++	+++	+++	Actin-mediated endocytosis and cathepsin B activity, oxidative stress	
	OH- ++++	++	++	Actin-mediated endocytosis and cathepsin B	

				activity, oxidative stress	
	OH- ++	+	+	Actin-mediated endocytosis and cathepsin B activity, oxidative stress	
	OH- +	+	+/-	Oxidative stress (actin-mediated endocytosis and cathepsin B activity not convincing)	
MWCNT	Raw	/	+++	Lysosomal damage and cathepsin B activity	(55)
	Purified (less Ni contamination)	/	++	lysosomal damage and cathepsin B activity	
	- (-COOH)	/	+	Cathepsin B activity	
Shape					
Chemical structure	Shape	Length/width (nm) (ratio)	Inflammasome activation	Identified mechanisms	Reference
Titanium rutile	Spicula	40/10 (4)	++	Actin-mediated endocytosis, lipid raft, lysosomal acidification and cathepsin B activity, oxidative stress	(41)
	Spheric	30-40 (≈ 1)	+	Actin-mediated endocytosis, lipid raft,	

				lysosomal acidification and cathepsin B activity, oxidative stress	
Poly(ethylene oxide)	Spherical with budding	NR	++	Actin-mediated endocytosis and cathepsin B activity	(42)
	Spherical	NR	+	Actin-mediated endocytosis and Cathepsin B activity	
	Spherical	NR	-	N.r.	
Gold	Rod	40/10 (4)	+	N.a.	(110)
	Spherical	40 (≈ 1)	-	N.a.	
	Cubic	40/40/40 (≈ 1)	-	N.a.	
Carbon	Needle-like MWCNT	13 000/>50 (<260)	+	Cathepsin B activity, oxydative stress, src/syk pathway and P2X7R activity	(54)
	Tangled MWCNT	10 000 - 50 000/8 – 15 (667-6250)	-	N.r.	
	Short MWCNT	1000 - 10 000/0,5 - 20/ (50-20000)	-	N.r.	
	Needle-like MWCNT	4150/150 (28)	+++	Actin-mediated endocytosis	(109)
	Needle-like HTCFNW	7600/586 (13)	++	N.a.	
	Needle-like HTCFNW	1800/365 (5)	+	Actin-mediated endocytosis	

N.a. : not assessed N.r. : not relevant

Priming cells to express pro-IL-1β : the role of alarmins

The inflammasome complex responds to particle-induced stressors by activating caspase-1 for the processing and release of biologically-active IL-1β. IL-1 signaling also requires other signals involving priming molecules for the transcription of pro-IL-1β (called signal 1). While the cellular perturbations leading to inflammasome and IL-1β activation after particle exposure are relatively well described, it is surprising to note that the precise mechanism(s) inducing pro-IL-1β expression still need to be identified in particle-induced lung responses. A variety of danger signals, also called alarmins, has been recognized as the first inflammatory signal elements strongly inducing pro-IL-1β production (see figure 2). These molecules are usually sequestered inside cells at homeostasis but released in the extracellular environment when the cell membrane is corrupted during necrosis, pyroptosis or if apoptotic bodies are not rapidly cleared and release their cytoplasmic content (secondary necrosis) (reviewed in (113)). In some cases, immune cells can also actively release alarmins. The cytokines IL-1α and IL-33 but also HMGB1 and certain heat shock (HSP) or S100 proteins are considered as potent alarmins upon particle exposure. They bind membrane receptors and trigger inflammatory pathways leading to NFκB or AP-1 activation and IL-1β gene transcription.

a. Interleukin-1α

IL-1α expression is constitutive in diverse types of cells and related to the NFκB/AP-1 pathway (reviewed in (114,115)). Akin IL-β, IL-1α is produced as a precursor. However, this pro-form is active and can bind IL-1RI to induce the production of inflammatory molecules. IL-1α lacks a secretory sequence signal and is released by an unconventional secretory pathway by simple diffusion across cell membrane upon membrane damage and necrosis or upon inflammasome activation. Several studies investigated IL-1α release in response to particles in LPS-primed cells (21,40,52,54). Less well described is the release of constitutive IL-1α cellular content in naive cells. Primary rat epithelial lung cells exposed to ultrafine carbon black released IL-1α independently of a concomitant gene expression. The release of IL-1α preceded and amplified the production of other pro-inflammatory molecules such as IL-6 (27). Fine fraction (PM2.5) and to a lesser extent coarse fraction (PM10) of pollution particulate matters induced IL-1α release from the

human bronchial epithelial cell line BEAS-2B (116). We observed that IL-1α was released from cellular stocks present in primary or cell line macrophages after exposure to nano- and micrometric silica as well as MWCNT. Importantly, pulmonary IL-1β release and neutrophil recruitment in response to silica instillation were strongly reduced when mice received IL-1α neutralizing antibodies or in IL-1α-deficient mice (117,118). Similarly, IL-1β detected in the peritoneal cavity following MSU injection was reduced in IL-1α-deficient mice (52). These findings strongly support the view that IL-1α represents a major signal that allows the expression of immature IL-1β.

b. HMGB1

HMGB1 is constitutively expressed in all cells and can be released following cell necrosis or secreted by activated immune cells. Extracellular HMGB1, alone or complexed to other pro-inflammatory molecules can bind the RAGE receptor or TLRs, trigger the NFκB and AP-1 pathway and induce pro-inflammatory cytokine production (119,120). Particle-induced HMGB1 release has been documented in human macrophages and bronchial epithelial cell lines treated with silica or in asbestos-treated mesothelial cells (14,25,30). Passive and active release of HMGB1 has also been reported in cultures of epithelial cell line or primary alveolar macrophages exposed to MWCNT. The presence of HMGB1 in the extracellular environment increased IL-1β secretion by MWCNT-treated alveolar macrophages. Interestingly, neutralization of extracellular HMGB1 by neutralizing antibodies reduced MWCNT-induced IL-1β secretion and inflammation *in vivo* (121). By using RAGE-deficient mice, Ramsgaard and colleagues also demonstrated that this receptor is involved in neutrophil recruitment following silica lung exposure (122). Thus HMGB1 represents an additional important alarmin that mediates the expression of pro-IL-1β.

c. Interleukin-33

Interleukin-33, a cytokine of the interleukin-1 family, is expressed by structural and inflammatory cells and, as a proform or after maturation, activates its receptor ST2 (123). Similar to interleukin-1α and β, precursor of this interleukin can be matured upon cleavage by several enzymes with various effects on its activity. Cleavage by caspase-1, 7 or 8 inactivates IL-33 whereas calpain and neutrophil- or mastocyte-derived proteases

increased its activity (124). IL-33 release in response to particle exposure has been reported in several studies. Alum has been shown to induce IL-33 release from THP-1 macrophages (125). MWCNT induced IL-33 release in supernatant of an epithelial cell line and in broncho-alveolar lavage fluid of mice (126-129). In conclusion, it is still unclear whether IL-33 is central to the induction of pro-IL-1β expression after particle exposure.

d. Other alarmins implicated in particle-priming

S100A8/S100A9 proteins are constitutively expressed by phagocytes that can release these proteins by non-classical pathways or by diffusion across cell membrane upon necrosis. Once in the extracellular environment, they can bind TLR4 or RAGE and induce NFκB and AP-1 pathway (reviewed in (130,131)). Increased S100A8 and S100A9 levels were detected in BAL of rats exposed to diesel exhaust particles or ZnO nanoparticles (132,133) and in lung tissue of mice exposed to SWCNT (134). Heat shock protein (HSP) form a group of proteins that can bind various kinds of receptors and stimulate NFκB and trigger pro-inflammatory cytokine production ((135,136) and reviewed in (137)). HSP60 release and subsequent TLR4 engagement have been implicated in the production of pro-IL-1β in monocytes exposed to polyethylene particles (138). Despite their well-recognized role in sterile inflammation, S100 and HSP proteins received little attention for their possible implication in IL-1β priming in the frame of particle-induced inflammation.

e. Other cytokines implicated in particle-priming

The IL-1RI signaling pathway induces NFκB and AP-1 activation and IL-1β gene expression (139). Constitutive expression of IL-1β has been described several times in non-immune cells (25,140-142) and its release could amplify IL-1β production by other cells. Particle-induced damage to structural cells could trigger IL-1β release by inflammasome activation or by diffusion of pro-IL-1β that could be matured by proteases (143-145). Kono and colleagues observed that while IL-1β was crucial in silica-induced inflammatory response, caspase-1-deficient mice demonstrated only a limited reduction of inflammatory parameters in comparison to cathepsin C-deficient mice. Cathepsin C is

necessary for activating serine proteases, and the authors postulated that its absence impaired extracellular IL-1β activation mediated by these proteases (146).

TNF-α is a powerful activator of NFκB/AP-1 and is known to induce IL-1β expression (147). Under resting conditions, TNF-α translation is repressed in most cells (148) but rapidly restored under stress conditions (149). In addition, a membrane-bound precursor of TNF-α can be processed by a TNF-α converting enzyme (TACE) to generate secreted mature TNF-α (150). Thus TNF-α can be produced and released rapidly, independently of transcriptional induction, to mediate early pro-IL-1β production. Release of TNF-α has been shown in response to different types of particles such as titanium nanoparticles, carbon nanotube, polymethylmethacrylate particles, PM10 ambient particulate matter, wood smoke and traffic particles *in vitro* or *in vivo* (151-156). Inhibition of TNF-α by neutralizing antibody has been shown to reduce IL-1β production by A549 cells and human bronchial epithelial cells exposed to PM10 (154). Interestingly, TNF-α has been shown to promote caspase-1 activation in response to silica (157).

Thus even if they are not considered to be constitutively expressed and stored such as classical alarmins, early release of IL-1β and TNF-α should be involved in priming step that allow immune cells to produce high amount of IL-1β.

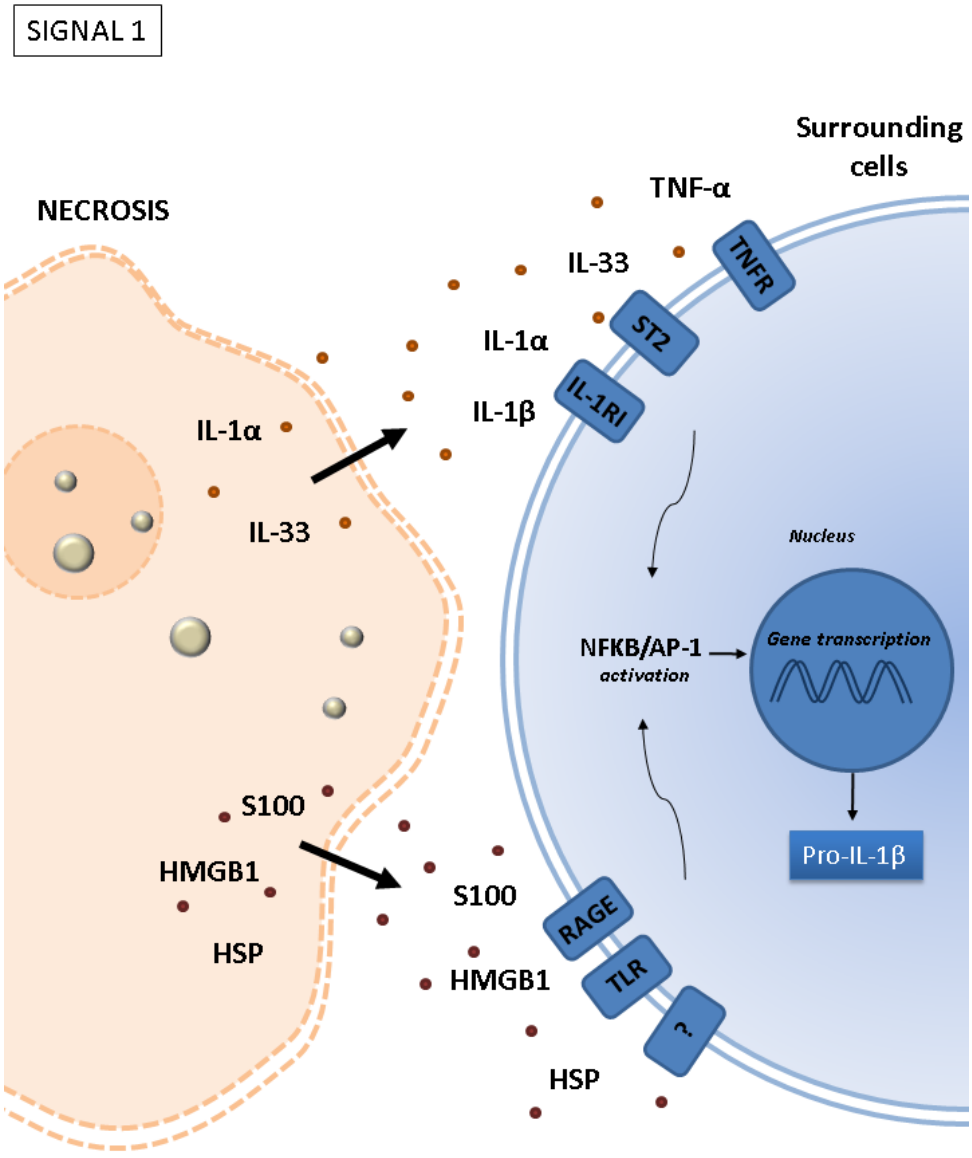


Figure 2 : **Process involved in particle-induced pro-IL-1β expression**

Pro-IL-1β production requires intermediary mediators. Silica-damaged cells release intracellular proteins called alarmins that possess inflammatory activities once present in the extracellular environment. HGMB1, S100 and HSP proteins bind to multiligand receptors such as RAGE or TLRs and stimulate NFκB/AP-1 pathway, leading to pro-IL-1β production. IL-1α and IL-33, two members of the IL-1 family, also pass across damaged cell membranes and bind their specific receptors, IL-1RI and ST2 respectively. Additionally, other cytokines that are not classified as alarmins but known to promote pro-IL-1β production via NFκB/AP-1 activation also participate in the expression of pro-IL-1β and synergize with alarmin, i.e. TNF-α and IL-1β itself.

General conclusions

After particle exposure, alarmins retained intracellularly as preexisting stocks in resident cells are released into the extracellular milieu. These first inflammatory mediators (signal 1, Figure 2) are potent activating stimuli required for macrophages and epithelial cells to express the biologically inactive precursor IL-1β (pro-IL-1β). This form is cleaved by silica-induced inflammasome assembly and activation prior its secretion as mature and bioactive IL-1β. This master cytokine is necessary to generate pulmonary neutrophil accumulation in response to particles.

The processes leading to inflammasome activation seems to be initiated following lysosomal destabilization and enzyme, ions or ROS release (signal 2, Figure 1). These events can result in mitochondrial damage, crucial to NLRP3 activation and inflammasome assembly. The particle characteristics decisive for lysosomolytic activity determine particle inflammasome activator potency and subsequent *in vivo* inflammatory reaction. Shape and size of the particle are important to determine particle internalization, mandatory for lysosomal alteration, as well as physical damage to lysosomal membrane. Surface area reactivity also determines lysosomal damage and subsequent inflammasome activation. In size-range that allows them to be internalized or to penetrate by diffusion, the smallest particles induce stronger inflammasome activation on a mass basis. When compared on a surface basis, this difference is generally reduced meaning that surface properties govern their inflammatory potential. Physical or chemical treatment aiming to reduce surface reactivity should be developed to control inflammogenicity of particles. In particular, nanoparticles can reach intracellular compartments and trigger metabolic processes. These particles induce toxicity and inflammasome activation by new pathways that are still to delineate.

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II. Objectives of the thesis

IL-1β plays a key role in the initiation and extension of particle-induced pulmonary inflammation (1-3). The potent inflammatory activity of this cytokine requires a multilevel fine tuning which needs to be better defined after particle exposure. The initial transcription of IL-1β gene and the subsequent production of intracellular inactive IL-1β precursor (pro-IL-1β) involve the recognition of endogenous danger signals (signal 1) released after tissue damage and stress. The maturation and secretion of IL-1β is induced by cellular events (signal 2) triggering the assembly of a multiproteic complex called inflammasome which consists of a NLR (Nod-like receptor), an adapter protein (ASC, Apoptosis-associated Speck-like protein containing CARD) and caspase-1. The subsequent activation of this latter enzyme is necessary for the extracellular release of active IL-1β.

In response to particles, the exact cellular events resulting in pro-IL-1β production (signal 1) remain enigmatic and undetermined since particles do not induce IL-1β transcription by themselves (4,5). In the first part of this thesis, we attempt to identify these mediator(s) in silica-treated mice and hypothesized that endogenous alarmins released from silica–damaged macrophages induce pro-IL-1β expression. Indeed, these danger signals strongly induce NFκB cascade and gene expression of several pro-inflammatory cytokines, in particular IL-1β. Among the diverse alarmins identified, we have predominantly investigated the role of IL-1α, IL-33 and HMGB1 because their release has already been reported in response to particles (6-8).

Although already well characterized, recent evidence has highlighted the complexity and diversity of the cellular events (signal 2) leading to inflammasome platform activation. For instance, it has been shown that volume changes and TRP (Transient Receptor Potential) channel activation in macrophages result in NRLP3 activation and IL-1β secretion (9-11). Interestingly, cell volume modifications are a hallmark of macrophage response to particles (12,13). The potential role of particle-induced cell volume changes and water flow in particle-induced inflammasome activation has not been delineated yet. By exploring aquaporin (AQP)-facilitating water movement, we determined in the second part of this work whether macrophage volume changes represent a necessary cell signal for activating inflammasome and IL-1β release after silica phagocytosis.

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III. Results

1. The alarmin IL-1α is a master cytokine in acute lung inflammation induced by silica micro- and nanoparticles

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PARTICLE AND FIBRE TOXICOLOGY

RESEARCH Open Access

The alarmin IL-1α is a master cytokine in acute lung inflammation induced by silica micro- and nanoparticles

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Abstract

Background: Inflammasome-activated IL-1β plays a major role in lung neutrophilic inflammation induced by inhaled silica. However, the exact mechanisms that contribute to the initial production of precursor IL-1β (pro-IL-1β) are still unclear. Here, we assessed the implication of alarmins (IL-1α, IL-33 and HMGB1) in the lung response to silica particles and found that IL-1α is a master cytokine that regulates IL-1β expression.

Methods: Pro- and mature IL-1β as well as alarmins were assessed by ELISA, Western Blot or qRT-PCR in macrophage cultures and in mouse lung following nano- and micrometric silica exposure. Implication of these immune mediators in the establishment of lung inflammatory responses to silica was investigated in knock-out mice or after antibody blockade by evaluating pulmonary neutrophil counts, CXCR2 expression and degree of histological injury.

Results: We found that the early release of IL-1α and IL-33, but not HMGB1 in alveolar space preceded the lung expression of pro-IL-1β and neutrophilic inflammation in silica-treated mice. *In vitro*, the production of pro-IL-1β by alveolar macrophages was significantly induced by recombinant IL-1α but not by IL-33. Neutralization or deletion of IL-1α reduced IL-1β production and neutrophil accumulation after silica in mice. Finally, IL-1α released by J774 macrophages after *in vitro* exposure to a range of micro- and nanoparticles of silica was correlated with the degree of lung inflammation induced *in vivo* by these particles.

Conclusions: We demonstrated that in response to silica exposure, IL-1α is rapidly released from pre-existing stocks in alveolar macrophages and promotes subsequent lung inflammation through the stimulation of IL-1β production. Moreover, we demonstrated that *in vitro* IL-1α release from macrophages can be used to predict the acute inflammogenic activity of silica micro- and nanoparticles.

Keywords: Alarmins, Inflammation, Neutrophils, Silica, Nanoparticles, IL-1 family, Inflammasome

Background

Inhalation of micrometric and nanometric particles can lead to pulmonary diseases characterized by inflammation, fibrosis and/or cancer. Pulmonary inflammation induced by inhaled particles is characterized by a marked accumulation of neutrophils which secrete large quantities of reactive oxygen metabolites, granule enzymes and pro-inflammatory mediators [1,2]. It has been demonstrated that the pro-inflammatory cytokine interleukin-1β (IL-1β) drives particle-induced inflammation by enhancing granulocyte migration and accumulation to inflammatory sites [3-5]. Mature IL-1β release in the extracellular environment requires several steps including the production of the biologically inactive IL-1β pro-form (pro-IL-1β) consecutive to Il1b gene transcription as well as its maturation and exocytosis through the NLRP3 inflammasome machinery. Pro-IL-1β production could result from cellular

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detection of pathogen components, PAMPs, or from pro-inflammatory cytokines. Also, IL-1β positively autoregulates its own synthesis via NFκb binding activity [6].

Several studies have demonstrated that K^+ efflux, cytosolic release of lysosomal cathepsins and mitochondria-derived factors such as reactive oxygen species (ROS) are involved in the NLRP3 inflammasome assembly and activation by micro- and nanoparticles [7-11]. It is, however, surprising that the signal(s) triggering NFκb-mediated up-regulation of pro-IL-1β after particle exposure remain(s) unknown [12,13]. The identification of a common element that initiates pro-IL-1β production in response to particles would be useful to understand their inflammatory properties and to predict their inflammatory potential.

The alarmin family comprises structurally distinct endogenous mediators including defensins, cathelicidins, eosinophil-associated ribonucleases, heat shock proteins (HSP), saposin-like granulysin, ion-binding proteins (e.g., S100 proteins and lactoferrin), and nucleotides/metabolites (e.g., uric acid) [14]. Additionally, some constitutive cytokines that possess intracellular functions such as IL-1α, IL-33 and High Mobility Group Box 1 (HMGB1) are also considered as alarmins [5,15-18]. Alarmins are passively released from necrotic cells upon infection and tissue injury or rapidly secreted by stimulated leukocytes. Alarmins play important intracellular roles in homeostasis and when released extracellularly strongly promote leukocyte cell recruitment and activation as well as tissue repair. This occurs for instance in response to sterile injury or infection [19].

IL-1α and IL-33 are produced as pro-form but contrary to IL-1β, these precursors are already active. Upon cell necrosis, the released IL-1α, IL-33 and HMGB1 alarmins alone or complexed to other molecules bind their respective receptors (IL-1R1, ST2 and RAGE, TLR2 or TLR4) and promote pro-inflammatory gene transcription via the NFκb pathway [16]. We thus hypothesized that lung injury induced by toxic particles leads to the release of intracellular alarmins which can induce pro-IL-1β production and subsequent inflammatory processes.

In this study, we identified the initial events leading to the lung inflammatory response to inhaled particles and found that the constitutively available alarmin IL-1α is rapidly released in alveolar space in response to silica exposure, before IL-1β induction and pulmonary neutrophil influx. We demonstrated that IL-1α can directly induce pro-IL-1β production by alveolar macrophages and that its neutralization impairs silica-induced lung IL-1β release and inflammation. Finally, we identified macrophages as a main source of IL-1α in the lung and developed an *in vitro* assay to evaluate the inflammogenic activity of nano- and micrometric particles based on their capacity to release IL-1α from macrophages.

Results

The early release of the endogenous IL-1α and IL-33 alarmins precedes silica-induced IL-1β production and neutrophilic inflammation in mice

In order to explore the implication of alarmins in particle-induced IL-1β production in the lung, we first measured in broncho-alveolar lavage fluid (BALF) and lung tissue the protein and gene expression of IL-1α, IL-33 and HMGB1 at different time points after an inflammatory dose of micrometric crystalline silica (DQ12, 2.5 mg) [20,21]. One hour after silica administration, IL-1α and IL-33 protein levels were already significantly increased in BALF. This release peaked at 6 and 12 hours and progressively returned to control values at 24 hours (Figure 1a and b). Silica did not affect BALF HMGB1 levels (Additional file 1: Figure S1a). An increase of lung IL-1α, IL-33 and HMGB1 transcript contents was only observed from 6 hours after silica administration and this effect was maintained up to 24 hours (Additional file 1: Figure S1d, e and f). These data suggest that pre-existing stocks of IL-1α and IL-33 protein are rapidly released in the lung after silica.

The early lung release (1 h) of IL-1α and IL-33 after silica preceded the increased expression of pro-IL-1β and the release of mature IL-1β. Indeed, the levels of lung IL-1β transcripts (Figure 1c) and BALF IL-1β protein (Additional file 1: Figure S1b) were mainly increased between 6 and 24 hours following instillation. Cellular lung inflammation was first monitored by BAL total cell and neutrophil (GR1+ cells) counts. Neutrophil accumulation was also quantified by assessing lung expression of CXCR2. Although the expression of this chemokine receptor has been reported in macrophages, CXCR2 is mainly expressed by recruited neutrophils and can be used as a biomarker of neutrophilic inflammation [22]. Akin biochemical parameters (Additional file 1: Figure S1c), cellular inflammation was obvious 6 hours after silica and persisted until 24 hours (Figure 1d to f). These data suggested that the rapid release of the intracellular stocks of IL-1α and IL-33 contributes to IL-1β production and neutrophilic inflammation following silica exposure.

The alarmin IL-1α induces pro-IL-1β production in alveolar macrophages

We next tested whether the alarmins IL-1α and IL-33 can directly activate the *in vitro* expression of pro-IL-1β. First, we determined the main cellular source of IL-1β in the lung of mice following silica exposure. IL-1β production is well defined in immune cells but other sources such as epithelial cells have been recently identified [23,24]. Therefore, we purified structural (epithelial cells and fibroblasts) and immune cells (i.e. T and B lymphocytes, dendritic cells and macrophages) from the lung of silica-treated mice and measured their pro-IL-1β

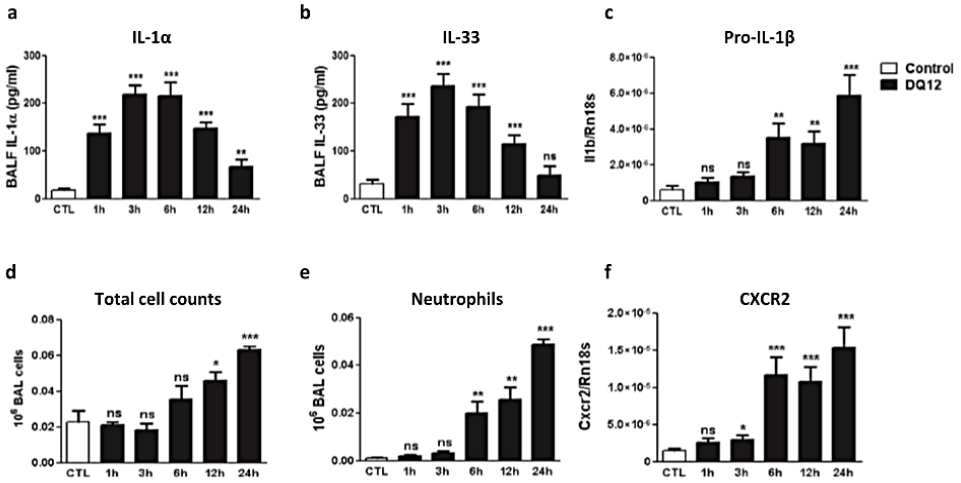


Figure 1 Silica induces IL-1α and IL-33 release in the lung before IL-1β production and neutrophilic inflammation. Levels of **(a)** IL-1α and **(b)** IL-33 in BAL fluid collected at different time points after silica (crystalline DQ12, 2.5 mg) or not (control). Pulmonary expression of **(c)** pro-IL-1β quantified by qRT-PCR at different time points after instillation of silica or not. Number of alveolar **(d)** total cells and **(e)** neutrophils ($GR1^+$ cells) assessed by flow cytometry. **(f)** Expression of the pulmonary neutrophilic CXCR2 marker quantified by qRT-PCR at different time points after silica or not. Values are means SEM of 3 to 8 animals. *p < 0.05, **p < 0.01 and ***p < 0.001 denote significant difference between animals treated with silica or not; ns, denotes no significant difference. P-values are estimated by *t*-test.

intracellular contents. Lymphocytes and structural cells produced little amount of pro-IL-1β after silica exposure. Alveolar macrophages and dendritic cells produced high levels of pro-IL-1β and were the major cell populations expressing IL-1β in silica treated mice (Figure 2a). We also verified that silica alone did not immediately stimulate pro-IL-1β synthesis in primary lung macrophage cultures (Figure 2b). Interestingly, recombinant IL-1α induced a dose-dependent pro-IL-1β production by alveolar macrophages as appreciated by ELISA (Figure 2c) and western blot analysis (Figure 2d). After recombinant IL-33 addition, a slight but not dose-dependent increase of pro-IL-1β levels was observed by ELISA (Figure 2e) but not by WB analysis (Figure 2f). As expected, the addition of recombinant mature IL-1β in macrophage cultures dose-dependently induced the expression of its pro-form (Figure 2g). At the same concentration, recombinant IL-1α and IL-1β but not IL-33 induced similar production of pro-IL-1β by alveolar macrophages (Figure 2h). Several forms of recombinant HMGB1 [25,26] had no effect on pro-IL-1β expression when added to macrophages (data not shown). Altogether, these results indicated that the alarmin IL-1α and mature IL-1β strongly stimulate pro-IL-1β production by alveolar macrophages.

The alarmin IL-1α is necessary for IL-1β production in response to silica particles in the mouse lung

Because IL-1α release preceded the expression of IL-1β in silica-treated mice (Figure 1a and c), we then delineated the implication of IL-1α on silica-induced pro-IL-1β production and mature IL-1β release by examining the secretion of IL-1β after silica treatment in mice lacking IL-1α. The genetic absence of IL-1α abrogated pro- and mature IL-1β expression (Figure 3a and b) in response to silica. Injection of neutralizing antibodies directed against IL-1α [27,28] also markedly decreased the levels of IL-1β transcript (Figure 3c) and protein (Figure 3d) in response to silica. These data strongly indicated that the rapid release of IL-1α promotes IL-1β production in the lung of silica-treated mice.

The alarmin IL-1α contributes to neutrophilic inflammation in response to silica particles in the lung of mice

We next determined the role of IL-1α in the development of lung inflammation induced by silica by examining neutrophil accumulation. First, we confirmed the pivotal role of IL-1β in lung inflammation development since neutrophil influx after silica was significantly impaired after IL-1β inactivation (KO and blocking antibodies, Figure 4a-f). Genetic deletion of Il1a resulted in

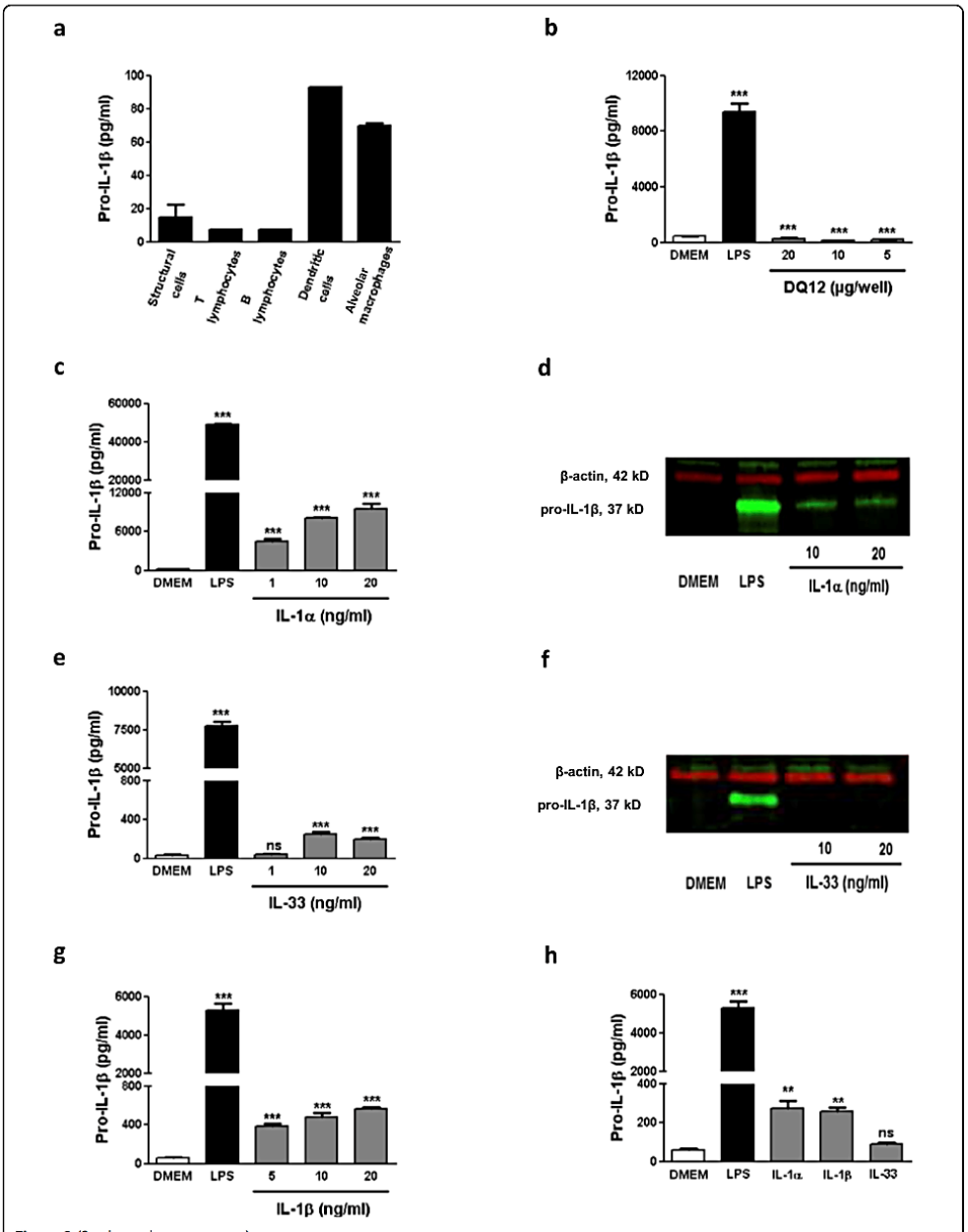


Figure 2 (See legend on next page.)

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Figure 2 IL-1-induced pro-IL-1β production in alveolar macrophages. (a) Intracellular levels of pro-IL-1β in structural cells ($CD45^-$ cells), T ($CD5^+$ cells) and B ($B220^+$ cells) lymphocytes, dendritic cells ($F4/80^-$ $CD11c^+$ cells) and alveolar macrophages ($F4/80^+$) purified from lungs 3 hours after silica instillation (crystalline DQ12, 2.5 mg). n = 2 to 6. **(b)** Intracellular levels of pro-IL-1β in primary cultured alveolar macrophages exposed overnight to LPS (0.1 μg/ml) or silica (DQ12). n = 4. Intracellular levels of pro-IL-1β in primary cultured lung macrophages exposed overnight to LPS (0.1 μg/ml) or recombinant IL-1α, IL-33 or IL-1β in different experiments (**(c)**, **(e)** and **(g)** respectively) or in the same experiment (10 ng/ml) **(h)**. n = 3 to 5. Western blot analysis of intracellular pro-IL-1β and β-actin in primary cultured alveolar macrophages exposed during 18 hours to LPS (0.1 μg/ml), **(d)** recombinant IL-1α or **(f)** IL-33. Values are means SEM. **p < 0.01 and ***p < 0.001 denotes significant difference between cells in DMEM and cells exposed to LPS, silica, recombinant IL-1α, recombinant IL-33 or recombinant IL-1β; ns, denotes no significant difference. P-values are estimated by *t*-test.

a deeper and more complete inhibition of cellular inflammation as appreciated by inflammatory cell and neutrophil accumulation (Figure 4a and b), CXCR2 expression (Figure 4c) as well as histological analyses (Figure 4f). Antibody inactivation confirmed the major implication of IL-1α in silica-induced neutrophilic inflammation (Figure 4d-f). Altogether, these data indicated that the alarmin IL-1α indirectly promotes particle-induced lung inflammation through the induction of IL-1β production but also possesses a direct inflammatory activity.

The *in vitro* release of IL-1α by macrophages upon particle exposure predicts the *in vivo* inflammatory activity of the particles

In vivo, IL-1α release upon silica exposure was strongly associated with the subsequent lung inflammatory response. We thus wondered whether the capacity of particles to induce IL-1α cell release determines their inflammatory activity and whether it can be used *in vitro* to predict their toxicity. To test this hypothesis, we first determined the cellular source of the alarmin IL-1α and assessed *in vivo* and *in vitro* its release in response to particles. Intracellular IL-1α content was measured in different structural and immune lung cell populations from nave or silica-treated mice. Among pulmonary resident cells, only macrophages contained significant amounts of IL-1α in control animals (Figure 5a). After silica treatment, alveolar macrophages contained and released high amount of IL-1α (Figure 5b and c). To a lesser extent, dendritic cells and B lymphocytes also produced and released IL-1α after silica instillation. Moreover, freshly purified alveolar macrophages from nave mice released IL-1 α in the medium in response

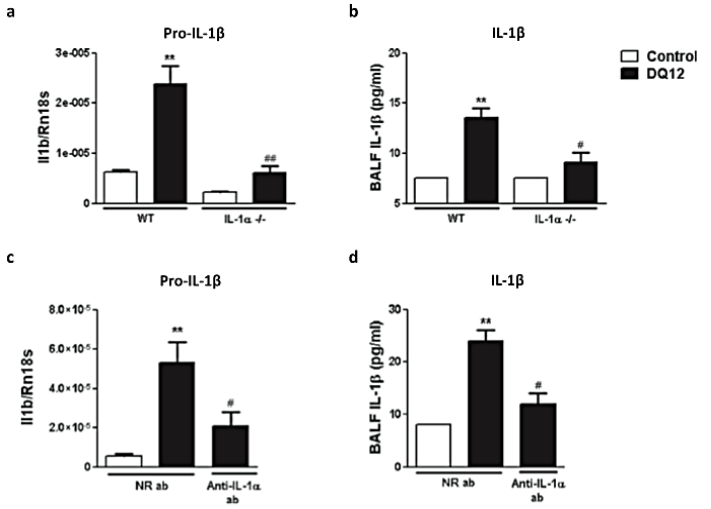


Figure 3 IL-1α inhibition reduces IL-1β production in lung of silica-treated mice. (a) Pulmonary expression of pro-IL-1β quantified by qRT-PCR and **(b)** levels of mature IL-1β in BAL fluid of WT and IL-1α KO mice, 24 hours after instillation of silica (crystalline DQ12, 2.5 mg) or not (control). **(c)** Pulmonary expression of pro-IL-1β quantified by qRT-PCR and **(d)** levels of mature IL-1β in BAL fluid 18 hours after silica or not, in mice treated with IL-1α neutralizing antibody or not. Values are means SEM of 3 to 8 animals. **p < 0.01 denote significant difference between animals treated or not with silica. # p < 0.05, ## p < 0.01 denote significant difference between silica-treated animals with decreased or unmodified IL-1α. P-values are estimated by *t*-test.

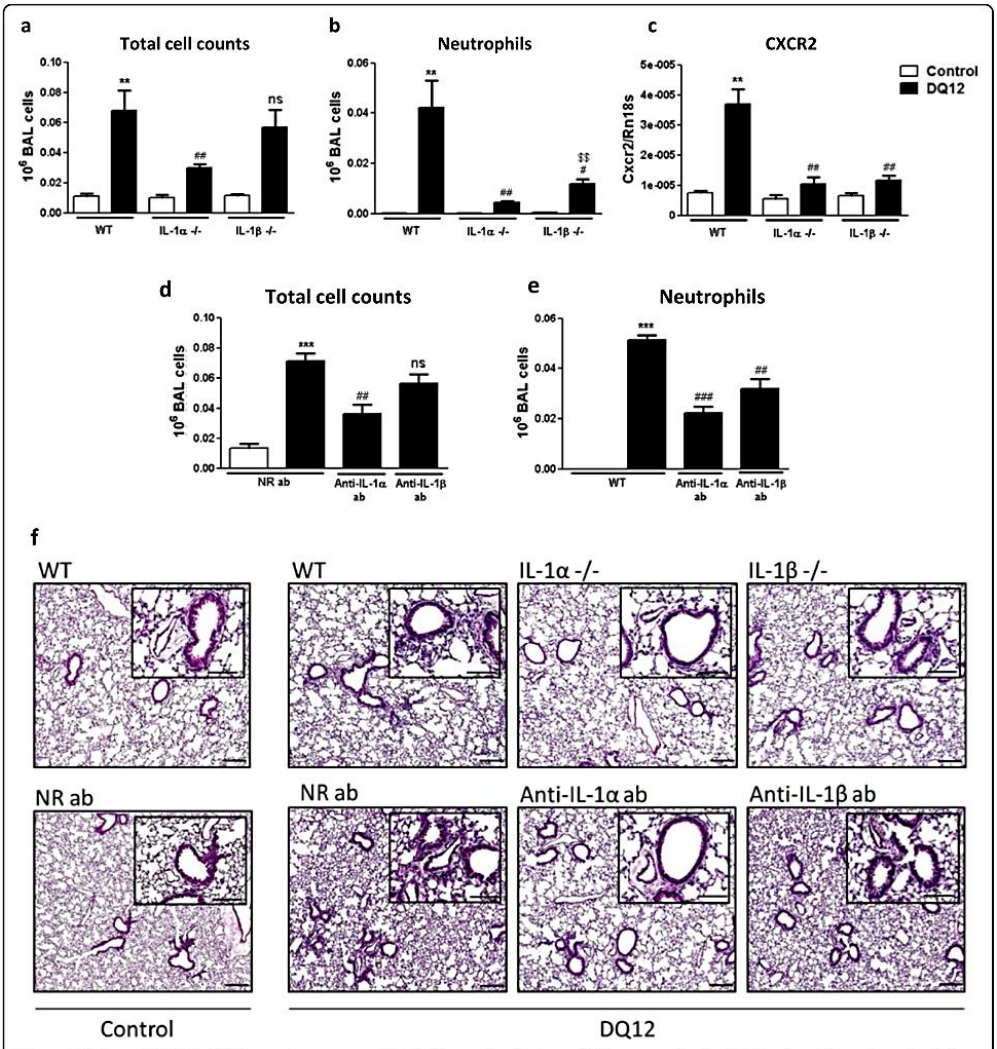


Figure 4 IL-1α and IL-1β inhibition reduces neutrophilic inflammation in lung of silica-treated mice. (a) Number of alveolar total cells in WT, IL-1α KO or IL-1β KO mice 24 hours after instillation of silica (crystalline DQ12, 2.5 mg) or not (control). **(b)** Number of alveolar neutrophils (GR1+ cells) assessed by flow cytometry and **(c)** expression of the pulmonary neutrophilic CXCR2 marker quantified by qRT-PCR in WT, IL-1α KO or IL-1β KO mice 24 hours after instillation of silica or not. Number of alveolar **(d)** total cells and **(e)** neutrophils in WT mice treated with anti-IL-1α or IL-1β neutralizing antibodies or not 18 hours after silica or not. **(f)** Hematoxylin and eosin-stained lung sections obtained from untreated WT mice or after silica instillation of WT, IL-1α KO and IL-1β KO mice or from untreated mice or after silica instillation of mice injected or not with anti-IL-1α or anti-IL-1β antibodies. Scale bars = 200 μm (large panels) and 100 μm (inserts). Values are means SEM of 3 to 5 animals. **p < 0.01 and ***p < 0.001 denote significant difference between animals treated with silica or not. # p < 0.05, ## p < 0.01 and ### p < 0.001 denote significant difference between silica-treated animals with decreased or unmodified IL-1α or IL-1β activity. $$ p < 0.01 denote significant difference in values between IL-1α KO and IL-1β KO silica-treated animals. Ns, denotes no significant difference between silica-treated animals with decreased or unmodified IL-1α or IL-1β activity. P-values are estimated by *t*-test.

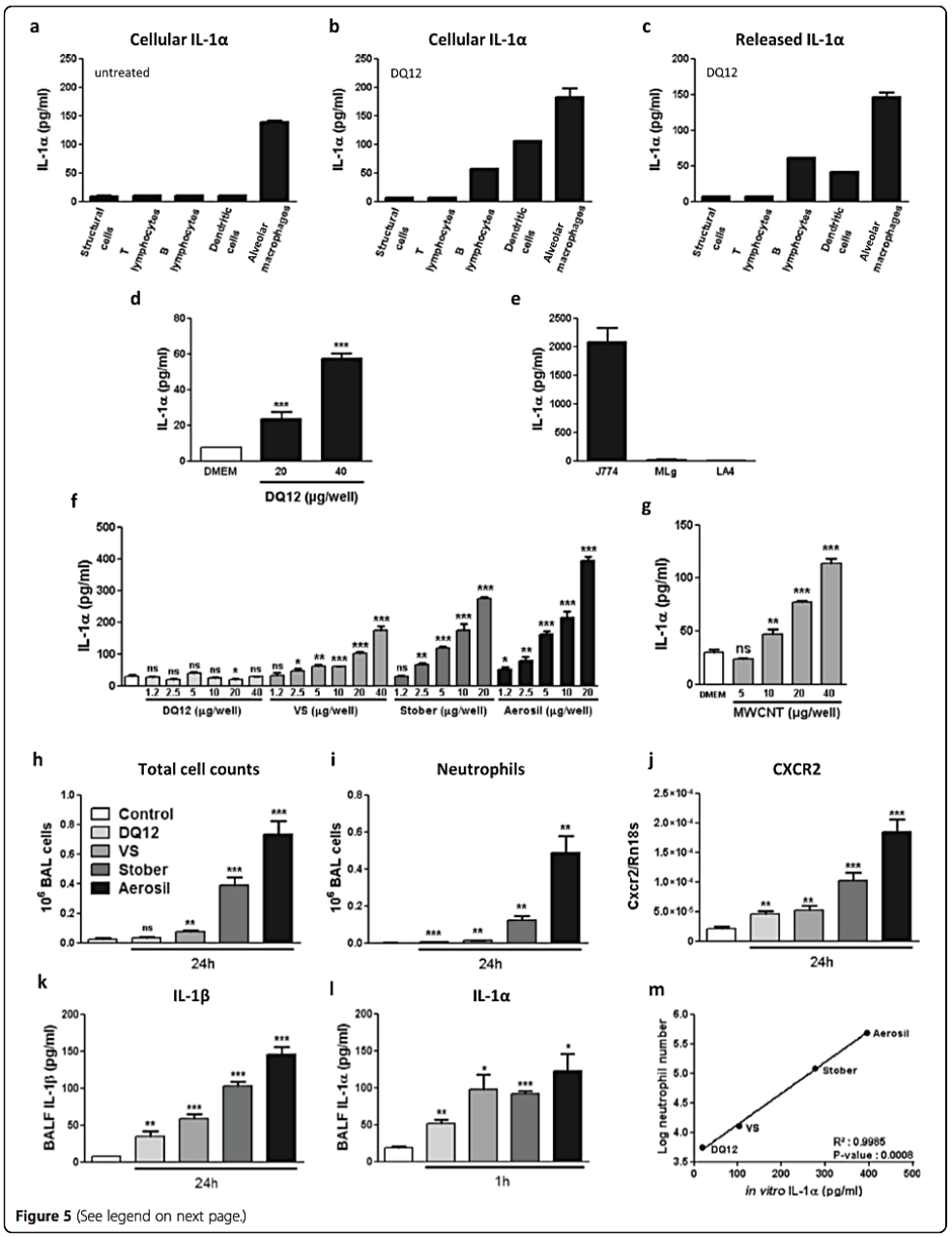


Figure 5 (See legend on next page.)

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Figure 5 IL-1α release by J774 macrophages after particle exposure predicts acute lung inflammation upon particle treatment in mice. Intracellular levels of IL-1α in structural cells ($CD45^-$ cells), T ($CD5^+$ cells) and B ($B220^+$ cells) lymphocytes, dendritic cells ($F4/80^-$ $CD11c^+$ cells) and alveolar macrophages ($F4/80^+$) freshly purified from **(a)** untreated lung or **(b)** 3 hours after silica instillation (crystaline DQ12, 2.5 mg). n = 1 to 6. **(c)** Release of IL-1α by structural cells ($CD45^-$ cells), T ($CD5^+$ cells) and B ($B220^+$ cells) lymphocytes, dendritic cells ($F4/80^-$ $CD11c^+$ cells) and alveolar macrophages ($F4/80^+$) purified from lungs 3 hours after silica instillation. n = 1 to 6 **(d)** Release of IL-1α by fresh primary lung macrophages ($CD45^+$ $F4/80^+$) exposed to silica (overnight). n = 4. **(e)** Intracellular levels of IL-1α in macrophage (J774), fibroblast (MLg) and epithelial (LA4) cell line. n = 2 to 6. **(f)** Release of IL-1α by J774 exposed overnight to increasing concentration of DQ12, Vitreous Silica (VS), Stber silica nanoparticles (Stober) and Aerosil 200. n =3 to 4. **(g)** Release of IL-1α by J774 exposed overnight to various concentration of MWCNT. n =3 to 4. **(h)** Number of alveolar total cells 24 hours after particle instillation (1.2 mg) or not. **(i)** Number of alveolar neutrophils ($GR1^+$ cells) assessed by flow cytometry and **(j)** expression of the pulmonary neutrophilic marker CXCR2 quantified by qRT-PCR in mice 24 hours after particle instillation or not. Levels of **(k)** mature IL-1β or **(l)** IL-1α in BAL fluid collected respectively 24 hours or 1 hour after instillation of particles or not. **(m)** Correlation between alveolar neutrophils (log $GR1^+$ cells) collected 24 hours after particle instillation and levels of IL-1α in supernatant of particle-exposed J774. Values are means ± SEM of 3 to 6 animals. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ denote significant difference between particle-treated and not animals; ns, denotes no significant difference. P-values are estimated by *t*-test. Correlation is calculated based on Pearson coefficient.

to silica exposure (Figure 5d). These data indicated that alveolar macrophages represent the main IL-1α-secreting lung cells and can be used to assess the alarmin IL-1α release in response to silica *in vitro*.

In order to develop an *in vitro* screening test, we next assessed the intracellular IL-1α content in murine macrophage (J774), fibroblast (MLg) and epithelial (LA-4) cell lines. Results on cell lines confirmed observations made on primary lung cells, i.e. that macrophages possess high constitutive IL-1α content, contrary to fibroblastic and epithelial cells (Figure 5e). We then compared IL-1α released by J774 in response to increasing amounts of different silica particles: micrometric crystalline silica (DQ12), micrometric amorphous silica (Vitreous Silica; VS), and two different amorphous nanosilica (Stber and Aerosil 200) (Figure 5f). While DQ12 silica induced a slight release of IL-1α in J774 macrophage supernatant, vitreous and Stber silica induced a significant IL-1α release from 2.5 μg of particle per well, while Stber-induced IL-1 α release was higher. Finally, Aerosil 200 already induced a significant increase of IL-1α levels at the dose of 1.2 μg/well. Based on these data, we concluded that the tested silica particles induce IL-1α release by J774 macrophages in the following order: DQ12 < VS < Stber < Aerosil 200. In parallel, mice were instilled with 1.2 mg of the 4 silica particles and lung IL-1α release, neutrophilic accumulation and IL-1β expression were evaluated. IL-1α concentrations assessed in BALF 1 hour after particle instillation were increased as *in vitro*: DQ12 induced a limited release of IL-1α, VS and Stber were associated with a moderate release of IL-1α while Aerosil induced the strongest IL-1α release (Figure 5l). Particle-induced inflammatory and neutrophilic accumulation (Figure 5h-j) and IL-1β production (Figure 5k) also correlated to *in vitro* IL-1α release. Correlation analyses between *in vitro* IL-1α release (20 μg of particle/well) and *in vivo* neutrophilic accumulation induced by the different silica particles confirmed the clear association between the *in vivo* lung inflammation intensity and the IL-1α release (Figure 5m, $R^2 = 0.9985$, p-value 0.0008). MWCNT, known for their lung inflammatory potential [29], also induced IL-1α release from macrophages (Figure 5g). In conclusion, IL-1α release by J774 macrophages was predictive of the acute lung inflammation consecutive to silica particle exposure.

Discussion

Recent evidence demonstrated that NLRP3 inflammasome activation by particles is pivotal in the release of the pro-inflammatory cytokine IL-1β and the development of lung inflammation [4,7,11]. Particles such as silica activate the inflammasome platform through potassium-, cathepsin- and/or ROS-dependent pathways and allow the release of the cleaved and active IL-1β. The early events leading to pro-IL-1β production following exposure to particles are, however, not identified yet. In models of sterile inflammation, damaged and necrotic cells release intracellular stores of biologically active alarmins that initiate subsequent pro-inflammatory cytokine transcription and neutrophil recruitment [30,31]. We thus postulated that early alarmin release due to particle-induced cell damage can induce pro-IL-1β production and subsequent inflammatory reaction. We discovered that IL-1α and IL-33 were released in response to particle instillation from preexisting cellular stocks. To our knowledge, this is the first time that early release of these alarmins is evaluated in the genesis of inflammation induced by inorganic particles.

Once in the extracellular environment, IL-1β and IL-1α both bind to the IL-1R1/IL-1rAcP complex and induce similar effects. However, contrary to IL-1β, IL-1α does not require maturation and/or exocytosis to be active; its precursors form can activate IL-1R1 or translocate to the nucleus where it will modify inflammatory gene transcription [32]. The role of the IL-1 family in particle-induced neutrophilic inflammation has been extensively investigated mainly by using IL-1 signaling pathway inhibition [3,11] i.e. IL-1Ra (IL-1receptor antagonist) administration [33] or inflammasome activation

impairment [3,7,34]. Due to their functional redundancy, these studies do not allow to discriminate between IL-1α or IL-1β implication. Some reports specifically demonstrated a tempering effect of IL-1β deficiency on particle-induced inflammation [4,5,35,36]. A dominant role of IL-1α compared to IL-1β has been demonstrated in nano-TiO2-induced peritonitis and lung neutrophilic inflammation by using IL-1R-, IL-1α- or inflammasome component-deficient mice [23]. In the present study, we newly demonstrate the implication of IL-1α in pro-IL-1β production consecutive to particle exposure. Indeed, IL-1α stimulated precursor IL-1β production by macrophages and the absence of this alarmin abrogated mature IL-1β release upon silica treatment in mice. This is in accordance with observations of Gross and co-authors who reported that absence of IL-1α reduced IL-1β levels measured in response to monosodium urate (MSU) intraperitoneal injection [5]. Similarly, IL-1α neutralizing antibodies decreased lung IL-1β induction due to cigarette smoke [37]. The effect of IL-1α on IL-1β induction was also directly observed on macrophages *in vitro* [5,15,32,38-40]. Interestingly, the implication of IL-1α as a dominant IL-1 cytokine has also been recorded in other murine models of toxic lung inflammation or peritonitis. Alveolar and peritoneal neutrophilic accumulation induced by bleomycin, cigarette smoke or MSU were reduced when IL-1α response was inhibited [5,27,37,41].

IL-1α is a constitutive and ubiquitary cytokine (reviewed in [32]) whose release is frequently assessed in epithelial cells [41,42] or macrophages [15,43,44]. In this paper, we established that among lung resident cells, macrophages represent a major source of available IL-1α. We also demonstrated their ability to release IL-1α in response to particle exposure. Resident macrophages have already been implicated in IL-1α release under inflammatory conditions in lung [37], but also in liver [45] and brain [46]. It has been recently shown that particles-induced pyroptosis, a lytic mode of cell death that exhibits cytoplasmic swelling and ruptures of the plasma membrane [47]. It is tempting to postulate that pyroptosis results in the release of IL-1α by dying macrophages. In addition, it is surprising to note that the release of IL-1α by alveolar macrophages exposed *in vitro* to silica particles does not result in a direct production of pro-IL-1β. IL-1α-releasing macrophages are dying cells and thus probably unable to newly produce pro-IL-1β. In vivo, extracellular IL-1α from necrotic macrophages could, however, trigger pro-IL-1β production by surrounding active macrophages which then propagate neutrophilic inflammation by processing IL-1β after silica endocytosis. Finally, amorphous silica particles are generally regarded as less harmful than the crystalline forms [48-50]. We showed that amorphous (nano)silica particles induced stronger IL-1α secretion and inflammation than crystalline particles. These observations are in line with the recent conclusions offered by several experimental studies indicating that some amorphous (nano)silica particles need to be considered as potent toxic entities [51-55].

We evidenced that IL-33 is released in the lung upon particle instillation. This alarmin did, however, not participate significantly in IL-1β production by lung cells. We also observed a strong induction of IL-33 mRNA 24 hours after silica instillation, suggesting a possible implication in later inflammatory processes. Indeed, recent studies have shown that IL-33 is released in response to particle administration [56,57] and is implicated in lung inflammation after 30 days [58-60].

As observed after cristobalite or asbestos exposure [24,33], it has been recently found that the alarmin HMGB1 was released in alveolar space after MWCNT treatment in mice [61]. The secreted HMGB1 enhanced IL-1β release from alveolar macrophages and its neutralization reduced lung IL-1β content and inflammation *in vivo* [61]. In contrast, we did not observe an increase of HMGB1 alveolar levels in response to silica and different recombinant forms of HMGB1 did not stimulate IL-1β production by alveolar macrophages *in vitro*. This apparent discrepancy could be explained by the type of pulmonary cells targeted by the particles. MWCNT may cause strong cell damage to epithelial cells while silica may preferentially affect macrophages. This could result in the activation of different endogenous danger signal and inflammatory pathways, i.e. related to HMGB1 or IL-1α, respectively. Importantly, Jessop and colleagues demonstrated that HMGB1 present in BAL fluid of MWNCT-treated mice induced IL-1β release by macrophages while recombinant failed. This suggest that the complexation with other inflammatory molecules [62] or the redox status of HMGB1 [25] are mandatory for HMGB1 inflammatory activity. These modifications may also prevent HMGB1 recognition by the ELISA assays and interfere with HMGB1 detection after particle treatment.

The activation of the NFkB pathway leading to il-1b gene expression is probably not limited to IL-1α. Indeed, the pro-inflammatory cytokine TNF-α is also known to induce NFkB activation and IL-1β production [33,63]. Also this cytokine can be released rapidly and independently of transcriptional induction [64]. It is well known that TNF-α is a key factor in the development of lung inflammation upon particle exposure [33,65-67]. As for IL-1α, we showed that TNF-α induced the production of pro-IL-1β in primary lung macrophages *in vitro* (Additional file 1: Figure 2), suggesting that a pleiad of pro-inflammatory mediators may indirectly amplify the production of immature IL-1β. Additional investigations are also needed to better delineate the role the alarmin S100 and heat shock proteins highly secreted following particle exposure [68-70].

Increased levels of IL-1 cytokines were reported in patients suffering from particle-associated inflammatory diseases such as asbestosis [71] and silicosis [72-74].

Targeting IL-1β, e.g. through the use of Anakinra (a recombinant non-glycosylated version of human IL-1Ra) is already in use for the clinical treatment of gout and other IL-1β-related autoimmune diseases [75]. Interestingly, the interaction of Anakinra with the IL-1 receptor prevents the biological functions of IL-1β but also of IL-1α. Our data extend this therapeutic possibility in particle-induced lung inflammation by showing that blocking the IL-1 α/β pathway significantly reduces the deleterious influx of neutrophils after particle exposure. It remains, however, to demonstrate whether IL-1 blockade could also be associated with a reduction of particle-induced lung fibrosis [48]. Indeed, we recently showed that mice lacking IL-1β, IL-1α or IL-1R and treated with DQ12 silica presented a reduction of chronic lung inflammation and granuloma formation compared to their WT counterparts. However, this strong effect on inflammatory development was not concomitantly accompanied by a reduction of lung fibrosis, suggesting that inhibiting IL-1-driven inflammation may not be sufficient to control the fibrotic lung disease after particle exposure [76].

Finally, we showed that IL-1α release by the macrophage J774 cell line is highly predictive of the acute inflammogenic potential of silica micro- and nanoparticles. These results are in agreement with other studies that demonstrated the predictive value of IL-1α release to screen the skin irritative potential of various chemicals [77-80]. Hence, the *in vivo* inflammatory response to silica can be predicted by a simple and quantitative model based on *in vitro* release of IL-1α by cell line macrophages. This model may serve in (nano)toxicology to predict the *in vivo* toxicity of new materials and particles and to reduce animal uses.

Conclusion

The present study clarifies the respective role of IL-1α and β in particle-induced lung inflammation. We found that the release of endogenous IL-1α represents an early and crucial event that determines lung inflammatory responses to particle in mice (Figure 6). Released IL-1α after particle exposure serves as an alarmin that triggers the expression and the secretion of IL-1β. Alveolar macrophages represent the major source of IL-1α and IL-1β and macrophage population can be used to develop *in vitro* assays useful for screening the acute inflammatory potential of various types of particles. Finally, our results emphasize the need to target both IL-1α and IL-1β for regulating particle-associated lung inflammatory diseases.

Materials and methods

Particles

Crystalline silica particles (DQ12; DMT GmbH and Co. KG, Essen, Germany), Vitreous silica (VS; a gift from Dr. Ghiazza, University of Torino, Italy), Aerosil 200 (Sigma-Aldrich, Bornem, Belgium) and multi-wall carbon nanotubes (MWCNT; MWNT-7, Mitsui, Tokyo, Japan) powders were sterilized by heating at 200c for 2h immediately before suspension. Sterile Stber particle suspension were synthesized based on Stber process and its characterization was described elsewhere [81]. Characteristics of the particles are listed in Table 1.

Animal model

Female C57BL/6 mice were obtained from Janvier (Le Genest-Saint-Isle, France). IL-1α-, IL-1β-, IL1-R1- and MyD88-competent and -deficient mice (in C57BL/6 background) were obtained from the Transgnose Institute (Orleans, France). Studies were performed on gender-matched littermates aged 8-12 weeks. The animals were housed in positive pressure air-conditioned units (25C, 50% relative humidity) on a 12-hr light/dark cycle and had access to standard diet and tap water ad libitum. The experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the institutional Ethics Committee. Suspensions of crystalline DQ12 silica particles in sterile water or 0.9% saline (Braun Medical, Diegem, Belgium) were injected directly into the lung by pharyngeal aspiration at a dose of 2.5 mg/mouse inducing robust lung inflammation [20,21]. Anti-IL-1α antibody (clone AL-161; e-Biosciences, San Diego, USA) and anti-IL-1β antibody (clone B-122; e-Biosciences) were injected into the peritoneal cavity at a dose of 80 μg/mouse 1 hour before silica instillation [5].

Broncho-alveolar lavage (BAL) and alveolar cell number

Mice were sacrificed with an intraperitoneal injection of sodium pentobarbital (20 mg/mice) and bronchoalveolar lavage was performed by cannulating the trachea and lavaging the lung 4 with 1ml of NaCl 0.9%. The broncho-alveolar lavage fluid (BALF) was centrifuged at 281g, 10 min, 4C (Centrifuge 5804R, Eppendorf, Hamburg, Germany). Cells recovered in BAL were counted then fixed in 1.25% paraformaldehyde and analyzed with FACS calibur (BD Biosciences) using FlowJo software. Neutrophils were identified by labelling with anti-GR1-PE (clone RB6-8C5, BD Biosciences, Erembodegem, Belgium) and their number calculated in function of total BAL cells counted with a Burker cell chamber. Fc receptors were blocked with anti-CD16/CD32 (clone 2.4G2, BD Biosciences) to reduce nonspecific binding.

Lung histopathology

Lungs were lavaged and perfused with 0.9% NaCl and superior left lung lobe was fixed in 3.6% formaldehyde solution (Sigma-Aldrich) during one night. Paraffin-embedded 5-μm sections were stained with hematoxylin

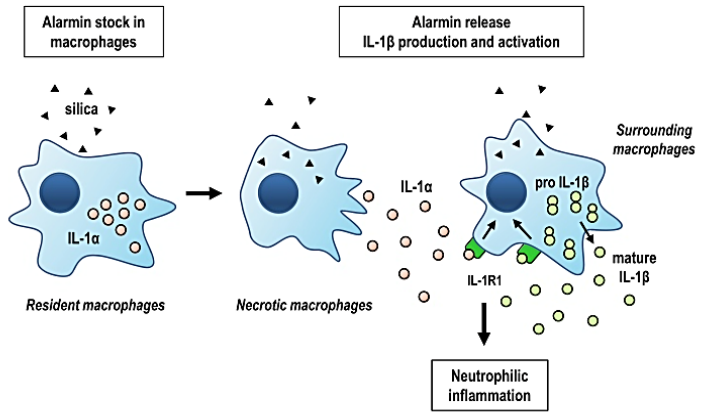


Figure 6 IL-1α released by alveolar macrophages upon particle exposure mediates neutrophilic inflammation directly and via IL-1β production. In homeostasis, IL-1α is constitutively expressed by resident alveolar macrophages and intracellularly retained as preexisting stocks. Released IL-1α into the extracellular milieu by necrotic macrophages serves as alarmin after silica exposure. IL-1α is a potent activating stimulus required for surrounding macrophages to express the biologically inactive precursor IL-1β (pro-IL-1β). This form is cleaved by silica-induced inflammasome assembly and activation prior its secretion as mature and bioactive IL-1β. IL-1α and β relayed by their receptor (IL-1R) are necessary to generate pulmonary neutrophil accumulation in response to silica particles.

and eosin. Images were acquired with a slide scanner SCN400 and analyzed with Digital Image Hub (Leica Microsystems, Diegem, BE).

RNA extraction and quantification

Total RNA extraction and quantification by qRT-PCR were performed as described [86]. Sequences of interest were amplified by PCR using the following forward primers (Invitrogen): CGG CTA CCA CAT CCA AGC AA (mouse Rn18s), GAC GGA CCC CAA AAG ATG AAG (mouse Il1b), GGA CTT CTC AAG ATC ATG GCT ACT T (mouse Cxcr2), TTG AAG ACC TAA AGA ACT GTT ACA GTG AA (mouse Il1a), GGA AAA GAC CAA GAG CAA GAC C (mouse Il33), TTT TGT CCA CAT GCC CTG C (mouse Hmgb1), and reverse primers: ATA CGC TAT TGG AGC TGG ATT ACC (mouse Rn18s), CTC TTC GTT GAT GTG CTG CTG TG (mouse Il1b), TAG TAG AGG TGT TTG CTG AAG ACG A (mouse Cxcr2), GCC ATA GCT TGC ATC ATA GAA GG (mouse Il1a), TTC TTC CCA TCC ACA CCG TC (mouse Il33), CTA ATA GTC CCA CGG TGT GAC AGT (mouse Hmgb1).

Cell purification

Mice treated with silica (2,5 mg/mouse) or not were sacrificed after 3 hours by intramuscular injection of 60 mg sodium pentobarbital. Lungs were perfused via the right heart ventricle with sterile NaCl 0.9%. One ml of enzyme mix containing 20 mg of pronase (Sigma-Aldrich) and 1mg of dnase (Worthington Biochemical Corporation,

Table 1 Physicochemical characterization of the particles

Name	Type	Crystallinity	Size TEM (nm)	Surface BET (m^2/g)
DQ12	Ground natural mineral	Crystalline	960[a]	7,4 -10,1[b]
VS	Ground fused silica	Amorphous	1600[c]	3.1[c]
Stber	Stber silica	Amorphous	12 [d]	400[d]
Aerosil 200	Fumed silica	Amorphous	12[e]	175-225[e]
MWCNT	Multi-wall carbon nanotubes	N/a	Length :5,700 3,700 [f] Width : 74 28 [f]	22[f]

(a) [82].
(b) [83].
(c) [51].
(d) [84].
(e) Data provided by the manufacturer (Sigma-Aldrich).
(f) [85].

Lakewood, USA) in HBSS (Invitrogen; Merelbeke; Belgium) with 1% of antibiotic antimycotic (AA) (fungizone (25 μg/mL), penicillin streptomycin (10000 U and 10000 μg/mL); Invitrogen) were infused in cannulated trachea. After 20 minutes, lungs were excised and placed into a tube chilled on ice with fetal bovine serum (FBS) (Invitrogen). Collected lungs were then crushed by repeated aspiration and expulsion in a 20 ml seringue and passed on a 70 μM filter. Structural cells were purified based on their absence of CD45 expression by using magnetic cell separation (MACS; Miltenyi Biotec) according to the manufacturer's protocol; T lymphocytes based on their CD5 expression; B lymphocytes based on their B220 expression; dendritic cells based on their absence of F480 and the presence of CD11c expression; and alveolar macrophages based on their F480 expression. Alveolar macrophages were also obtained from lung cell suspensions grown in $75cm^2$ tissue culture flask in DMEM (Invitrogen) supplemented with 10% FBS and 1% of AA, detached using trypsin (Invitrogen) and purified based on their CD45 expression by using magnetic cell separation (MACS) according to the manufacturer's protocol.

Pro-IL-1β and IL-1α content in lung cells

For ELISA measurement of intracellular or released pro-IL-1β or IL-1α, fresh lung cells purified from nave or silica treated mice, primary cultured macrophages, J774, MLg or LA4 (10^6 cells/well in 96-well plate) were exposed overnight with LPS (0,1μg/ml; Enzo Life Sciences, Antwerpen, Belgium), DQ12, mouse recombinant IL-1α, IL-33, IL-1β, TNF-α (R&D systems, Minneapolis, USA) or HMGB1 (chemokine, non-oxydable and cytokine form; HMGBiotech, Milano, Italia) when necessary. For intracellular cytokine measurement, cell pellets were lysed by addition of 100 μl of triton X-100 0,1%. The ex vivo released of IL-1α were quantified by ELISA in the supernatants of cells purified from silica-treated lungs maintained in culture during 24 hours in 100 μl of DMEM. For *in vitro* IL-1α release in response to particles, fresh alveolar $F4/80^+$ macrophages (10^6 cells/well in 96-well plate) or J774 cells (0.4 10^6 cells/well in 96-well plate) were incubated overnight with particles dispersed in 100 μl of DMEM and IL-1α was measured in the supernatant.

For western blot analysis, cells were lysed on ice in 200 μl Triton lysis buffer (TLB) (1% Triton, 25mM Tris pH 7.4, 150mM NaCl + anti-protease tablets from Roche Applied Science at 2 mg/ml). Protocol for Western blot is described elsewhere [84].

Enzyme-linked immunosorbent assays (ELISA)

ELISA kits were used to measure IL-1β, IL-1α, IL-33 (DY401, R&D Systems, Wiesbaden-Nordenstadt, Germany), HMGB1 (Cedarlane Laboratories USA Inc., Burlington, USA) and pro-IL-1β (e-Biosciences). Assays were run according to the manufacturer's protocols with a detection limit of 5 pg/ml for IL-1β, IL-1α, IL-33, 0,2 ng/ml for HMGB1 and 25 pg/ml for pro-IL-1β

Statistics

Results were analyzed by *t*-test. Statistical significance was considered at $P < 0.05$.

Additional file

Additional file 1: Figure S1. Levels of (a) HMGB1, (b) IL-1β and proteins (c) in BAL fluid collected at different time points after silica (crystalline DQ12, 2.5 mg) or not (control). Pulmonary expression of (d) pro-IL-1α, (e) pro-IL-33 and (f) HMGB1 quantified by qRT-PCR at different time points after instillation of silica or not. Values are means SEM of 3 to 8 animals. *p <0.05, **p < 0.01 and ***p < 0.001 denote significant difference between animals treated with silica or not; ns, denotes no significant difference. P-values are estimated by t-test. **Figure S2.** (a) Intracellular levels of pro-IL-1β in primary cultured alveolar macrophages exposed overnight to LPS (0.1 μg/ml) or recombinant TNF-α. n = 3 to 5. (b) Western blot analysis of intracellular pro-IL-1β and β-actin in primary cultured lung macrophages exposed during 18 hours to LPS (0.1 μg/ml) or recombinant TNF-α. Values are means SEM. **p < 0.01 and ***p < 0.001 denotes significant difference between cells in DMEM and cells exposed to LPS or recombinant TNF-α. P-values are estimated by t-test.

Competing interests
The authors declare that they have no competing interests.

Authors contributions
VR, AA and FH conceived and designed the experiments. RD, FU, YY, and MP performed the experiments. IC and BR provided mouse strains. VR, AA, DL and FH analyzed the data. VR, DL and FH wrote the manuscript. All authors read and approved the final manuscript.

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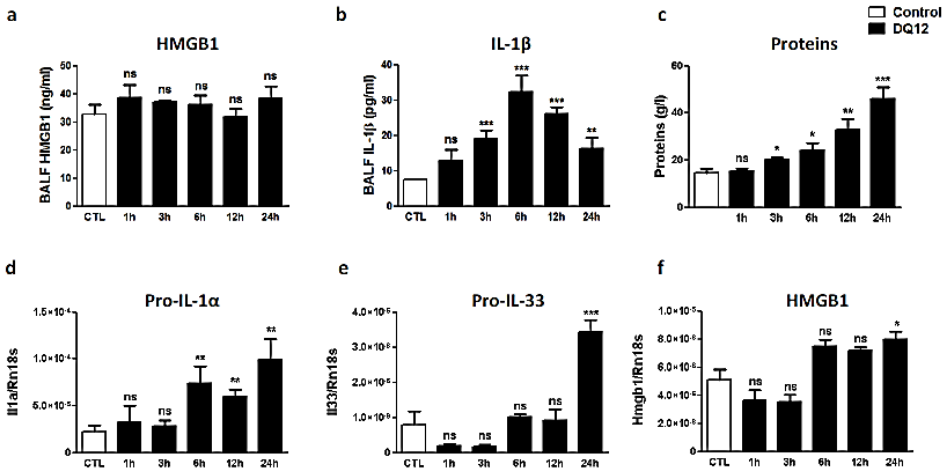
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Supplemental legends

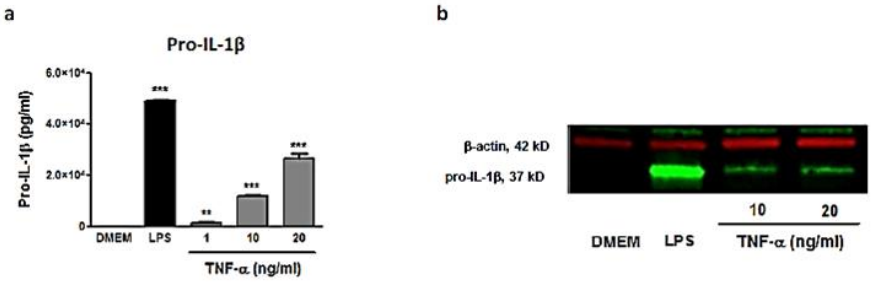
Supplemental figure 1 : Levels of **(a)** HMGB1, **(b)** IL-1β and proteins **(c)** in BAL fluid collected at different time points after silica (Crystalline DQ12, 2.5 mg) or not (control). Pulmonary expression of **(d)** pro-IL-1α, **(e)** pro-IL-33 and **(f)** HMGB1 quantified by qRT-PCR at different time points after instillation of silica or not. Values are means ± SEM of 3 to 8 animals. *p < 0.05, **p < 0.01 and ***p < 0.001 denote significant difference between animals treated with silica or not; ns, denotes no significant difference. P-values are estimated by t-test.

Supplemental figure 2 : (a) Intracellular levels of pro-IL-1β in primary cultured alveolar macrophages exposed overnight to LPS (0.1 µg/ml) or recombinant TN-α. n = 3 to 5. **(b)** Western blot analysis of intracellular pro-IL-1β and β-actin in primary cultured lung macrophages exposed during 18 hours to LPS (0.1 µg/ml) or recombinant TNF-α. Values are means ± SEM. **p < 0.01 and ***p < 0.001 denotes significant difference between cells in DMEM and cells exposed to LPS or recombinant TNF-α. P-values are estimated by t-test.

Supplemental Figure 1



Supplemental Figure 2



2. Critical role of aquaporins in interleukin-1β (IL-1β)-induced inflammation

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Critical Role of Aquaporins in Interleukin 1β (IL-1β)-induced Inflammation*

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Background: Aquaporins are channels permeable to water, and they are essential for immune cell migration.
Results: We demonstrate that aquaporin-mediated water fluxes are necessary for the NLRP3 inflammasome-dependent release of mature IL-1β *in vitro* and *in vivo*.
Conclusion: Aquaporins are implicated in the mechanisms of proinflammatory cytokine secretion during inflammation.
Significance: The discovery of a new function for AQPs opens new diagnostic and therapeutic opportunities in inflammatory disorders.

Rapid changes in cell volume characterize macrophage activation, but the role of water channels in inflammation remains unclear. We show here that, *in vitro*, aquaporin (AQP) blockade or deficiency results in reduced IL-1β release by macrophages activated with a variety of NLRP3 activators. Inhibition of AQP specifically during the regulatory volume decrease process is sufficient to limit IL-1β release by macrophages through the NLRP3 inflammasome axis. The immune-related activity of AQP was confirmed *in vivo* in a model of acute lung inflammation induced by crystals. AQP1 deficiency is associated with a marked reduction of both lung IL-1β release and neutrophilic inflammation. We conclude that AQP-mediated water transport in macrophages constitutes a general danger signal required for NLRP3-related inflammation. Our findings reveal a new function of AQP in the inflammatory process and suggest a novel therapeutic target for anti-inflammatory therapy.

Because of their ability to trigger or dampen inflammatory processes, macrophages are key effectors of innate immunity. They possess a wide range of specific receptors recognizing endogenous and foreign patterns indispensable to organize adequate clearance and immune responses. Upon detection of threat signals, macrophages elicit immune reactions, trying to eliminate danger and to inform other cells by releasing cytokines and chemokines. In particular, the macrophage-derived cytokine IL-1β drives inflammation by enhancing granulocyte differentiation, migration, and accumulation to inflammatory sites. This cytokine has been implicated in lung inflammatory reactions in response to bacteria, viruses, inorganic particles, and drugs (1–4).

The release of mature IL-1β by macrophages requires the assembly of a multiproteic inflammasome complex, of which the caspase 1 component is the final effector. The NLRP3 (NOD-like receptor protein 3) inflammasome is the most fully characterized inflammasome. It can be activated by a wide array of stimuli, including extracellular ATP, bacterial toxins such as nigericin, monosodium urate crystals, and environmental and industrial particles such as silica (5). How those structurally and chemically very different stimuli can activate this inflammasome in macrophages is still under investigation, but common cellular stress signals are likely involved. It has been established that ionic movements represent a common event required for inflammasome activation by diverse stimuli. A drop in cytosolic K^+ is necessary and sufficient for caspase 1 activation and IL-1β release by macrophages (5). Cell distension and subsequent regulatory volume decrease (RVD)[2] mediated by activation of volume sensor systems such as transient receptor potential (TRP) channels have also been associated with inflammasome activation in macrophages (6–8). How-

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[2] The abbreviations used are: RVD, regulatory volume decrease; TRP, transient receptor potential; AQP, aquaporin; BAL, bronchoalveolar lavage; ASC, apoptosis-associated speck-like protein containing a CARD; ngp, neutrophilic granule protein; DQ12, crystalline silica (α-quartz); SNP, silica nanoparticles.

ever, the potential importance of channel-mediated water transport as a general pathway of inflammasome activation remains unknown.

Aquaporins (AQPs) are a family of channels that facilitates water transport across cell membranes in response to osmotic gradients. Aquaporin 1 (AQP1), the archetype of all aquaporins, is selectively permeable to water and involved in rapid cellular water fluxes and cell volume regulations (9). Although AQPs are primarily expressed in epithelial and endothelial cells, these channels are also expressed in erythrocytes and immune cells such as macrophages, dendritic cells, and thymocytes (10–15). AQPs are implicated in the phagocytic functions, activation, and migration of immune cells (10, 12, 14).

Here we explore the involvement of AQPs in mature IL-1β release by macrophages. We show that AQP-related cell water transport triggered by diverse soluble and particulate NLRP3 activators is sensed as a danger signal by macrophages and that AQPs are implicated in NLRP3 inflammasome activation *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Animal Model—Female C57BL/6 mice were obtained from Janvier (Le Genest-Saint-Isle, France). Wild-type ($AQP1^{+/+}$) and knockout ($AQP1^{-/-}$) mice (16) were obtained from A. S. Verkman (University of California, San Francisco, CA) and developed as a specific pathogen-free colony at the Université Catholique de Louvain Medical School (Brussels, Belgium). Studies were performed on gender-matched littermates aged 8–12 weeks. The animals were housed in positive pressure, air-conditioned units (25 °C, 50% relative humidity) on a 12-h light/dark cycle and had access to standard diet and tap water *ad libitum*. The experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the institutional ethics committee. A suspension of crystalline silica particles (DQ12, d50 = 2.2 μm, a gift from Dr. Ambruster, Essen, Germany) in sterile 0.9% saline (Braun Medical, Diegem, Belgium) was injected directly into the lung by pharyngeal aspiration at a dose of 2.5 mg/mouse. To sterilize and inactivate any trace of endotoxins, particles were heated at 200°c for 2 h immediately before suspension and administration. Bleomycin (Aventis, Brussels, Belgium) was suspended in sterile 0.9% NaCl, and 1.25 units/kg body weight was injected directly into the lung by pharyngeal aspiration. The experimental protocol was approved by the local ethical committee for animal research at the Université Catholique de Louvain (2010/UCL/MD/034) and conformed to the Belgian and European Community regulations (LA1230312 and CEE no. 86/609).

Bronchoalveolar Lavage (BAL) and Alveolar Neutrophil Number—Mice were sacrificed after 6 h with an intraperitoneal injection of sodium pentobarbital (20 mg/mice), and bronchoalveolar lavage was performed by cannulating the trachea and lavaging the lung four times with 1 ml of NaCl 0.9%. The BAL fluid was centrifuged at 281 × *g* for 10 min at 4 °C (centrifuge 5804R, Eppendorf, Hamburg, Germany). Cells recovered in BAL were fixed in 1.25% paraformaldehyde and analyzed with FACSCalibur (BD Biosciences) using FlowJo software. Neutrophils were identified by labeling with anti-GR1-PE (clone RB6-8C5, BD Biosciences), and their number was calculated in a function of total BAL cells counted with a Burker cell chamber. Fc receptors were blocked with anti-CD16/CD32 (clone 2.4G2, BD Biosciences) to reduce nonspecific binding.

Lung Histopathology—Lungs were lavaged and perfused with 0.9% NaCl, and the superior left lung lobe was fixed in 3.6% formaldehyde solution for one night. Then, paraffin-embedded, 5-μm sections were stained with hematoxylin and eosin. Images were acquired with a microscope (Zeiss, Jena, Germany, model Jenalumar) equipped with an Apochromat ×12.5/0.35 for pictures in a large panel and a Planachromat fl ×50/0.95 for pictures in the insets (Carl Zeiss, Munich, Germany). The microscope was connected to a Coolpix 4500 camera (Nikon, Kingston upon Thames, UK).

RNA Extraction and Quantification—Total RNA extraction and quantification by quantitative PCR were performed as described previously (17). Sequences of interest were amplified by PCR using the following forward primers (Invitrogen): CGG CTA CCA CAT CCA AGC AA (mouse 18 S RNA), GAC GGA CCC CAA AAG ATG AAG (mouse IL-1β), CCA GCC AGA GTG GAA CTT TCG (mouse NLRP3), GGA GCT CAC AAT GAC TGT GCT TA (mouse ASC), GGA CTT CTC AAG ATC ATG GCT ACT T (mouse CXCR2), GAC TGC GAC TTC CTG GAG GAT (mouse ngp) and reverse primers: ATA CGC TAT TGG AGC TGG ATT ACC (mouse 18s RNA), CTC TTC GTT GAT GTG CTG CTG TG (mouse IL-1β), TAC AAA TGG AGA TGC GGG AGA (mouse NLRP3), CTG CCA CAG CTC CAG ACT CTT(mouse ASC), TAG TAG AGG TGT TTG CTG AAG ACG A (mouse CXCR2), and GTA TCC TCT CGA CTG CAA TCC CTG (mouse ngp).

ELISA—ELISA kits were used to measure IL-1β and IL-6 (R&D Systems, Wiesbaden-Nordenstadt, Germany). Assays were run according to the protocols of the manufacturer with a detection limit of 15 pg/ml. The IL-1β assay selectively measured the mature form of IL-1β (17 kDa) (18).

Cell Purification—Mice were sacrificed by intramuscular injection of 60 mg sodium pentobarbital. For peritoneal macrophages, the peritoneal cavity was lavaged with 10 ml of sterile NaCl 0.9% and centrifuged (280 × *g*, 10 min, 4 °C). Macrophages were purified from peritoneal lavage on the basis of their F4/80 expression by using magnetic cell separation (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the protocol of the manufacturer. For lung macrophages, lungs were perfused via the right heart ventricle with sterile NaCl 0.9%, and then 1 ml of enzyme mix containing 20 mg of Pronase (Sigma-Aldrich, Bornem, Belgium) and 1 mg of DNase (Worthington Biochemical Corp.) in Hanks' balanced salt solution (Invitrogen) with 1% of antibiotic antimycotic (Fungizone (25 μg/ml), penicillin-streptomycin (10,000 units and 10,000 μg/ml); Invitrogen) was infused in the cannulated trachea. After 20 min, lungs were excised and placed into a tube chilled on ice with FBS (Invitrogen). Collected lungs were then crushed by repeated aspiration and expulsion in a 20-ml syringe and passed on a 70-μM filter. The filtered fraction was grown in a 75-cm^2 tissue culture flask in DMEM (Invitrogen) supplemented with 10% FBS and 1% of antibiotic antimycotic and then detached using trypsin (Invitrogen). Lung macrophages were purified on the basis of their CD45 expression by using magnetic cell sep-

aration (Miltenyi Biotec) according to the protocol of the manufacturer. Macrophages were primed for 18 h by exposure to 0.1 μg/ml of purified LPS (Enzo Life Sciences, Antwerpen, Belgium) in complete medium.

Inflammasome Activation—LPS-primed cells (200,000 cells/well in a 96-well plate, 7×10^6 cells/well in a 6-well plate) were exposed to an inflammasome activator (200 μl) for 30 min for ATP (5 mM), 1 h for nigericin (20 μM), Stöber silica nanoparticles (SNP) (100 μg/ml) and hypotonic shock, and 3 h for DQ12 (400 μg/ml). Supernatant was collected for mature IL-1β measurement, and the cell pellet was used to quantify active caspase 1. The Stöber-based synthesis process and characterization of SNP are described elsewhere (19). SNP diameter of 12.2 nm or 100 nm were obtained by transmission electron microscopy or by dynamic light scattering in DMEM, respectively. The total surface area evaluated by the BET (Brunauer, Emmet, and Teller) model was 400 m^2/g. Isotonic and hypotonic media were prepared on the basis of Perregaux (6). The isotonic solution contained 0.9 M $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 2.7 mM KCl, 5 mM glucose, 20 mM Hepes, and 137 mM NaCl. In hypotonic solutions, NaCl was absent (47 mosmol/kg H_2O) or at a concentration reduced to 3.75 mM (53 mosmol/kg H_2O), 7.5 mM (60 mosmol/kg H_2O), 15 mM (66 mosmol/kg H_2O), or 30 mM (100 mosmol/kg H_2O). All solutions were prepared in LPS-free water (Baxter, Braine-l'Alleud, Belgium) and filtered on a 0.22-μm filter. Hypertonic solutions were obtained by adding 100 mM NaCl (415 mosmol/kg H_2O) or 200 mM mannitol to DMEM. When a pretreatment was required, cells were first incubated with $HgCl_2$ 30 min prior to the activator. $HgCl_2$ was maintained in the medium until the end of the experiment. For mercurial AQP inhibition after hypotonic shock, $HgCl_2$ was added at different time points following hypotonic shock and maintained till the end of the experiment (60 min after the shock). For TRP inhibition, Ruthenium Red (Sigma-Aldrich) was added when hypotonic shock was applied and maintained until the end of the experiment (60 min after the shock). For the cytotoxicity assay, cells were incubated in DMEM with 2% WST1 (Roche Applied Science, Vilvoorde, Belgium) for 15 min.

Active Caspase 1 Measurement—After treatment with an inflammasome activator, cells were incubated with green fluorescent peptide, 5-carboxyfluorescein-Tyr-Val-Ala-Asp-fluoromethyl ketone, according to the recommendations of the manufacturer (Immunochemistry Technologies). Fluorescence was measured by using a SPECTRAmax GEMINI XS microplate spectrofluorometer (Molecular Devices). For the flow cytometry assay, 10^6 cells were exposed to hypotonic medium during 30 or 60 min in microtubes and then incubated with the probe according to the recommendation of the manufacturer and analyzed by using a FACSCalibur (BD Biosciences).

Size Modification Assessment—For microscopical analysis of size modification, LPS-primed cells (300,000 cells/well in a 48-well plate) were exposed to isotonic or hypotonic medium, and pictures were taken after the required time of incubation under optimal culture conditions. Images were acquired with a Zeiss AxioObserver.z1 equipped with a T° and pO2/pCO2 regulation chamber and an EC "Plan-Neofluar" ×10/0.30 objective (Carl Zeiss). The microscope was connected to an AxioCam camera using AxioVision software (Carl Zeiss). For flow cytometry assessment of cell volume modification, LPS-primed cells were incubated in microtubes in isotonic or hypotonic medium during the desired time with or without mercury treatment. Cells were then fixed by addition of paraformaldehyde directly to the medium and analyzed with FACSCalibur.

Western Blot Analysis—After treatments, supernatants of LPS-primed macrophages ($7\ 10^6$ cells/well in 6-well plates) were collected, and cell pellets were lysed on ice in Triton lysis buffer (1% Triton, 25 mM Tris (pH 7.4), 150 mM NaCl, and antiprotease tablets (Roche Applied Science) at 2 mg/ml). The supernatant was concentrated by methanol-chloroform precipitation and then suspended in 30 μl of Laemmli buffer (Bio-Rad). The supernatant and cell samples were then sonicated for 5 min in a bath (Bransonic-12, Geneva, Switzerland), heated at 99 °C for 5 min, and centrifuged for 10 min at 14,000 rpm. Samples were loaded onto a 4–20% polyacrylamide gel (Mini-Protean TGX, Bio-Rad) and transferred to a PVDF membrane (Millipore) by electroblotting. Membranes were incubated overnight at 4 °C with primary antibody (0.2 μg/ml, polyclonal goat anti-IL-1β antibody, catalog no. AF-401-NA, R&D Systems; 2 μg/ml, polyclonal rabbit anti-caspase 1 p10 antibody, Santa Cruz Biotechnology, Santa Cruz, CA or 1 μg/ml, monoclonal mouse anti-β-actin antibody, catalog no. A1978, Sigma-Aldrich). After washing, membranes were incubated for 1 h with RDye infrared secondary antibodies (1:15,000; donkey IgG anti-goat, donkey IgG anti-rabbit, or donkey IgG anti-mouse, Westburg, Leusden, The Netherlands). Detection was performed with an Odyssey CLx Western blot detection system (Westburg).

Statistics—Results were analyzed by Student Newman-Keuls test. Statistical significance was considered at $p < 0.05$.

RESULTS

AQP-facilitated Water Transport Induces NLRP3 Inflammasome-related IL-1β Activation and Release in Macrophages—Because macrophage swelling is commonly recorded following exposure to different NLRP3 inflammasome activators (8), we analyzed the importance of water transport in IL-1β release *in vitro*. We first contained cell swelling in ATP-activated lung macrophages by exposure to increased extracellular tonicity (20). Under extracellular hyperosmolar conditions, ATP-induced IL-1β release by LPS-primed lung macrophages was reduced significantly (Fig. 1*a*). The absence of cytotoxicity because of increased extracellular osmolarity was verified by measuring cellular mitochondrial activity (Fig. 1*d*). To determine whether this effect was specific to inflammasome activation, we also measured the levels of the proinflammatory cytokine IL-6, which does not directly require inflammasome activation for its secretion. Unlike IL-1β, the release of IL-6 was not modified by extracellular hyperosmolar conditions (Fig. 1*e*), suggesting that swelling is not required for the release of other proinflammatory cytokines that do not directly depend on inflammasomes for their release. The inhibitory effect of an extracellular hyperosmolarity on ATP-induced inflammasome activation was confirmed in peritoneal macrophages on the basis of the same protocol (Fig. 1*b*). IL-1β release by peritoneal macrophages was also reduced when osmolarity was increased

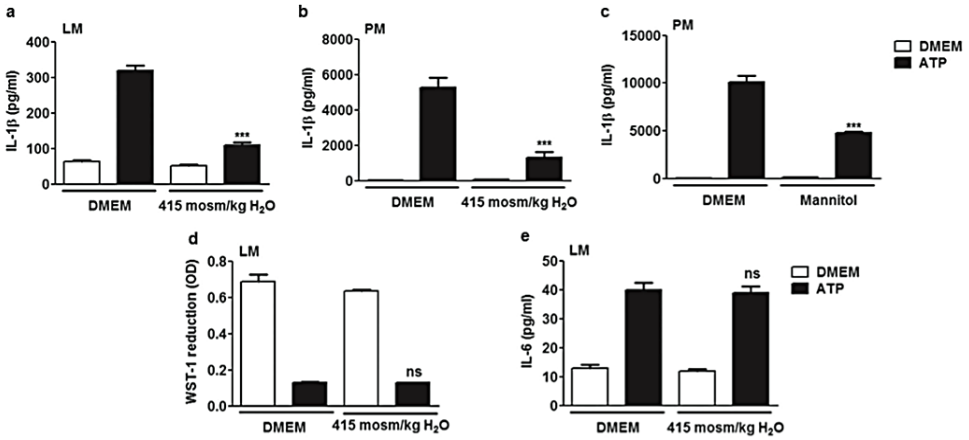


FIGURE 1. **Water entry is necessary for IL-1β release in macrophages.** Release of IL-1β by lung macrophages (*LM*) (*a*) or peritoneal macrophages (*PM*) (*b*) primed with LPS (0.1 μg/ml, overnight) and subsequently exposed or not exposed to ATP (5 mM) with addition or no addition of NaCl to the medium (415 mosmol/kg H_2O). $n = 4$ replicates/condition. *c*, release of IL-1β by peritoneal macrophages exposed or not exposed to ATP (5 mM) with addition or no addition of mannitol to the medium (200 mM). $n = 3$ or 4. *d*, mitochondrial activity assessed by optical density (*OD*) of reduced WST-1 and release of IL-6 by lung macrophages exposed or not exposed to ATP (5 mM) with addition or no addition of NaCl to the medium (*e*). $n = 3$ or 4. Data are means ± S.E. ***, $p < 0.001$ upon exposure to hypotonic ATP with or without NaCl or mannitol addition; *ns*, no significant difference. *p* values were calculated by Student Newman-Keuls test.

by mannitol addition (Fig. 1*c*). These results show that rapid cell swelling is necessary for IL-1β release in macrophages.

To study water channel implication, a model of osmotic swelling-induced NLRP3 inflammasome activation and IL-1β release was used (7). IL-1β release was assessed in lung macrophages exposed for 60 min to hypotonic solutions with decreasing osmolarity. Reducing medium osmolarity to 100 and 66 mosmol/kg H_2O had no significant impact on mature IL-1β detected in cell supernatant, but more drastic hypotonic conditions (from 60 to 47 mosmol/kg H_2O) progressively induced significant IL-1β release (Fig. 2*a*). To test the involvement of water channels in that process, we treated cells 30 min before the hypotonic shock with mercury, which pharmacologically inhibits most AQPs by interacting with an extracellular cysteine residue (21). The implication of AQPs in osmotic swelling was studied 2 min after exposure of lung macrophages to 60 mosmol/kg H_2O. At this time point, the size of cells exposed to hypotonic medium was increased compared with isotonic medium. This swelling was reduced in mercury-treated cells (Fig. 2*b*). AQP inhibition decreased the release of IL-1β by lung macrophages submitted to hypotonic shock (Fig. 2*c*). We also explored the response of peritoneal macrophages treated according to the same protocol. The reduction of IL-1β release was confirmed when AQPs were inhibited in peritoneal macrophages submitted to hypotonic shock (Fig. 2*d*). This effect was supported by Western blot data (Fig. 2*e*). The release of mature IL-1β (*IL-1β p17*) by peritoneal macrophages in response to hypotonic shock was reduced upon mercury exposure. In parallel, the decrease of the intracellular pool of the IL-1β proform (*IL-1β p37*) consecutive to hypotonic shock was moderated by AQP blockade (Fig. 2*e*). Mercurial AQP inhibition also resulted in reduced caspase 1 activation consecutive to hypotonic shock in lung macrophages (Fig. 2*f*). Together, these results demonstrated that rapid cell swelling via AQPs leads to NLRP3 inflammasome-related release of mature IL-1β by macrophages.

The requirement of AQPs was extended to other soluble and insoluble NLRP3 inflammasome activators. The concentration of IL-1β detected in the supernatant of lung macrophages after silica (*DQ12*) and ATP was significantly lower when AQPs were inhibited (Fig. 2, *g* and *i*). Similarly, mercury inhibition of AQP led to decreased silica-induced (Fig. 2*h*) and ATP-induced (Fig. 2*j*) IL-1β release in peritoneal macrophages. The implication of AQP in caspase 1 activation (active caspase 1, *casp1 p10*) and mature IL-1β release was confirmed by Western blotting in ATP-treated peritoneal macrophages (Fig. 2*k*). AQP blockade also decreased the ATP-induced reduction of the intracellular IL-1β proform. The absence of cytotoxicity (Fig. 2*l*) and IL-6 release modifications (Fig. 2*m*) in the presence of mercury was verified in lung macrophages exposed to ATP. Together, these data indicate that rapid water transport via AQPs leads to IL-1β activation and release by lung and peritoneal macrophages after exposure to soluble and particulate NLPR3 stimuli.

AQP-facilitated RVD Is Sufficient to Induce the NLRP3 Inflammasome-related Release of Mature IL-1β in Macrophages—We next investigated which phase of the AQP-dependent volume changes (initial swelling or subsequent volume restoration) mediates inflammasome activation in macrophages. First, the kinetics of volume modifications, caspase 1 activation, and IL-1β release after hypotonic shock in lung macrophages were monitored. Cell swelling was obvious 2 min after exposure to hypotonic medium and sustained until 10 min. The subsequent size reduction was already perceptible 20 min after hypotonic shock, and the return to the initial size was achieved after 45 and 60 min (Fig. 3, *a–c*). IL-1β excretion was

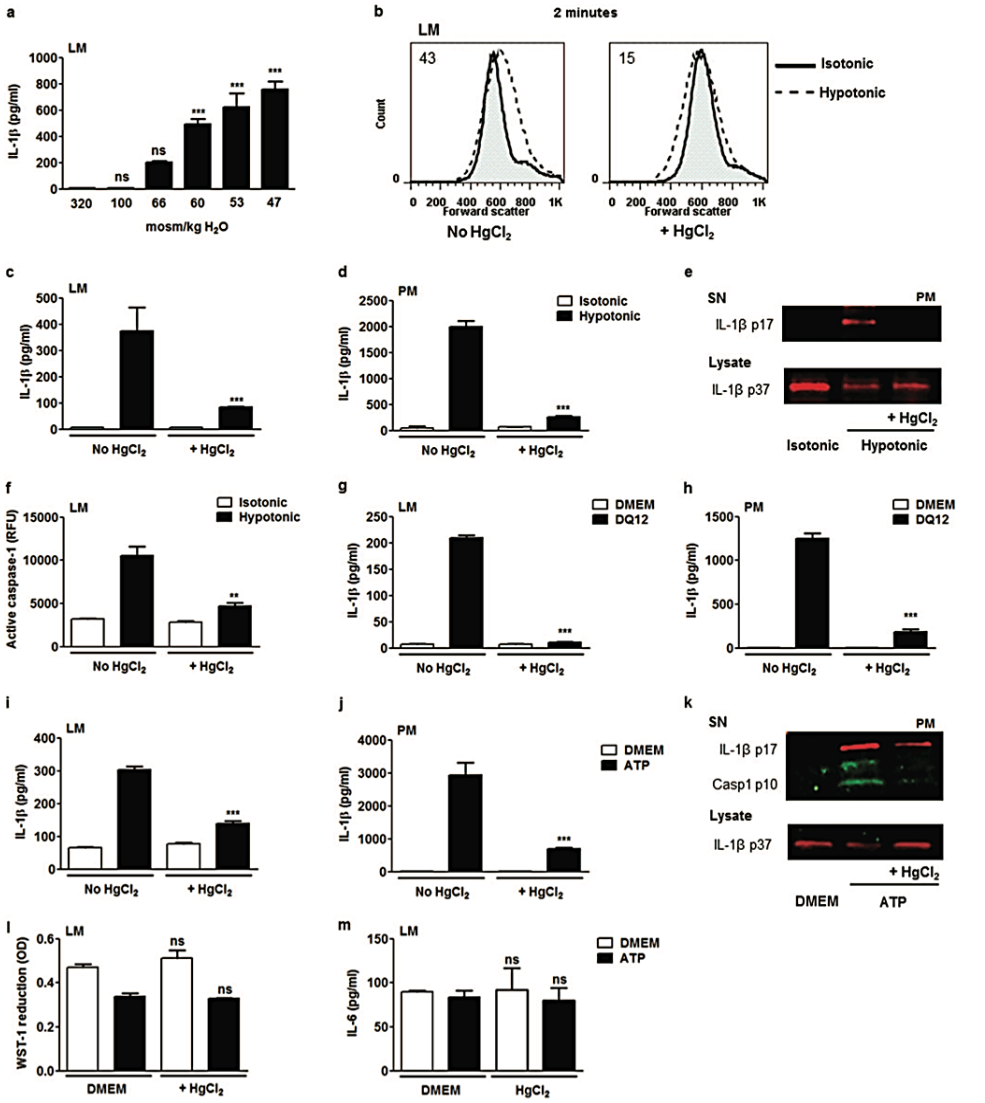
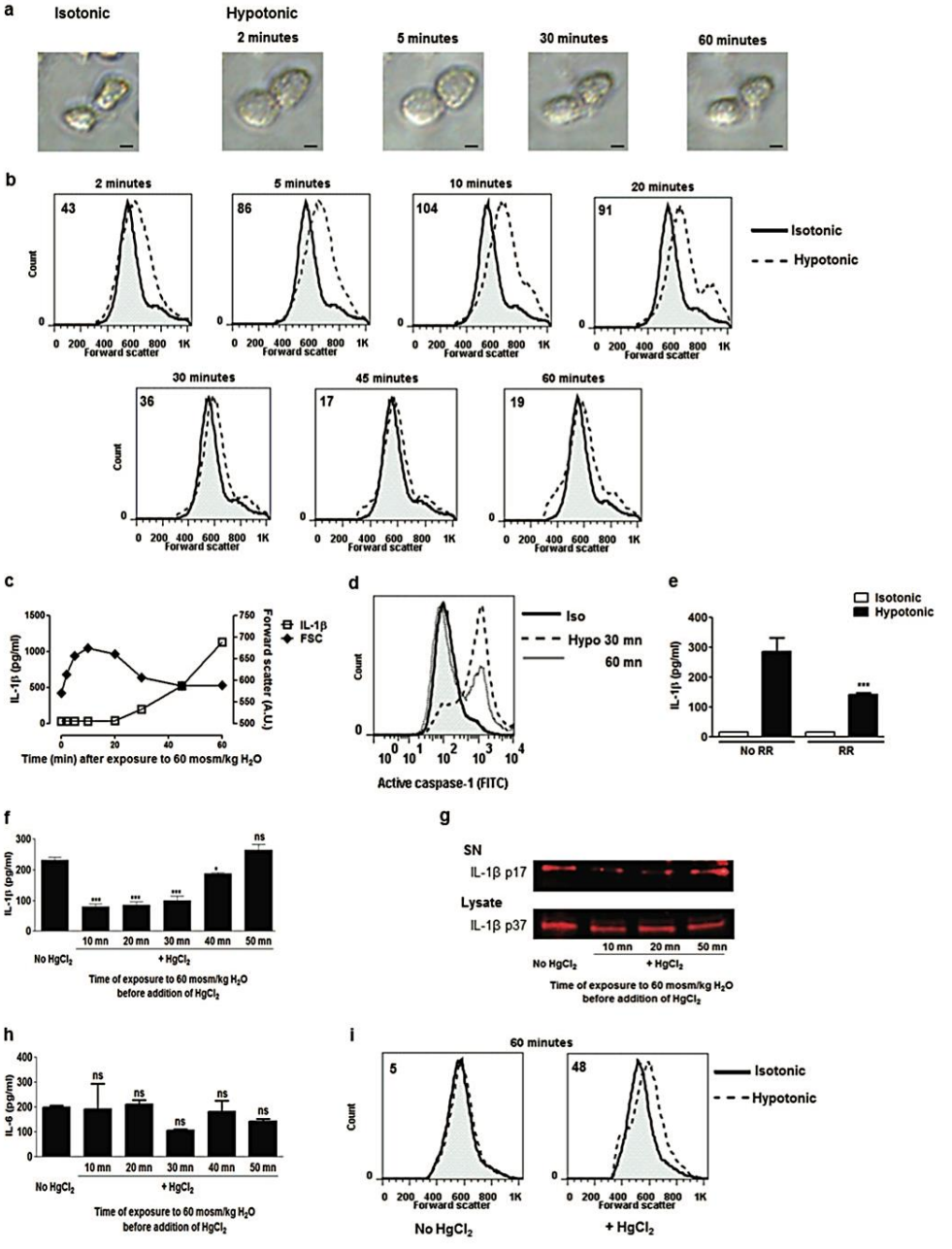


FIGURE 2. **AQP-facilitated water transport induces NLRP3 inflammasome-related caspase-1 activation and mature IL-1β release.** *a*, IL-1β release by lung macrophages (*LM*) primed with LPS (0.1 μg/ml) exposed to isotonic (320 mosmol/kg H_2O) or hypotonic medium (100 mosmol/kg H_2O to 47 mosmol/kg H_2O). $n = 3$ replicates/condition. *b*, flow cytometry forward scatter plots of lung macrophages subjected or not subjected to hypotonic shock (60 mosmol/kg H_2O) in the absence or presence of $HgCl_2$ (10 μM). Values indicated in the plots are differences between median forward scatter of cells exposed or not exposed to hypotonic shock. Levels of released IL-1β by lung (*c*) or peritoneal macrophages (*PM*) (*d*) exposed or not exposed to hypotonic shock (in the absence or presence of $HgCl_2$ (10 μM). $n = 3$. *e*, Western blot analysis of mature IL-1β (*IL-1β p17*) in the supernatant (*SN*) and pro-IL-1β (*IL-1β p37*) in cell lysate of peritoneal macrophages exposed or not exposed to hypotonic shock in the absence or presence of $HgCl_2$. *f*, fluorimetric measurement of cellular active caspase 1 in lung macrophages exposed or not exposed to hypotonic shock in the absence or presence of $HgCl_2$ (10 μM). $n = 3$ or 4. Release of IL-1β by lung (*g*) or peritoneal macrophages (*h*) exposed or not exposed to silica (400 μg/ml) in the absence or presence of $HgCl_2$ (10 μM)). $n = 3$ or 4. IL-1β release by lung (*i*) or peritoneal macrophages (*j*) exposed or not exposed to ATP (5 mM) in the absence or presence of $HgCl_2$. $n = 3$ or 4. *k*, Western blot analysis of mature IL-1β and active caspase 1 (*Casp1 p10*) in the supernatant and pro-IL-1β in cell lysate of peritoneal macrophages exposed or not exposed to ATP in the absence or presence of $HgCl_2$. *l*, mitochondrial activity assessed by optical density (*OD*) of reduced WST-1 and release of IL-6 by lung macrophages exposed or not exposed to ATP (5 mM) in the absence or presence of $HgCl_2$ (10 μM) (*m*). $n = 3$ or 4. Data are means ± S.E. **, $p < 0.01$; ***, $p < 0.001$ upon exposure to hypotonic medium or ATP or no exposure, with $HgCl_2$ or no exposure; *ns*, no significant difference. *p* values were calculated by Student Newman-Keuls test.


a
Isotonic
Hypotonic
2 minutes
5 minutes
30 minutes
60 minutes
b
2 minutes
5 minutes
10 minutes
20 minutes
43
86
104
91
Count
Forward scatter
Isotonic
Hypotonic
30 minutes
45 minutes
60 minutes
36
17
19
c
IL-1β (pg/ml)
Forward scatter (A.U.)
IL-1β
FSC
Time (min) after exposure to 60 mosm/kg H2O
d
Iso
Hypo 30 mn
60 mn
Count
Active caspase-1 (FITC)
e
Isotonic
Hypotonic
IL-1β (pg/ml)
No RR
RR
f
IL-1β (pg/ml)
10 mn
20 mn
30 mn
40 mn
50 mn
No HgCl2
+ HgCl2
Time of exposure to 60 mosm/kg H2O
before addition of HgCl2
g
SN
IL-1β p17
Lysate
IL-1β p37
10 mn
20 mn
50 mn
No HgCl2
+ HgCl2
Time of exposure to 60 mosm/kg H2O
before addition of HgCl2
h
IL-6 (pg/ml)
10 mn
20 mn
30 mn
40 mn
50 mn
No HgCl2
+ HgCl2
Time of exposure to 60 mosm/kg H2O
before addition of HgCl2
i
60 minutes
5
48
Count
Forward scatter
Isotonic
Hypotonic
No HgCl2
+ HgCl2

detected 30 min after hypotonic shock when the cell RVD process started (Fig. 3*c*). Similarly, the active caspase 1 content in macrophages was detected 30 min after hypotonic shock and increased up to 1 h (Fig. 3*d*). The implication of cell volume sensors in IL-1β excretion was assessed by using a nonspecific TRP inhibitor, Ruthenium Red. TRP inhibition resulted in decreased IL-1β release by lung macrophages in response to hypotonic shock (Fig. 3*e*). These observations suggested that IL-1β release by macrophages arise from the RVD process consecutive to the initial cell swelling.

To clarify the role of AQPs in the RVD-related shrinkage process, $HgCl_2$ was added after cell swelling (*i.e.* at 10, 20, 30, 40, or 50 min) upon hypotonic stress. Inhibition of AQPs after cell swelling and when RVD was initiated was sufficient to reduce mature IL-1β release (Fig. 3, *f* and *g*) without affecting the intracellular IL-1β proform pool. This effect was not related to mercurial cytotoxicity (data not shown) nor IL-6 release modifications (Fig. 3*h*). To confirm the inhibitory effect of mercury on RVD, cell size was assessed 1 h after the shock when AQPs were inhibited 10 min after the shock (Fig. 3*i*). These data indicate that volume restoration following cell swelling is implicated in NLRP3 inflammasome-related IL-1β release and that this process involves AQPs.

AQP1 Mediates NLRP3-related Caspase 1 Activation and Mature IL-1β Release in Macrophages—We further investigated the implication of AQPs in NLRP3 inflammasome activation by targeting AQP1, a water-specific channel expressed by immune cells (11, 12, 15). Cell swelling consecutive to hypotonic shock was reduced in AQP1-deficient cells (Fig. 4*a*). AQP1 deficiency also reduced the release of mature IL-1β by lung (Fig. 4, *b* and *c*) and peritoneal (Fig. 4*d*) macrophages submitted to hypotonic shock. Similarly, caspase 1 activation because of hypotonic shock was impaired in AQP1-deficient lung macrophages (Fig. 4*e*). These results demonstrate that AQP1 leads to a rapid cell volume modification necessary for NLRP3 inflammasome activation and IL-1β release in response to osmotic shock.

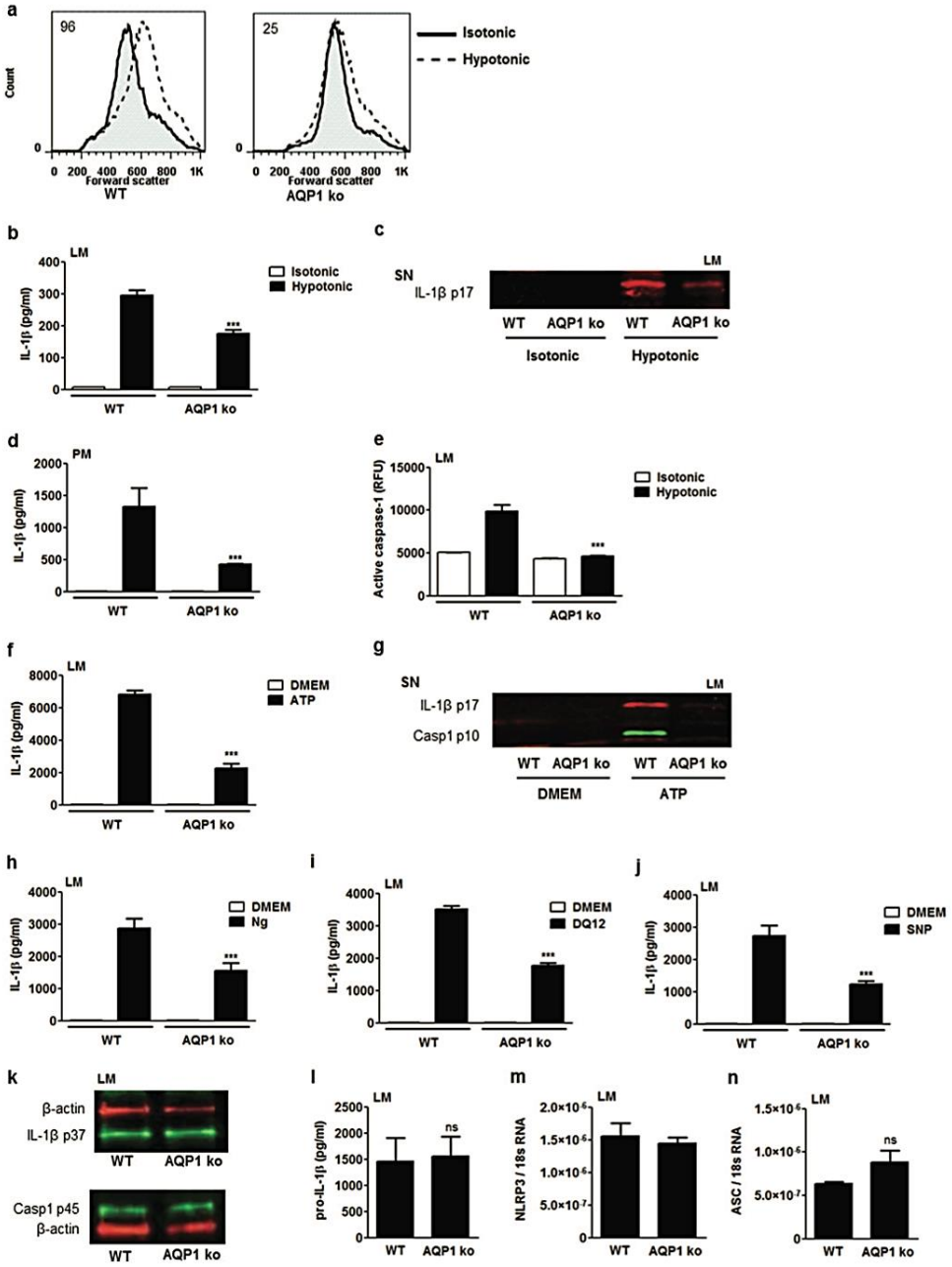
The implication of AQP1 in NLRP3 inflammasome activation was extended to soluble and non-soluble inflammasome activators. AQP1 deletion decreased mature IL-1β release and caspase 1 activation by lung macrophages exposed to ATP (Fig. 4, *f* and *g*). The concentration of IL-1β detected in the supernatant of AQP1-deficient lung macrophages after nigericin, silica, and SNP was also significantly lower in comparison to cells from WT mice (Fig. 4, *h–j*). We then verified whether AQP1 deletion could affect the expression of NLRP3 inflammasome components (Fig. 4, *k–n*). AQP1 deficiency did not modify the pool of the IL-1β proform after LPS stimulation (Fig. 4, *k* and *l*). Similarly, no decrease in the expression of procaspase 1 (Fig. 4*k*, *casp1 p45*), NLRP3 (Fig. 4*m*), or ASC (Fig. 4*n*) was found in AQP1-deficient cells. Thus, these data indicate that AQP1 contributes to IL-1β activation and release by macrophages after exposure to NLRP3 soluble and particulate stimuli.

AQP1 Mediates Crystal-induced NLRP3 Inflammasome-dependent Lung Inflammation in Mice—To verify whether aquaporin-mediated inflammasome activation has relevance in inflammation, we assessed the acute lung response of mice deficient in AQP1 to silica particles, a danger signal inducing NLRP3 inflammasome assembly and mature IL-1β release *in vivo* (22). Silica-induced lung inflammation is mainly characterized by an early accumulation of neutrophils and an increase of proinflammatory mediators, both believed to be mediated by IL-1β (1, 23). DQ12 was administered in the lung of $AQP1^{+/+}$ and $AQP1^{-/-}$ mice, and acute lung inflammatory parameters were compared. Early neutrophilic inflammation was quantified by counting alveolar neutrophils and assessing the lung expression of specific markers such as ngp and CXCR2 (24). The cellular response to silica was evidenced by an increased number of $GR1^+$ cells in BAL fluid (Fig. 5*a*) and expression of neutrophilic markers in WT mice (Fig. 5, *b* and *c*), whereas lower levels of neutrophils as well as ngp and CXCR2 transcripts were detected in the lung of $AQP1^{-/-}$ mice. Silica-induced neutrophilic inflammation was also investigated histologically (Fig. 5, *e–h*). In hematoxylin and eosin-stained lung sections obtained from untreated mice, neutrophils were absent in WT and $AQP1^{-/-}$ samples (Fig. 5, *e* and *f*). When treated with silica, WT mice presented a more pronounced neutrophilic infiltration than AQP1 deficient mice (Fig. 5, *g* and *h*). Inflammasome activation was evaluated by the measurement of mature IL-1β in the alveolar space. The BAL fluid concentration of this cytokine was increased significantly after silica in WT mice and, to a lower extent, in AQP1-deficient animals (Fig. 5*d*). In contrast to silica particles, lung inflammation induced by the antibiotic bleomycin (not considered a direct NLRP3 activator) was not reduced in AQP1-deficient mice (data not shown). In agreement with *in vitro* observations, we concluded that AQP1 contributes to IL-1β release and neutrophilic inflammation in response to silica particles *in vivo*.

DISCUSSION

Innate immune cells possess diverse sensors recognizing molecular patterns related to pathogenic or endogenous danger indicators, with most sensors recognizing a limited number of patterns. Inflammasomes can activate caspase 1 in response to

FIGURE 3. **AQPs mediate IL-1β activation during the cell volume reduction process in macrophages.** Shown are optical microscopy (*Scale bar* = 4 μm) (*a*) and flow cytometry forward scatter plots (*b*) of lung macrophages primed with LPS (0.1 μg/ml) at different time points after hypotonic shock (60 mosmol/kg H_2O). Values indicated in the flow cytometry plots are differences between median forward scatter of cells exposed or not exposed to hypotonic shock. *c*, IL-1β release (*left y axis*) and flow cytometry forward scatter (*FCS*) (*right y axis*) of lung macrophages at different time points after hypotonic shock. $n = 4$ replicates/condition. *d*, flow cytometry plot of intracellular active caspase 1 content in lung macrophages 30 or 60 min after hypotonic (*Hypo*) shock (*Iso*, isotonic). Shown is the IL-1β release by lung macrophages 1 h after hypotonic shock in the absence or presence of Ruthenium Red (*RR*, 30 μM) (*e*) or $HgCl_2$ (10 μM, added 10, 20, 30, 40, or 50 min after hypotonic shock) (*f*). $n = 3$ or 4. *g*, Western blot analysis of mature IL-1β (*IL-1β p17*) in the supernatant (*SN*) and pro-IL-1β (*IL-1β p37*) in cell lysate of lung macrophages exposed or not exposed to hypotonic shock in the absence or presence of $HgCl_2$ (10 μM, added 10, 20, or 50 min after hypotonic shock). *h*, IL-6 release by lung macrophages 1 h after hypotonic shock in the absence or presence of $HgCl_2$ (10 μM, added 10, 20, 30, 40, or 50 min after hypotonic shock). $n = 3$ or 4. *i*, flow cytometry forward scatter plots of lung macrophages 1 h after hypotonic shock in the presence or absence of $HgCl_2$ (10 μM, added 10 min after hypotonic shock). The values indicated in the plots are differences between median forward scatter of cells exposed or not exposed to hypotonic shock. Data are means ± S.E. *, $p < 0.05$; ***, $p < 0.001$ upon exposure to hypotonic medium with Ruthenium Red or $HgCl_2$ or no exposure; *ns*, no significant difference. *p* values were calculated by Student Newman-Keuls test.


a
96
25
Isotonic
Hypotonic
Count
0
0 200 400 600 800 1K
Forward scatter
WT
AQP1 ko
b
LM
IL-1β (pg/ml)
Isotonic
Hypotonic
***
WT
AQP1 ko
c
SN
LM
IL-1β p17
WT AQP1 ko WT AQP1 ko
Isotonic
Hypotonic
d
PM
IL-1β (pg/ml)
e
LM
Active caspase-1 (RFU)
f
DMEM
ATP
g
IL-1β p17
Casp1 p10
h
Ng
i
DQ12
j
SNP
k
β-actin
IL-1β p37
Casp1 p45
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pro-IL-1β (pg/ml)
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NLRP3 / 18s RNA
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ASC / 18s RNA

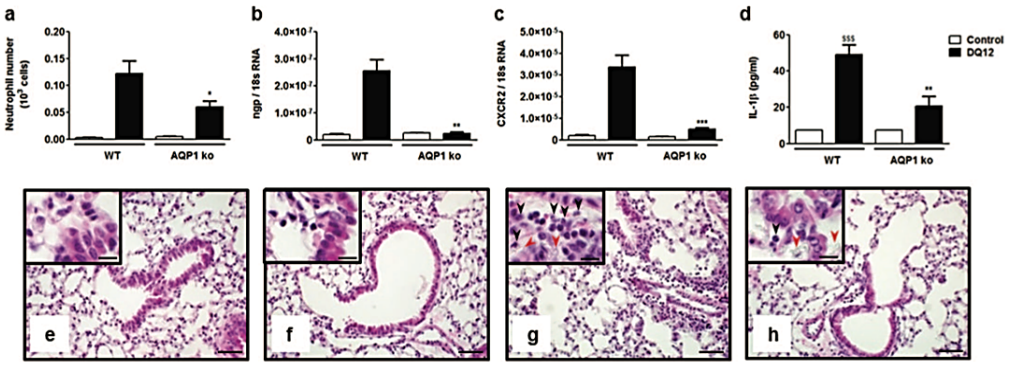


FIGURE 5. **Acute lung inflammatory response to silica particles is reduced in AQP1-deficient mice.** *a*, number of alveolar neutrophils ($GR1^+$ cells) assessed by flow cytometry and expression of the pulmonary neutrophilic markers ngp (*b*) and CXCR2 (*c*) quantified by quantitative RT-PCR in WT and AQP1 KO mice 6 h after instillation of silica (*DQ12*, 2.5 mg) or no instillation (*control*). *d*, levels of mature IL-1β in BAL fluid collected 6 h after instillation of silica or no instillation. Data are means ± S.E. $n = 3$ to 6 animals/condition. *e–h*, hematoxylin and eosin-stained lung sections obtained from WT (*e* and *g*) or AQP1 KO mice (*f* and *h*), collected 6 h after instillation of silica (*g* and *h*) or no instillation (*e* and *f*). *Black arrows* identify neutrophils, and *red arrows* identify silica deposition. Scale bars = 50 μm (*large panels*) and 20 μm (*insets*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ in silica-treated WT and AQP1 KO mice. *p* values were calculated by Student Newman-Keuls test.

a great variety of stimuli, including ATP, bacterial toxins, viral components, crystals, and particles (2, 15, 20, 22). The identification of a common element among these diverse stimuli that initiates inflammasome assembly may have a wide applicability in a range of inflammatory diseases (5).

Cell swelling is a hallmark of inflammatory conditions (25, 26). In particular, swelling has been linked to macrophage activation and to its ability to induce inflammation by cytokine release (27–29). Interestingly, macrophage swelling and following RVD are recorded in association with NLRP3 inflammasome activation and IL-1β release in response to different stimuli (6–8, 15, 30). In this study, we show the importance of AQP in cell volume changes, mainly RVD, for mature IL-1β release *in vitro* and *in vivo*. In particular, we demonstrate the implication of AQP1 in the development of neutrophilic inflammation. Rapid AQP-mediated water transport is thus sensed as a danger signal by the cell to trigger caspase 1 activation and mature IL-1β release in response to a variety of stimuli. The implication of AQPs in cell RVD and inflammasome activation is, however, not limited to AQP1. Mercury blocks most AQPs. Thus, its inhibitory effect on inflammasome activation could be related to different AQPs. Indeed, AQP9 is known to be expressed in leukocytes, and activation of the mononuclear cells following an intravenous endotoxin challenge results in modified expression of this water channel (13). Also, AQP9 inhibition has been linked to reduced inflammasome activation in response to monosodium urate crystals (15).

Several interactions of AQP with orchestrators of inflammasome activation can be proposed. Cells possess different volume sensor systems, such as the calcium-permeable channel protein TRP, driving RVD in macrophages. The activation of these channels and the associated ionic changes activate TAK1, a MAPKK kinase, necessary for hypotonicity-, alum-, ATP- and nigericin-induced mature IL-1β release and inflammation (7, 8). In this study, we confirmed the implication of TRP in IL-1β release by macrophages in response to hypotonic shock. Interestingly, it has been observed that hypotonicity in salivary gland cells increased the association of TRPV4 and AQP5. In this case, TRP activation required AQP (31). The RVD process implies an efflux of intracellular K^+ that has been shown to be necessary for inflammasome activation in response to various stimuli, including hypotonic shock (5, 7, 20). In this study, we found that intracellular potassium decrease was not reduced by AQP1 deficiency in lung macrophages exposed to hypotonic shock (data not shown). Thus, the implication of AQP1 in IL-1β activation is not related to potassium efflux. Finally, it has been observed that inhibition of cell swelling and water flux by using mercury and phloretin reduced the capacity of macrophages to

FIGURE 4. **AQP1 contributes to swelling, caspase 1 activation, and mature IL-1β release in macrophages.** *a*, flow cytometry forward scatter plots of WT or AQP1 KO lung macrophages (*LM*) primed with LPS (0.1 μg/ml) and subsequently subjected or not subjected to hypotonic shock (60 mosmol/kg H_2O). The values indicated in plots are differences between median forward scatter of cells exposed or not exposed to hypotonic shock. *b*, IL-1β released by WT and AQP1 KO lung macrophages exposed or not exposed to hypotonic medium for 1 h. $n = 3$ replicates/condition. *c*, Western blot analysis of mature IL-1β (*IL-1β p17*) in the supernatant (*SN*) of WT and AQP1 KO lung macrophages exposed or not exposed to hypotonic medium. *d*, IL-1β released by WT and AQP1 KO peritoneal macrophages (*PM*) exposed or not exposed to hypotonic medium (100 mosmol/kg H_2O) for 1 h. $n = 3$. *e*, fluorimetric measurement of cellular active caspase 1 in WT and AQP1 KO lung macrophages exposed or not exposed to hypotonic medium (60 mosmol/kg H_2O) for 1 h. $n = 3$. *f*, IL-1β release by WT and AQP1 KO lung macrophages primed with LPS (0.1 μg/ml) and subsequently exposed or not exposed to ATP (5 mM). $n = 3$ or 4. *g*, Western blot analysis of mature IL-1β and active caspase 1 (*Casp1 p10*) in the supernatant of WT and AQP1 KO lung macrophages exposed or not exposed to ATP. Shown is the IL-1β release by WT and AQP1 KO lung macrophages primed with LPS (0.1 μg/ml) and subsequently exposed or not exposed to nigericin (*Ng*, 20 μM) (*h*), silica (*DQ12*, 400 μg/ml) (*i*), or SNP (100 μg/ml) (*j*). $n = 3$ or 4. *k*, Western blot analysis of intracellular pro-IL-1β (*IL-1β p37*), procaspase 1 (*Casp1 p45*), and β-actin in LPS-primed WT and AQP1-deficient lung macrophages. *l*, intracellular levels of pro-IL-1β in WT and AQP1 KO lung macrophages, quantified by ELISA. $n = 3$ or 4. Expression of NLRP3 (*m*) and ASC transcripts (*n*) quantified by quantitative RT-PCR in WT and AQP1 KO lung. $n = 3$ or 4. Data are means ± S.E. ***, $p < 0.001$ in WT or AQP1 KO cells; *ns*, no significant difference. *p* values were calculated by Student Newman-Keuls test.

release mature IL-1β after monosodium urate crystals exposure (15). The authors concluded that water entry reduced intracellular potassium concentration to critical levels necessary for inflammasome assembly in this model. During the release process of mature IL-1β, active caspase 1/ASC aggregates abutted the external surfaces of actin filaments (32). Thus, it is also possible that AQP-mediated water efflux regulates caspase 1 processing and mature IL-1β release by modulating actin cytoskeletal rearrangements (33).

A possible implication of AQPs in inflammatory diseases has been proposed because of their role in cell migration and edema formation (34). At the alveolar level, AQP1 and AQP5 are the most abundant, with an endothelial or epithelial distribution, respectively (35, 36). It has been shown that AQP5 knockout mice develop less inflammation in a model of house dust mite-induced asthma (37). We observed that AQP1 deficiency reduces neutrophilic inflammation as well as cytokine release in silica-induced lung inflammation. Besides the respiratory tract, AQPs have been associated with inflammation in other organs (38, 39). In a model of peritoneal dialysis, reduced accumulation of leukocytes was observed in mice genetically deficient in AQP1 (40). AQP4-deficient mice developed less inflammation in a model of autoimmune encephalomyelitis (41). In light of our results, we propose that, in addition to their possible role in cell migration and edema formation, AQP-mediated water fluxes in macrophages, subsequent RVD, and, finally, mature IL-1β release contribute to the inflammatory responses in these experimental models.

In humans, AQP alterations have also been correlated with several inflammatory diseases. Overexpression of AQP1 has been evidenced in autoimmune and alcoholic pancreatitis (42), in Alzheimer disease (43), as well as in rheumatoid and psoriatic arthritis cases (44). AQP4 expression is enhanced in the brain of Alzheimer disease patients and cerebral amyloid angiopathy patients (43, 45). AQP5 polymorphisms have been associated with chronic obstructive pulmonary disease and chronic inflammation in smoking patients and survival rate in severe sepsis (46, 47). These data further support a role of AQPs in human inflammatory diseases. Hemorrhagic shock and sepsis result in multiple organ failure, in particular acute lung injury, which may lead to death. IL-1β and NLRP3 activation have been implicated in this process (48, 49). Interestingly, hypertonic solution has been shown to reduce inflammation and to increase the vital prognosis of these patients (50). The anti-inflammatory mechanism of this hypertonic resuscitation procedure is still unclear but, in light of our data, we postulate that it reduces AQP-mediated water transport and, consecutively, restricts inflammasome activation. Hence, AQPs should be considered as a new target in inflammation-associated disorders.

In conclusion, we demonstrated that rapid AQP-mediated cell swelling and mainly subsequent volume restoration are sensed as danger signals by macrophages and trigger NLRP3 inflammasome activation. AQP-mediated water movements appear as the common element unifying the variety of NLRP3 inflammasome activators. *In vivo*, AQPs contribute to inflammation by mediating mature IL-1β release.

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IV. Discussion

Interleukin-1β (IL-1β) plays an important role in numerous acute and chronic inflammatory diseases (1). The production of inactive IL-1β precursor (pro-IL-1β) is induced by endogenous danger signals (also termed DAMPs for Damage-Associated Molecular Pattern molecules) activating NFκB and AP-1 transcription factors and il-1b transcription in macrophages. Cleavage and secretion of active IL-1β from pro-IL-1β involve the assembly of a multiproteic complex called inflammasome which consists of a Nod-like receptor (NLR), an adapter protein (ASC, Apoptosis-associated Speck-like protein containing CARD) and caspase-1 (a cysteine-aspartic acid protease).

Accumulation of neutrophils during the lung responses to micro- and nanoparticle such as silica, asbestos and carbon nanotubes is regulated, as least in part, by the release of mature IL-1β (2-4). By identifying the molecules inducing IL-1β maturation, Tschopp and colleagues discovered ten years ago that urate, alum and calcium crystals but also silica or asbestos promote inflammation by their capacity to stimulate inflammasome and IL-1β processing in exposed macrophages (3-6). This pioneer team, with others, showed that silica or asbestos-induced pulmonary neutrophilic inflammation, pro-inflammatory cytokine release and granuloma formation were strongly impaired in mice lacking NLRP3 or IL-1R (3,4). These observations were extended to other particles since lung inflammation induced by nanoparticles was also limited in the absence of IL-1β processing and signaling (7). The functions of IL-1β during the establishment of pulmonary inflammation are now defined. IL-1β directly stimulates epithelial cells and macrophages to secrete pro-inflammatory chemokines and cytokines (8). IL-1β also regulates the inflammatory functions of Th17 lymphocytes by increasing their secretion of the neutrophilic cytokine IL-17 (9,10).

While the exact cellular mechanisms leading to IL-1β secretion are finely depicted after particle exposure (see the Introduction), the danger signals that initiate pro-IL-1β production need to be identified. Alarmins constitute a group of endogenous molecules constitutively expressed and stocked in tissue-resident cells. When they are released after cellular damages, extracellular alarmins serve to inform adjacent cells of injury or infection and orchestrate appropriate immune responses (11).

In the first presented study (see article 1), we attempted to characterize DAMP release in mice treated with silica particles and determine their capacity to elicit lung IL-1β expression. We discovered that IL-1α functions as the major alarmin controlling IL-1β

production in response to particles. Moreover, we demonstrated that silica-damaged lung macrophages represent the main source of extracellular IL-1α and IL-1β. IL-1α is possibly released from particle-exposed macrophages by leakage across cell membrane inherent to necrosis, pyroptosis and secondary necrosis or by secretion involving calpain and/or caspase-1 (7,12-15). Interestingly, advanced caspase-1, -3 and -9-mediated cell death have been clearly observed in alveolar macrophages rapidly after silica exposure, suggesting that both pyroptotic and apoptotic processes are involved in the release of IL-1α (16,17). IL-1α-releasing macrophages are dying cells and thus probably unable to newly produce pro-IL-1β. Extracellular IL-1α released from damaged macrophages could, however, trigger pro-IL-1β production by surrounding active macrophages which then propagate neutrophilic inflammation by processing IL-1β after silica endocytosis. We have recently identified a particular population of lung macrophages with low capacity to phagocyte particles but possessing high ability to produce IL-1α. These macrophages could be able to produce both IL-1α and IL-1β after endocytosis of non-cytotoxic dose of silica for instance. In this context, IL-1α also promotes the proliferation of these macrophages, amplifying macrophages accumulation and potentially IL-1β secretion (18). Finally, while IL-1α mediates inflammation by regulating IL-1β production, we also revealed that IL-1α itself participates in the lung neutrophil recruitment after silica treatment. Specific activity of IL-1α on lung inflammation should be explained by its involvement in alveolar macrophage activation and proliferation or by paracellular activity related to its membrane-associated form (18,19). Alternatively, a pro-inflammatory activity should be associated with its reported pro-inflammatory intracrine activity (20).

The mechanism leading to IL-1β production is probably complex and likely implicates a network of molecules instead of a unique central mediator (i.e. IL-1α). Indeed, recent studies exploring DAMPs after particle exposure demonstrated that the alarmins IL-33 and HMGB1 were also involved in lung inflammation caused by fiber-shaped carbon nanotubes and asbestos (21-25). It is thus probable that the type of particles, the targeted lung cells and the stage of inflammatory responses are all important parameters determining DAMPs release and activity. In addition, other molecules competent for inducing pro-IL-1β production such as S100 and heat shock proteins are also secreted in response to particle treatment and could be involved in inflammation as well (8,26,27). Also, TNF-α is another cytokine commonly implicated in particle-induced inflammation (28,29). Remarkably, the

release of TNF-α in external cell environment can result from the shedding of membrane-bound TNF-α precursor by TACE (Tumor necrosis factor α (TNFα)-converting enzyme) (30). High levels of BAL TNF-α are detected 1 hour after silica instillation in mice (not shown) and TNF-α strongly induces pro-IL-1β expression by lung macrophages (cfr article 1, supplemental figure 2). These observations support the view that several mediators rapidly secreted in response to particles amplify IL-1β production. Finally, mature IL-1β mediates its own production by binding IL-1RI receptor (31). Supposedly, IL-1β is not expressed under resting conditions and the role of IL-1β on its own synthesis has not been explored during early inflammatory steps after particle exposure. Surprisingly, we were able to detect mature IL-1β in BAL fluid of silica-treated mice before its transcription (cfr article 1 figure 1 and supplementary fig 1). Some studies have reported a constitutive expression of IL-1β in non-immune cells explaining this early release during inflammation (32-35). The primary presence of mature IL-1β is attributed to pro-IL-1β released from damaged structural cells and processed by extracellular enzymes or inflammasome complexes.

Change in cell volume is a hallmark of inflammasome activation and regulatory volume decrease (RVD)-associated TRP channel activation and K^+ efflux regulate IL-1β processing in macrophages (36). Flow cytometry analysis before and during inflammasome activation revealed macrophage shrinkage in response to silica treatment *in vitro* (data not shown). AQPs constitute a family of specific water channels mainly present at sites where rapid fluid movements are necessary to maintain fluidic homeostasis. Aquaporins are also required for water movements, volume adjustments and shape modifications in immune cells (37). During this work (see article 2), we assessed the possible implication of AQP-mediated water fluxes and cell shrinkage in particle-induced IL-1β cleavage and secretion. We newly demonstrated that AQPs, in particular AQP1, regulate macrophage shrinkage and are critical for IL-1β processing in response to micro- and nanosilica. How AQP-mediated water efflux participates to particle-induced inflammasome activation remains to be determined. AQPs are involved in cytoskeleton plasticity and endocytosis, suggesting that AQPs are potentially required for particle phagocytosis, phagolysosome formation and subsequently inflammasome activation (38-40). Alternatively, the regulation of the intracellular cytoskeleton plasticity by AQP could be necessary for inflammasome component mobility and assembly (40). Importantly, AQP1 are common constituents of

intracellular vesicle membranes and regulate vesicle swelling and membrane stability (41,42). We can thus speculate that AQPs are essential in particle-induced lysosomal swelling and rupture associated with inflammasome activation. AQP activity has also been reported necessary for TRP-mediated calcium influx, involved in both IL-1α and IL-1β maturation and secretion (14,43). Interestingly, AQP1-deficient mice released less IL-1α in the alveolar space in response to silica particles (data not shown), supporting a role for AQPs in the release of alarmins in treated mice. Interestingly, AQP1 can be increased by NFκB activation (44) and has been involved in M1 inflammatory polarization of macrophages (unpublished data). These data suggest a positive feed-back loop between IL-1 and AQP that sensitizes macrophage to danger signals associated with cell volume modification.

The discovery that micro- and nanoparticles activate inflammasome machinery is regarded as an important step in the identification of new targets and new biomarkers to treat and detect particle-induced pulmonary inflammatory diseases. Our data support that simultaneous blockade of IL-1α and IL-1β abrogates silica-induced neutrophil accumulation in the lungs of mice. Anakinra is a recombinant, nonglycosylated form of the human interleukin-1 receptor antagonist (IL-1Ra). It blocks the biologic activity of IL-1α and IL-1β by competitively inhibiting their binding to the interleukin-1 type I receptor (IL-1RI). Targeting the IL-1 pathway through the use of Anakinra is already in use in the clinical treatment of gout and other autoimmune diseases and could also be proposed in particle-induced lung diseases. Our data extend this therapeutic possibility by showing that IL-1α and IL-1β blockade strongly reduce lung neutrophilic inflammation. Abrogation of AQP1 also impaired IL-1β release and lung inflammation in mice treated by silica. AQPs and water movement could thus be considered as new targets in particle-induced lung diseases and neutralization of AQP-mediated water movement by using antibodies or specific small molecule inhibitors could represent a new therapeutic option to control lung inflammation after particle exposure (45).

While compelling *in vitro* and *in vivo* evidence support a crucial role of IL-1β during particle-induced neutrophilic inflammation, Th17 responses and granuloma formation (9,10,46,47), its exact implication in fibrogenesis and cancerogenesis remains unclear. Indeed, tumor development and cancer lesions were not compromised in mice lacking NLRP3 after peritoneal injection of asbestos fibers (48). Similarly, silica- or carbon

nanotube-induced lung fibrosis was not prevented in mice deficient in NLRP3, IL-1β, IL-1R or MyD88 (9,10). These results suggest that IL-1β and inflammasome components are dispensable in cancer and fibrosis induced by particles and call for caution with the use of anti-IL-1 as a clinical strategy to limit these chronic lung disorders.

Summary figure

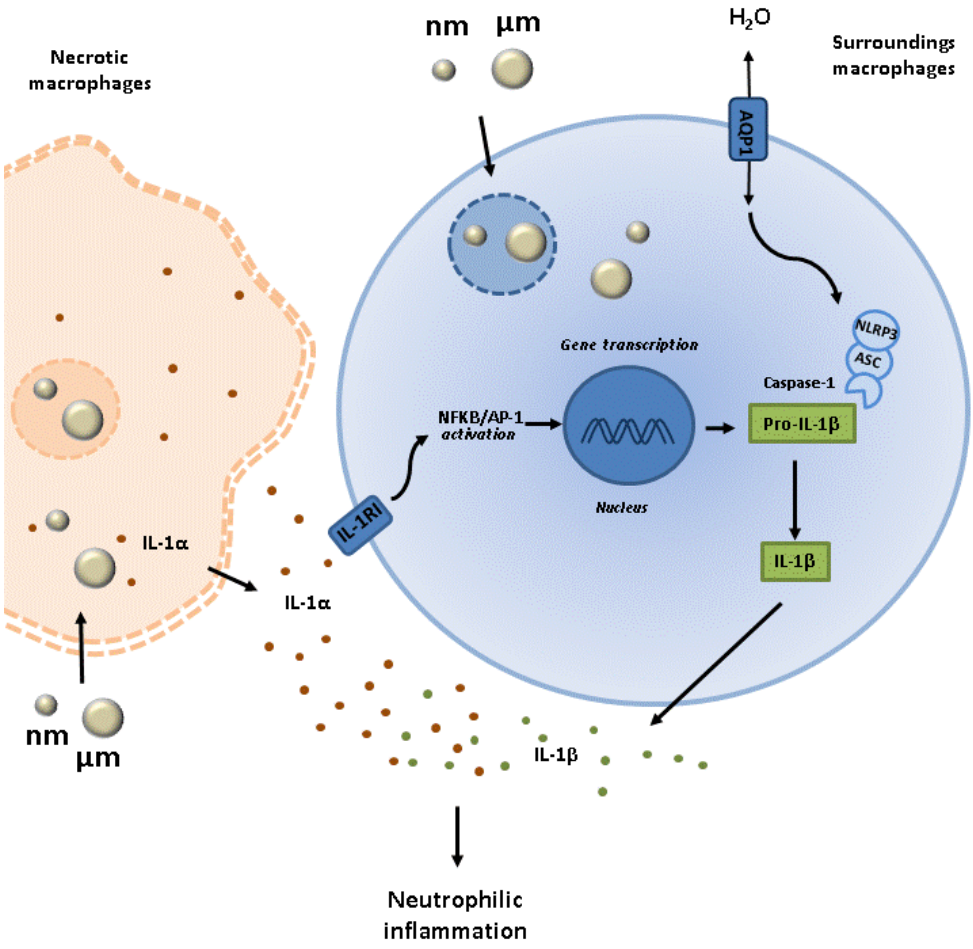


Figure 3: In this experimental work, we found that lung macrophages damaged by micro- and nanosilica release constitutive IL-1α. This alarmin induces pro-IL-1β production in surrounding macrophages. Aquaporin (AQP)-mediated water movements and macrophage shrinkage are implicated in the process leading to maturation and secretion of IL-1β consecutive to particle endocytosis and inflammasome engagement. The marked and sustained release of IL-1α and IL-1β by activated macrophages actively participates in the establishment of neutrophilic inflammation in response to particle exposure.

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