Cell stress increases ATP release in NLRP3 inflammasome-mediated autoinflammatory diseases, resulting in cytokine imbalance

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Cell stress is implicated in triggering bouts of systemic inflammation in patients with autoinflammatory disorders. Blood monocytes from patients affected by NLRP3-mediated cryopyrin-associated periodic syndromes (CAPS) release greater amounts of IL-1β than monocytes from unaffected subjects. Here we show that stress lowers the threshold of activation; blood monocytes from CAPS patients maintain the high levels of secreted IL-1β (fivefold) and IL-18 (10-fold) when stimulated with 1,000-fold less LPS than that required for full IL-1β secretion in control subjects. Unexpectedly, IL-1α secretion is increased 10-fold, indicating that inflammatory episodes in CAPS may not be entirely a result of IL-1β but may also involve IL-1α. In CAPS monocytes, LPS induces the externalization of copious amounts of ATP (10-fold), which drive IL-1β, IL-18, and IL-1α release via activation of the P2X purinoceptor 7. This enhanced ATP release appears to be the link between cell stress and increased cytokine secretion in CAPS. In the later phase after LPS stimulation, CAPS monocytes undergo oxidative stress, which impairs production of the anti-inflammatory IL-1 receptor antagonist (IL-1Ra). Remarkably, IL-1Ra secretion is fully restored by treatment with antioxidants. In two patients with the same NLRP3 mutation, but different disease severity, monocytes from the mildly affected patient exhibited more efficient redox response, lower ATP secretion, and more balanced cytokine production. Thus, the robustness of the individual antioxidant response increases the tolerance to stress and reduces the negative effect of the disease. Pharmacologic block of P2X purinoceptor 7 and improved stress tolerance may represent novel treatment strategies in stress-associated inflammatory diseases.

interleukin 1 family | primary monocytes | reactive oxygen species | redox stress

Cryopyrin-associated periodic syndromes (CAPS) are autoinflammatory diseases linked to mutations in the gene *NLRP3*; the disease is characterized by recurrent episodes of fever and systemic inflammation (1). The pathophysiology of CAPS is mainly caused by the dysregulated secretion of IL-1β, which has been validated by dramatic therapeutic responses to the blocking of the IL-1 receptor with anakinra or the neutralization of IL-1β with canakinumab (2, 3). The NLRP3 inflammasome is a multiprotein complex that requires activating signals to assemble and generate active caspase-1, which in turn converts the inactive IL-1β and IL-18 precursors into their mature active forms (4). Extracellular ATP is a common inflammasome-activating event (5). ATP is released during inflammation by activated platelets, dying leukocytes, and injured parenchymal cells and binds to P2X purinoceptor 7 (P2X7R) on inflammatory cells, triggering a series of intracellular processes, only partially understood, that nevertheless lead to inflammasome activation (6). Human monocytes from healthy subjects stimulated by Toll-like receptor (TLR) agonists secrete their endogenous ATP, which autocrinally activates P2X7R (7, 8), thereby partially liberating monocytes from requiring exogenous ATP. ATP secretion requires reactive oxygen species (ROS) production (9), which is induced in inflammatory cells by triggering TLR (10–12).

Although in some cell types, including mouse macrophages or human myelomonocytic cell lines, ROS inhibitors such as diphenyleneiodonium [DPI (13)] impair pro-IL-1β synthesis and NLRP3 priming (14, 15) in primary human monocytes, pro-IL-1β biosynthesis and intracellular accumulation are unaffected by treatment with DPI (12, 15), indicating that ROS inhibition in monocytes hinders IL-1β secretion by acting at the posttranslational level.

Primary monocytes from patients affected by autoinflammatory diseases are under stress (16–20). In CAPS patients, blood monocytes display levels of both ROS and antioxidants considerably higher than in healthy subjects (16); this basal stress causes an aberrant redox response to TLR stimulation, with further increase of ROS followed by collapse of intracellular antioxidant systems, resulting in net oxidative stress. A functional consequence of oxidative stress is the impaired production of cytokines downstream of IL-1, such as IL-1 receptor antagonist (IL-1Ra) and IL-6 (18).

Here, we identify a series of events induced by stress in monocytes from CAPS patients that contribute to the pathogenesis of the disease. First, we provide evidence that increased ATP secretion secondary to LPS-induced ROS is significantly contributing to the increased secretion of IL-1β and IL-18 and, unexpectedly, IL-1α. We also show that stress at baseline lowers the threshold of activation, with secretion of these three cytokines induced by markedly lower concentrations of LPS,

Significance

Single amino acid mutations in NLRP3 in patients with cryopyrin-associated periodic syndromes (CAPS) lead to oversecretion of IL-1β, resulting in severe inflammatory manifestations. How this occurs is unclear. We show here that cellular stress in blood monocytes contributes to CAPS pathophysiology, allowing copious release of ATP in response to minute concentrations of inflammatory stimuli. As a consequence, the released ATP enhances NLRP3-mediated secretion of IL-1β and IL-18 by activating P2X purinoceptor 7. Later, CAPS cells undergo oxidative stress, which impairs secretion of the anti-inflammatory IL-1 receptor antagonist, worsening the clinical picture. Antioxidants rescue the reduced production of the antagonist. Blocking extracellular ATP and improving stress tolerance may represent novel therapeutic strategies in autoinflammatory diseases.

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concentrations unable to activate secretion in monocytes from healthy subjects. Moreover, we demonstrate that the presence of antioxidants restores the secretion of the anti-inflammatory cytokine IL-1Ra, which is inhibited by oxidative stress. In addition, comparative studies in two patients from the same family, with the same NLRP3 mutation, suggest that the basal degree of stress and the efficiency of redox response of the individual patients influence the severity of the disease phenotype.

Results

LPS-Activated CAPS Monocytes Release Higher Amounts of ATP Than Healthy Monocytes, Resulting in P2X7R-Mediated Increased Secretion of IL-1β, IL-18, and IL-1α. CAPS is associated with mutant NLRP3, suggesting the loss of regulation of IL-1β secretion occurs at the processing level; that is, posttranslationally (1–3). This view was confirmed by the finding that the expression of IL-1β, negligible in unstimulated monocytes from both healthy donors and CAPS patients, is induced by LPS at comparable levels (Fig. 1*A*). Similarly, the baseline expression of NLRP3 was a little higher in CAPS monocytes, but was less induced by LPS than in healthy monocytes (Fig. 1*B*).

To investigate the mechanism underlying the posttranscriptional increase of IL-1β secretion, we compared ATP secretion by monocytes from normal subjects and CAPS patients after LPS stimulation. As shown in Fig. 1*C*, CAPS monocytes release levels of ATP dramatically higher (10-fold) than monocytes from healthy subjects. The increase in ATP release was paralleled by increased secretion of IL-1β (fivefold; Fig. 1*D*). LPS-induced ATP release is prevented by the ROS inhibitor DPI (Fig. 1*C*), indicating that ATP secretion is induced by ROS. Preventing ROS production in monocytes also prevents the secretion of IL-1β (Fig. 1*D*) without affecting expression and biosynthesis of the precursor cytokines (Fig. 1*E*), confirming previous observations (12, 15). Oxidized ATP (oATP), a P2X7R blocker (21), strongly inhibits IL-1β secretion (Fig. 1*D*), further demonstrating the direct involvement of externalized ATP for inflammasome activation through engagement of P2X7R.

Secretion of IL-18, which is also processed by caspase-1, and of IL-1α, which instead does not require caspase-1-mediated cleavage, were also increased in LPS-stimulated CAPS monocytes compared with monocytes from healthy subjects and were blocked by either DPI or oATP (Fig. 1 *F* and *G*).

The half-life of extracellular ATP is generally short as a result of the presence of ectonucleotidases such as CD39 and CD73, which rapidly and efficiently hydrolyze ATP to adenosine (22). To understand whether the high levels of extracellular ATP present in supernatants of LPS-stimulated CAPS monocytes depend on increased release or deficient hydrolysis, we analyzed the expression and activity of CD39 and CD73. As shown in Fig. 2 *A* and *B*, both molecules are present in monocytes from healthy subjects and CAPS patients at comparable levels. Incubation in the presence of the specific ecto-ATPase inhibitor 6-N,*N*-diethyl-D-β-γ-dibromomethylene adenosine triphosphate [ARL 67156 (7, 23)] resulted in the detection of more ATP in supernatants of monocytes from both healthy and CAPS subjects, ruling out functional defects on ectonucleotidases (Fig. 2*C*) and indicating that the high extracellular ATP is actually a result of increased secretion by stressed CAPS monocytes. Interestingly, whereas in healthy monocytes the increase of extracellular ATP caused by ARL 67156 treatment was paralleled by enhanced IL-1β secretion, ARL 67156 failed to induce IL-1β in CAPS monocytes, despite the rise in extracellular ATP (Fig. 2*D*).

CAPS Monocytes Display a Lower Threshold for LPS-Induced Inflammasome Activation and IL-1β, IL-18, and IL-1α Secretion. CAPS and healthy monocytes were then stimulated with decreasing concentrations of LPS. As shown in Fig. 3*A*, similar levels of intracellular pro-IL-1β were induced by LPS at 100, 0.1, and 0.01 ng/mL. However, monocytes from control subjects secreted markedly less IL-1β in response to low concentrations of LPS (Fig. 3*B*). For example, at 0.01 ng/mL, IL-1β released was only 25% of that induced by 100 ng/mL, and no IL-1β was detected with lower concentrations of LPS. In contrast, the release of IL-1β by CAPS monocytes started to decline in response to LPS at 0.01 ng/mL and was still present at 1 pg/mL (Fig. 3*B*).

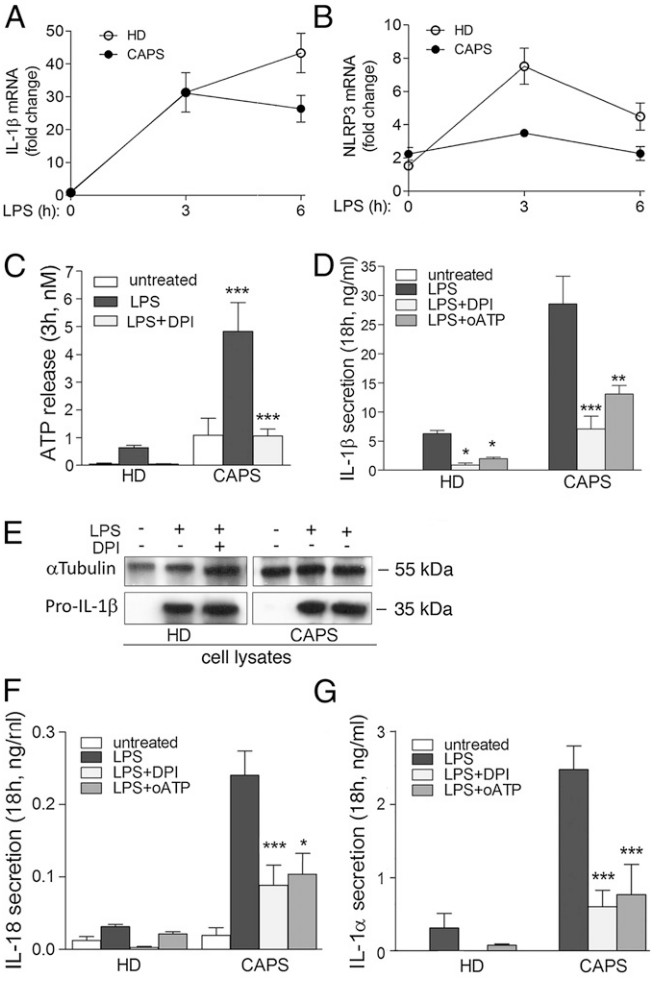

Fig. 1. Increased levels of IL-1β, IL-18, and IL-1α secretion by CAPS monocytes depend on increased release of ATP. (*A* and *B*) RT-PCR of IL-1β (*A*) and NLRP3 (*B*) mRNA at various times from the exposure to LPS. Data are expressed as fold changes versus untreated monocytes at time 0. Mean ± SD [healthy donors (HD) and CAPS, $n = 3$]. (*C*) ATP release by monocytes incubated without or with 100 ng/mL LPS, in the absence or presence of DPI (20 μM). Data are expressed as nM, mean ± SEM (HD, $n = 6$; CAPS, $n = 4$). (*D*) IL-1β secreted by monocytes cultured 18 h with or without 100 ng/mL LPS, in the presence or absence of DPI (20 μM) or oATP (300 μM). Data are expressed as ng/mL, mean ± SEM (HD, $n = 6$; CAPS, $n = 4$). (*E*) Pro-IL-1β in monocyte cell lysates from one HD (HD1) and one CAPS (CAPS3; Table S1) patient at 3 h from exposure to 100 ng/mL LPS. (*Upper*) α-tubulin is shown as loading control. (*F* and *G*) IL-18 (*F*) and IL-1α (*G*) secreted by monocytes cultured as described in *D* (HD and CAPS, $n = 3$).

We investigated whether the discrepancy between similar levels of pro-IL-1β synthesis but distinct IL-1β secretion by monocytes from healthy and CAPS subjects was a result of differences in the levels of the LPS-induced ATP release. As shown in Fig. 3*C*, in healthy monocytes, 0.01 ng/mL of LPS was unable to induce the secretion of ATP and, consequently, of IL-1β (Fig. 3*B*). In contrast, in CAPS monocytes, LPS at 0.01 ng/mL still induced a release of ATP three times higher than that detected in control monocytes exposed to the standard dose of LPS (2.8 ± 0.5 vs. 1.0 ± 0.2 nM; Fig. 3*C*); this amount of secreted ATP was able to drive high levels of secreted IL-1β (Fig. 3*B*).

Similar to IL-1β, IL-18 and IL-1α secretion, in response to low concentrations of LPS, was impaired in healthy monocytes but was maintained in CAPS (Fig. 3 *D* and *E*).

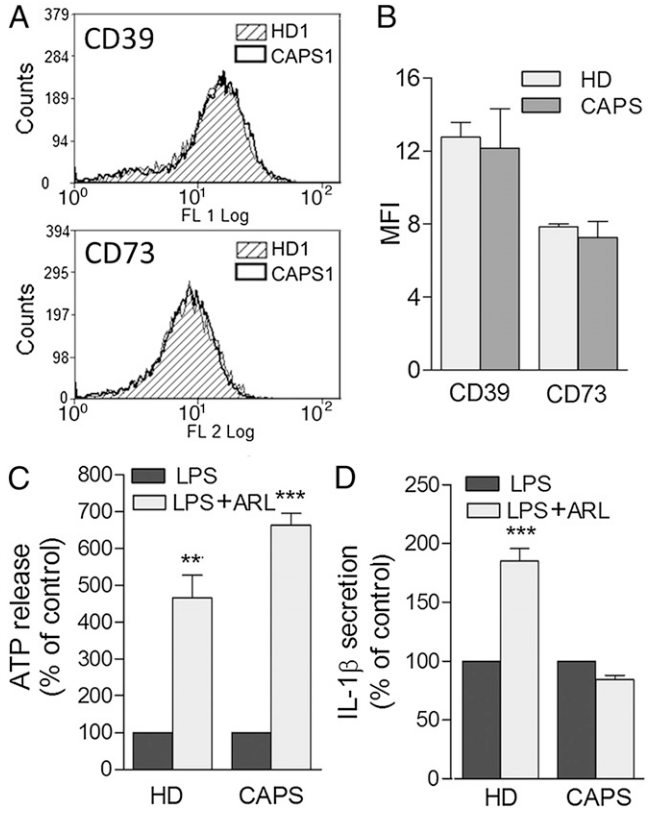

Fig. 2. Increased release of ATP by monocytes from CAPS patients is not related to functional defects in ectonucleotidases. (*A*) Cell surface expression of CD39 and CD73 by monocytes from HD (one representative subject of four tested is shown) and CAPS patient (one representative subject of two tested is shown). (*B*) For each analysis in *A*, mean fluorescence intensity (MFI) were quantified (mean ± SEM). (*C* and *D*) ATP (*C*) and IL-1β (*D*) quantified in supernatants of HD or CAPS monocytes, cultured 3 h (*C*) or 18 h (*D*) with LPS in the absence or presence of ARL 67156 (ARL; 200 μM). Data expressed as percentage of release by ARL 67156-treated versus untreated cells (HD, $n = 4$; CAPS, $n = 2$).

LPS-Induced Oxidative Stress in CAPS Monocytes Is Prevented by the Reducing Agent DTT That Increases IL-1Ra Production. The stress state of CAPS monocytes at baseline is characterized not only by the increased ROS levels (Fig. 4*A*) but also by the overexpression of xc^-, a major antioxidant system in monocytes (24). Thus, unstimulated CAPS monocytes release higher levels than healthy monocytes of free cysteine, a marker of the xc^- system (Fig. 4*B*). However, whereas in monocytes from healthy subjects, the xc^- system is up-regulated after LPS stimulation, in CAPS monocytes, the antioxidant response collapses after exposure to LPS, as indicated by the decrease of cysteine release (Fig. 4*B*), and oxidative stress then occurs (16, 18). A functional consequence of oxidative stress is global translational arrest (25). Accordingly, the secretion of IL-1Ra, a second-wave cytokine induced later than IL-1 after TLR triggering, is impaired in CAPS monocytes (Fig. 4*C*) (18). To investigate whether relief from stress in CAPS cells can rescue IL-1Ra production, stimulation with LPS was performed in the presence of the reducing agent DTT. Remarkably, whereas IL-1Ra secretion by healthy monocytes was marginally modulated by the reducing agent, DTT significantly induced the secretion of the cytokine in monocytes from CAPS patients (Fig. 4*C*).

Monocyte Redox State and Redox Response Correlate with the Disease Severity in Two Relatives Sharing the Same NLRP3 Mutation. According to these data, it is likely that a different antioxidant capacity of monocytes may result in greater or lesser disease severity in different CAPS patients. Therefore, we studied cytokine production, redox state, and ATP release in monocytes from two relatives (patients 5 and 6; Table S1) sharing the NLRP3 T348M mutation and correlated these parameters with the gravity of their disease. The daughter displayed an early disease onset at birth, characterized by daily urticarial skin rash and persistent systemic inflammation, and developed a progressive hearing loss since the age of 4 y. She presented an overlap phenotype between chronic infantile neurological cutaneous articular syndrome and Muckle-Wells syndrome. Conversely, the father displayed a typical Muckle-Wells syndrome phenotype with a late disease onset at the age of 17 y and with a milder disease course, mainly characterized by recurrent episodes of skin rash and arthralgia. Neurosensorial hearing loss presented at the age of 27 y. In line with the more severe disease, monocytes from the daughter secreted more IL-1β than the father, in response to the standard concentration of LPS (Fig. 5*A*). Moreover, at lower concentrations of LPS, the levels of IL-1β secretion by the daughter were unchanged, whereas in the father's monocytes, a decrease was observed, although it was less evident than in monocytes from healthy donors (Fig. 5*A*). In addition, the impairment in the production of IL-1Ra was more pronounced in the monocytes from the daughter (Fig. 5*B*).

The reduced dysregulation in cytokine production by the father's monocytes compared with those of his daughter was paralleled by a more balanced redox state and response. ROS

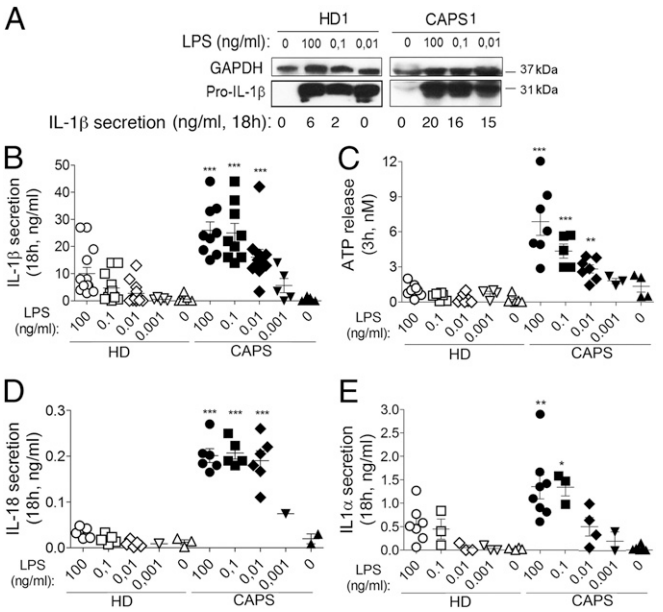

Fig. 3. Low concentrations of LPS induce IL-1β, IL-18, and IL-1α secretion by CAPS monocytes. (*A*) Pro-IL-1β in monocyte cell lysates from one HD (HD1) and one CAPS (CAPS1; Table S1) at 3 h from exposure to LPS at 100, 0.1, or 0.01 ng/mL, as indicated. (*Upper*) GAPDH is shown as loading control. The secreted IL-1β (ng/mL) at 18 h from stimulation is indicated. One representative experiment of three performed is shown. (*B*) IL-1β secreted by monocytes form 11 HD and nine CAPS cultured 18 h in the absence or presence of LPS at 100, 0.1, or 0.01 ng/mL. Monocytes from five CAPS and five HD were also exposed to 0.001 ng/mL of LPS. (*C*) ATP released by monocytes from HD or CAPS patients stimulated 3 h with LPS at 100 ng/mL (HD, $n = 8$; CAPS, $n = 7$), 0.1 ng/mL (HD, $n = 6$; CAPS, $n = 5$), or 0.01 ng/mL (HD, $n = 8$; CAPS, $n = 7$), 0.001 (HD, $n = 4$; CAPS, $n = 3$). (*D*) IL-18 secreted by monocytes cultured 18 h in the absence (HD, $n = 3$; CAPS, $n = 2$), or presence of LPS at 100 ng/mL (HD and CAPS, $n = 6$), 0.1 ng/mL (HD and CAPS, $n = 5$), 0.01 ng/mL (HD and CAPS, $n = 6$), or 0.001 ng/mL of LPS (HD and CAPS, $n = 1$). (*E*) IL-1α secreted by monocytes cultured 18 h in the absence (HD and CAPS, $n = 6$) or presence of LPS at 100 ng/mL (HD, $n = 7$; CAPS, $n = 8$), 0.1 ng/mL (HD, $n = 3$; CAPS, $n = 3$), 0.01 ng/mL (HD, $n = 3$; CAPS, $n = 4$), or 0.001 ng/mL of LPS (HD, $n = 3$; CAPS, $n = 2$). Significance refers to IL-1β (*B*), ATP (*C*), IL-18 (*D*), or IL-1α (*E*) secretion by CAPS versus HD monocytes at the same LPS concentrations.

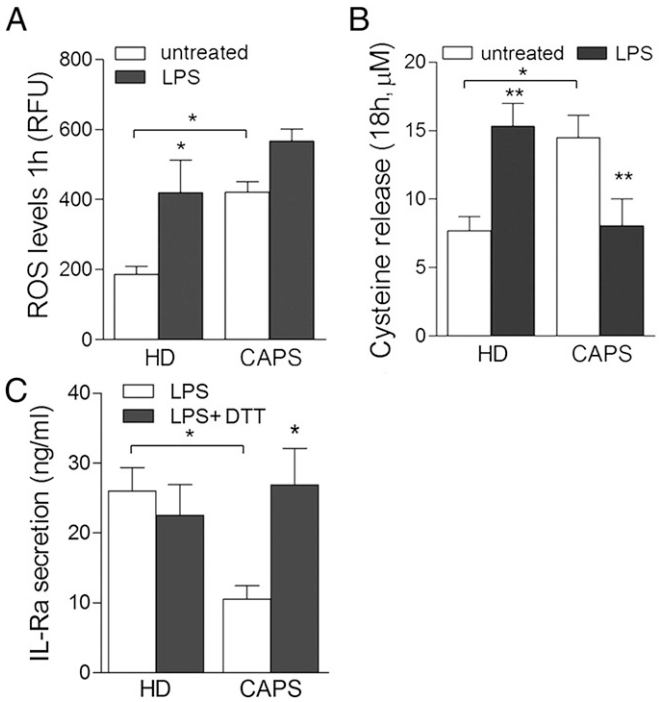

Fig. 4. LPS-induced oxidative stress in CAPS monocytes is prevented by the antioxidant DTT that rescues IL-1Ra secretion. (*A*) ROS levels in HD and CAPS monocytes were evaluated at baseline (untreated) or after 1 h of exposure to 100 ng/mL LPS. Data are expressed as relative fluorescence units (RFU); mean ± SEM (HD, $n = 4$; CAPS, $n = 3$). (*B*) Cysteine release by HD and CAPS monocytes was evaluated in supernatants after 18 h of culture in medium alone (untreated) or with LPS at 100 ng/mL. Data are expressed as μM, mean ± SEM (HD, $n = 8$; CAPS, $n = 7$). (*C*) IL-1Ra quantified by ELISA in supernatants of monocytes from four HD and four CAPS stimulated 18 h with 100 ng/mL LPS in the absence or presence of 1 mM DTT.

production at baseline and after LPS stimulation was higher in both CAPS patients than in controls but was statistically significant only in the daughter compared with healthy monocytes (Fig. 5*C*). As in all CAPS (15), antioxidant levels, including cysteine release (Fig. 5*D*) and intracellular superoxide dismutase 1 and thioredoxin content, were higher in the circulating monocytes from the two patients (Fig. 5*E*). In addition, in the daughter's monocytes, LPS stimulation failed to induce a significant antioxidant response, which indicates the insurgence of oxidative stress, whereas monocytes from the father displayed a small up-regulation of the three antioxidant markers (Fig. 5 *D*–*E*). Finally, monocytes from the daughter released higher levels of ATP than those from her father (Fig. 5*F*).

Discussion

In many inherited diseases, the clinical picture does not depend uniquely on the mutated gene but is modulated by complex genetic and environmental factors that are only partially understood (26, 27). Here we show that redox distress, present in circulating monocytes expressing mutated NLRP3 (16, 18), is a contributing factor and participates in the pathophysiology of CAPS by promoting abundant ATP secretion in response to TLR4 triggering by low concentrations of LPS. Although CAPS is a rare disease, we were fortunate to have the monocytes from a cohort of CAPS patients and performed all studies on freshly obtained circulating blood monocytes to avoid long-term cell cultures that modify the cell redox state and response (15), and may thus provide misleading data.

How ROS induce ATP release remains to be clarified. A recent study on keratinocytes proposes that ROS production induces ATP release by opening Pannexin hemichannels (28). As Pannexin 1 also mediates ATP exit in monocytes (29) and is implicated in IL-1β release (30), a ROS-dependent, Pannexin-mediated mechanism may operate also in monocytes.

The levels of ATP detected in supernatants of LPS-stimulated monocytes from CAPS patients are markedly higher (more than 10-fold) than in monocytes from healthy subjects. As CAPS and normal monocytes express comparable levels of functional CD39 and CD73, the higher extracellular ATP in CAPS is likely a result of increased secretion, rather than defective hydrolysis by ectonucleotidases. Together with the observation that compounds blocking P2X7R or hydrolyzing extracellular ATP inhibit IL-1β secretion in CAPS, these data indicate that the loop of ATP release, P2X7R triggering, and inflammasome activation, which controls TLR-mediated IL-1β secretion in normal

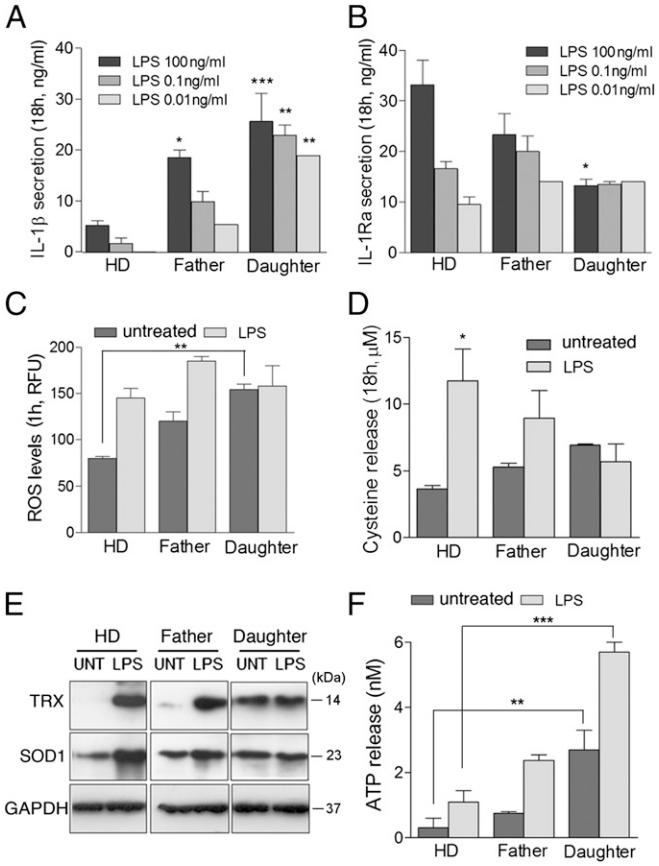

Fig. 5. Different degree of cell stress and cytokine secretion dysregulation by father and daughter expressing the same NLRP3 mutation. (*A* and *B*) IL-1β (*A*) and IL-1Ra (*B*) secretion in supernatants of monocytes from HD ($n = 5$) and two CAPS patients (father and daughter, patients 6 and 5 in Table S1) stimulated with LPS at 100, 0.1, and 0.01 ng/mL. Data are expressed as ng/mL (data obtained from three HD and from three different experiments with the single patients, mean ± SEM; for stimulation with 0.01 ng/mL of LPS, a single experiment in triplicate was performed with patient cells). The significance refers to IL-1β (*A*) or IL-1Ra (*B*) secretion by monocytes from the two patients versus HD monocytes at the same LPS doses. (*C* and *D*) ROS levels (*C*) and cysteine release (*D*) by unstimulated or LPS-stimulated (100 ng/mL) monocytes from HD and the two patients. Data are expressed as RFU or μM (mean ± SEM; HD, $n = 3$ in *C* and $n = 5$ in *D*; patients 6 and 5: three different experiments). (*E*) Western blot analysis of thioredoxin (TRX), superoxide dismutase 1 (SOD1), and GAPDH as loading control in cell lysates from monocytes from a representative HD (of three performed) and the two patients, untreated or stimulated 18 h with 100 ng/mL of LPS as indicated. Samples were loaded on the same gel, but blots have been cut to eliminate uninteresting lanes between the relevant ones. (*F*) ATP release was quantified as in the legend to Fig. 1. Data are expressed as nM, mean ± SEM (HD, $n = 3$; patients 6 and 5: three different experiments).

monocytes (7–9), also is required in CAPS monocytes, where it is strongly amplified because of the huge externalization of ATP (Fig. S1).

Although freshly drawn peripheral blood mononuclear cells from healthy subjects contain active caspase-1 (31), extracellular ATP becomes necessary for LPS-induced processing and release of IL-1β after cell adherence to plastic, and the ATP requirement increases with the differentiation to macrophages (30). Despite monocytes secrete their endogenous ATP (7), macrophages require exogenous ATP (15, 31); in all cases, IL-1β secretion is reduced in the presence of oxidized ATP (7, 31), supporting the critical role for autocrine/paracrine ATP in the release of IL-1β. In the present study, blocking the ecto-ATPase activity increased both extracellular ATP and IL-1β in healthy monocytes, but only ATP was increased in CAPS monocytes. Our interpretation is that the amount of ATP released by CAPS monocytes exceeds the amount of ATP required to trigger the maximal IL-1β secretion; thus, a further increase of extracellular ATP is without effect. Notably, these data explain the lack of responsiveness of CAPS monocytes to exogenous ATP as an inducer of IL-1β secretion (32); ATP released by monocytes from CAPS patients after TLR triggering is sufficient to drive the processing and secretion of a large percentage of the total amount of the IL-1β precursor. Hence, additional exogenous ATP is not effective.

Increased ATP release, with consequent enhanced IL-1β secretion, also is observed in monocytes from healthy subjects under oxidative stress (9). However, although concomitant triggering of multiple TLRs with high concentrations of agonists is required to induce oxidative stress in healthy monocytes (9), in CAPS, exposure to LPS alone, even at lower concentrations, is sufficient. This is likely because unstimulated monocytes from CAPS patients are already under stress that reduces the threshold for LPS-mediated inflammasome activation.

The low threshold of LPS activation may explain the recurrent episodes of systemic inflammation in the absence of a recognizable clinical trigger in CAPS; for example, undetectable stimuli, such as mild trauma or upper respiratory viral infections, may activate IL-1β secretion. A similar hyper-responsiveness to low concentrations of LPS has been described in monocytes from the tumor necrosis factor receptor-associated periodic syndrome (TRAPS; see ref. 33), which, similar to CAPS monocytes, are under stress and display higher levels of ROS than control monocytes (17). It is conceivable that a state of stress is present also in monocytes from other autoinflammatory diseases that express a mutated gene in monocytes. This is true for familial Mediterranean fever syndrome (20) and NLRP12-associated periodic syndrome (34), the monocytes of which are under stress, although to a lesser extent than in CAPS. Why and how mutations cause cell stress is unclear. Because mitochondria are the first target of ROS, and their oxidation further induces ROS production (35), it is conceivable that mitochondria-derived ROS contribute a great extent to cell stress in autoinflammatory diseases. In agreement, the involvement of mitochondrial ROS have been proposed in TRAPS (17) and in CAPS (18). Also in other inherited diseases not directly involving inflammatory cells, such as Duchenne muscular dystrophy (36), the loss of proteostasis due to the presence of a mutated protein is likely sufficient to induce oxidative stress that in turn drives inflammation. The inflammatory response is stronger if the mutated protein is present in professional inflammatory cells, such as in the case of autoinflammatory diseases. In fact, the stress state at baseline clearly helps the insurgence of oxidative stress on TLR triggering. Because P2X7R activation triggers ROS production (37–39), oxidative stress is further facilitated by the amplified ROS generation induced by the high autocrine/paracrine secretion of ATP that overcomes the antioxidant defenses. In CAPS, the mutant NLRP3 displays a lower binding affinity than wild-type NLRP3 for cAMP, an inhibitor of NLRP3 activation, thereby being more susceptible to activation (40). Increased ROS may cause a decrease in cAMP, as described in lung inflammation (41), thus providing a link between the two mechanisms.

We show here that not only IL-1β, but also IL-18 and IL-1α, secretion is enhanced by ROS-induced ATP in CAPS monocytes (Fig. S1). IL-18, as IL-1β, is processed by inflammasome-activated caspase-1, but IL-1α is not. However, IL-1α and other leaderless secretory proteins not cleaved by caspase-1 are secreted after inflammasome activation through an undefined mechanism or mechanisms (42, 43). IL-1α secretion is increased even by low doses of LPS, implying its involvement in the promotion and progression of inflammatory episodes in CAPS, and should be considered when IL-1 blockade is applied as a therapeutic strategy.

An additional finding of this study is that treatment with antioxidants such as DTT, which detoxify ROS, preventing oxidative stress, restores the production of the anti-inflammatory cytokine IL-1Ra, which is impaired in CAPS monocytes (Fig. S1). This observation confirms our previous findings that oxidative stress is responsible for the deficient production of cytokines downstream of IL-1 by CAPS monocytes (18) and indicates that avoiding oxidative stress could maintain the production of regulatory cytokines, helping to control inflammation (44).

The rescue of IL-1Ra production by antioxidants suggests that a genetic or acquired resistance to oxidative stress may result in a less-severe disease phenotype, as proposed for chronic inflammation-mediated diseases such as diabetes (45). Indeed, we found a correlation among stress state, degree of antioxidant response, and severity of the disease in two patients sharing the same NLRP3 mutation and exposed to the same in vitro conditions. These observations support the relevance of the individual tolerance to disease and pave the way to new therapeutic opportunities to combat autoinflammatory diseases by improving the patient response to mutation-dependent stress.

Materials and Methods

Patients. Ten CAPS patients positive for mutations of the *NLPR3* gene, seven of whom were affected by chronic infantile neurological cutaneous articular syndrome, two by Muckle-Wells syndrome, and one by familial cold autoinflammatory syndrome, were enrolled in the study (Table S1). At the time of the study, two patients were not receiving treatment and displayed elevation of acute phase reactants and variable clinical manifestations (Table S1). Eight patients were receiving IL-1 blockers and exhibited a complete control of disease activity. Twelve age-matched healthy controls were studied in parallel. Blood samples were taken after informed consent by patients or parents. The informed consent was approved by the "G. Gaslini" Ethical Board.

Chemicals. The 5,5′-dithiobis-(2-nitrobenzoic acid), DTT, cysteine, DPI, LPS, oATP, and ARL67156 were from Sigma-Aldrich, and the 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) was from Invitrogen.

Cell Preparation and Culture. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn heparinized blood by Ficoll-Paque (Sigma-Aldrich) gradients. Flow cytometry analyses using phycoerythrin-conjugated anti-CD14 mAb (Life Technologies) revealed that CD14+ monocytes varied between 10% and 20%, both in patients and controls. PBMC were enriched by adherence, as described (9, 12, 15, 16, 18, 20), and were activated with different doses of LPS (from *Escherichia coli* 0111:B4; Sigma-Aldrich) at 37 °C in RPMI 1640 medium supplemented with 5% (vol/vol) FCS (Euroclone).

Real-Time PCR. Total mRNA isolated from monocytes using TriPure Isolation Reagent (Roche Applied Science) was reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed using Platinum SYBR green qPCR SuperMix-UDG (Invitrogen). The specific primers for IL-1β, NLRP3, and GAPDH have been described elsewhere (9, 12, 20). Target gene levels were normalized to that of GAPDH mRNA, and relative expression was determined using the ΔCt method, as described (9, 12, 15, 20).

Measurement of ATP. Extracellular ATP concentration was determined in 3 h supernatants with the ATP Bioluminescence Assay Kit HS II (Roche), as described (9).

Determination of Free Cysteine in Culture Media. Supernatants from 18 h of culture were reacted with 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid), and the

absorption was measured at 412 nm (9, 12, 15, 16, 18, 20). Cysteine was used as standards.

ELISA Analyses. Cytokine content in 18 h supernatants was determined by ELISA from R&D Systems (IL-1β, IL-1α, IL-1Ra) and from MBL (IL-18).

Western Blot Analysis. Triton X-100 cell lysates from monocytes were resolved on 12% SDS/PAGE and electrotransferred. Filters were probed with 3ZD anti-IL-1β mAb (IgG1; obtained from the National Cancer Institute Biological Resources Branch), anti-human thioredoxin mAb (clone 2B1; gift from F. Clarke, University of Brisbane, Australia), rabbit anti-human superoxide dismutase Ab (Stressgen), anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb (Novus Biologicals), and anti human α-tubulin mAb (Sigma-Aldrich), followed by the relevant secondary Ab (Dako) and developed with ECL-plus (BioRad), as described (12, 16).

Flow Cytometry. Monocytes were stained with anti-human CD39 (eBioscience) or CD73 (Becton Dickinson) Ab, analyzed with a CyAn flow cytometer (Beckman Coulter) and by the Summit V4.3 software (DAKO) (9).

Intracellular ROS Detection. PBMC (0.5×10^6 cells in 0.2 mL of RPMI 1640 medium plus 5% (vol/vol) FCS) were plated in 96-well plates. Adherent cells were loaded with 10 μM H_2DCF-DA for 30 min before or after 1.5 h of stimulation with LPS. At the end of incubation, cells were lysed in 50 μL 0.2% Triton X-100 in PBS. Fluorescence was measured in cell lysates with a microplate fluorometer (excitation, 480 nm; emission, 530 nm). Data were normalized versus the protein content of cell lysates, measured by the Lowry method (9, 12, 15, 16, 18, 20).

Statistical Analysis. Data were analyzed by using the ANOVA test, followed by Bonferroni post test, using GraphPad software. Significance is expressed as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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