LETTERS

Gout-associated uric acid crystals activate the NALP3 inflammasome

Fabio Martinon[1], Virginie Pétrilli[1], Annick Mayor[1], Aubry Tardivel[1] & Jürg Tschopp[1]

Development of the acute and chronic inflammatory responses known as gout and pseudogout are associated with the deposition of monosodium urate (MSU) or calcium pyrophosphate dihydrate (CPPD) crystals, respectively, in joints and periarticular tissues. Although MSU crystals were first identified as the aetiological agent of gout in the eighteenth century[1] and more recently as a 'danger signal' released from dying cells[2], little is known about the molecular mechanisms underlying MSU- or CPPD-induced inflammation. Here we show that MSU and CPPD engage the caspase-1-activating NALP3 (also called cryopyrin) inflammasome, resulting in the production of active interleukin (IL)-1β and IL-18. Macrophages from mice deficient in various components of the inflammasome such as caspase-1, ASC and NALP3 are defective in crystal-induced IL-1β activation. Moreover, an impaired neutrophil influx is found in an *in vivo* model of crystal-induced peritonitis in inflammasome-deficient mice or mice deficient in the IL-1β receptor (IL-1R). These findings provide insight into the molecular processes underlying the inflammatory conditions of gout and pseudogout, and further support a pivotal role of the inflammasome in several autoinflammatory diseases.

The notion of autoinflammatory diseases delineates a heterogeneous group of pathologies characterized by spontaneous periodic inflammation and fever in the absence of infectious or autoimmune causes[3]. Hereditary periodic fevers, systemic onset juvenile idiopathic arthritis, Still's disease, Behçet's disease and the metabolic disorders gout and pseudogout are examples of such inflammatory maladies. Increased production of the inflammatory cytokine IL-1β was recently identified as the cause of several autoinflammatory diseases, providing clear evidence for a pivotal role of this cytokine in triggering autoinflammation[4–8]. IL-1β, also known as the endogenous pyrogen, is a highly inflammatory cytokine whose production is tightly controlled by at least three distinct steps[9]. The first step involves the production of the pro-IL-1β protein (p35); this is followed by cleavage of the precursor pro-IL-1β to produce the active IL-1β protein (p17), and finally IL-1β is released into the extracellular environment. The middle step, processing of pro-IL-1β, involves the activation of a caspase-1-activating complex, the best characterized being the inflammasome[10,11].

Upon activation, the inflammasome is formed by a member of the NALP protein family, such as NALP1, NALP2 or NALP3, and the adaptor protein ASC that connects the NALPs with caspase-1 (ref. 12). Signals and mechanisms leading to inflammasome activation are still poorly understood. Muramyl dipeptide (MDP), a degradation product of the bacterial cell wall component peptidoglycan and contaminant of crude lipopolysaccharide (LPS), was recently shown to activate a NALP3 inflammasome[13] through the leucine-rich repeat domain of NALP3, suggesting that NALPs, like Toll-like receptors (TLRs), are fundamental for microbial detection[14]. However, the inflammasome is also proficient in sensing stress or endogenous danger signals, such as extracellular ATP or hypotonic stress[10,11,15]. Recently, MSU crystals were identified as a danger signal formed after release of uric acid from dying cells[2]. This observation, and the well-known role of uric acid crystals in gouty arthritis[16], prompted us to investigate whether MSU crystals could activate the inflammasome.

Cells from the differentiated monocytic cell line THP1 were incubated with MSU crystals. Maturation of IL-1β was indeed detected after stimulation with as little as 10 $\mu g\,ml^{-1}$ of the crystals (Fig. 1a). The caspase-1 dependency of the pro-IL-1β cleavage was confirmed by addition of the caspase-1 inhibitor zYVAD-fmk, which completely blocked MSU-induced IL-1β activation (Fig. 1a). CPPD, another type of pathogenic crystal involved in calcium pyrophosphate deposition disease, also known as pseudogout, was as active as MSU (Fig. 1b). Crystal-induced IL-1β processing was specific for pathogenic agents, as the non-inflammatory allopurinol or diamond crystals and particulate elements such as zymosan and aluminium powder failed to induce pro-IL-1β processing (Fig. 1c), despite their similar size and/or chemical composition. Compared to the known activators of the inflammasome (that is, crude LPS, ATP), MSU and CPPD were more active[11,13] (Fig. 1c). This superior potency was particularly evident when analysing processing of pro-IL-18, the second known substrate of caspase-1 (Fig. 1c). Previously, we demonstrated that the inflammatory caspases are cleaved and released along with active IL-1β after activation of the inflammasome[13]. This was also observed when cells were treated with MSU and CPPD (Fig. 1c, d). In order to exclude the possibility that crystal-mediated activation of caspase-1 is a unique property of the THP1 cell line only, MSU and CPPD were added to purified human monocytes. As shown in Fig. 1d, a strong response to both pathogenic crystals was also elicited in primary cells.

In order to provide direct evidence for the involvement of the inflammasome in crystal-induced inflammation, we analysed peritoneal macrophages (PMΦs) derived from mice deficient in various key proteins of the inflammasome complex or other proinflammatory pathways. Given the absence and/or rapid degradation of pro-IL-1β in PMΦs *ex vivo*, and because we failed to see any direct induction of the transcription or translation of pro-IL-1β by MSU or CPPD, we stimulated TLR4 in PMΦs with highly purified LPS to induce the synthesis of the cytokine[11,13]. Consistent with our previous findings in human monocytes, murine PMΦs stimulated with MSU or CPPD activated caspase-1 and secreted mature IL-1β (Fig. 2a). Maturation was abolished in PMΦs from caspase-1-deficient mice, confirming the specificity of the activation. As expected, MyD88-deficient PMΦs did not produce mature IL-1β due to their defective TLR signalling, resulting in a failure to produce pro-IL-1β after LPS pre-stimulation (Fig. 2a). Nevertheless, $MyD88^{-/-}$ PMΦs still activated caspase-1 (Fig. 2a), further suggesting that this activation is

[1]Department of Biochemistry, University of Lausanne, Chemin des Boveresses 155, 1066 Epalinges, Switzerland.

(PAMPs)) provide signals that alert our immune system to danger and promote the innate generation of immunity[27]. However, PAMPs (non-self) are not the only triggers of innate immunity. Innate immunity is able to recognize abnormal self or danger signals, such as uric acid released by injured cells[2,28]. How these danger signals are recognized by cells is mostly unknown, but based on our results inflammasomes probably constitute some of the long-sought proximal sensors for stress or danger signals designed to initiate inflammation.

In addition to gouty inflammation, the NALP3 inflammasome is also implicated in other autoinflammatory diseases. Specific gain-of-function mutations in the NALP3 protein lead to three related familial autoinflammatory diseases: Muckle–Wells syndrome, familial cold autoinflammatory syndrome and chronic infantile neurologic cutaneous and articular syndrome[4,29]. In patients with these diseases, mutations in NALP3 lead to a constitutive processing of IL-1β[30]. In the case of gout and pseudogout, aberrant NALP3 inflammasome activation is not genetic, but mediated by local deposition of crystals. Importantly, inflammation in hereditary periodic fevers patients with mutations in NALP3 can be markedly improved by treatments designated to block IL-1β[20,21]. Owing to the similarity between NALP3-mediated hereditary periodic fevers and gout and pseudogout, we can anticipate that similar treatments could benefit gout and pseudogout patients. It is also reasonable to foresee that further identification of additional inflammasome-activating endogenous danger signals will probably shed some light on the molecular aetiology of other autoinflammatory diseases such as systemic onset juvenile idiopathic arthritis and Behçet's disease that share similarity with hereditary periodic fevers, gout or pseudogout.

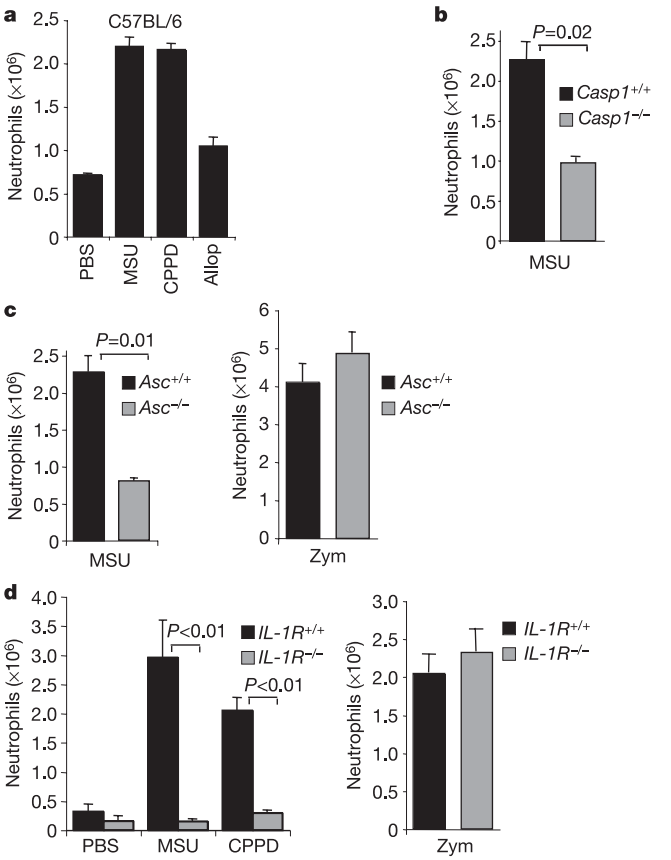

Figure 4 | Role of the inflammasome in a mouse model of crystal-mediated peritonitis. a–d, The indicated wild-type or mutant mice received 0.5 ml (intraperitoneally) of sterile PBS alone or supplemented with 1 mg of the indicated crystals or 0.2 mg of zymosan. Neutrophil influx was quantified 6 h later (values are ±s.e.m. of $n = 4$–6 mice per group). Unpaired Student's *t*-test was used to calculate *P* values. Allop, allopurinol.

METHODS

Primary human monocyte and THP1 preparation and stimulation. THP1 cells were stimulated for 3 h with 0.5 μM of PMA the day before stimulation, as described[10]. This treatment increases the phagocytic properties of the cells and induces a constitutive production of pro-IL-1β. Human monocytes were purified as described previously[30]. All cells were stimulated in OptiMEM medium as indicated. Human mature IL-1β was detected with a specific antibody directed against the cleaved epitope (D116) from Cell Signaling.

Mouse macrophage preparation. Eight-to-twelve-week-old mice of indicated genotypes were injected intraperitoneally with 4% thioglycollate solution, and macrophages were collected by peritoneal lavage 3 days later. Cells were plated at the density of 7×10^5 cells in 12-well dishes and non-adherent cells were removed after 3 h. Cells were cultured in RPMI complemented with 10% FCS, sodium pyruvate, penicillin/streptomycin and L-glutamine. All cells were stimulated in OptiMEM medium.

***In vivo* mouse peritonitis model.** Peritonitis was induced by injection of 1 mg of crystals or 0.2 mg of zymosan in 0.5-ml sterile PBS. After 6 h, mice were killed by CO_2 exposure and peritoneal cavities were washed with 10 ml of PBS. The lavage fluids were analysed for PMN recruitment by FACS using the neutrophil marker Ly-6G (1A8, BD Biosciences).

Mice and reagents. NALP3 targeting vector (Supplementary Fig. 1) was electroporated into C57BL/6 embryonic stem (ES) cells (Ozgene). Homologous recombinant ES cells were identified by Southern blot analysis and microinjected into C57BL/6 blastocysts. Offspring were backcrossed to C57BL/6 mice and germline transmission was confirmed by PCR of tail genomic DNA. Additional details on mice, preparation of crystals and reagents are given in the Supplementary Information.

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