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Basic Calcium Phosphate Crystals Induce Monocyte/Macrophage IL-1β Secretion through the NLRP3 Inflammasome In Vitro

Borbała Pazar,* Hang-Korng Ea,†‡ Sharmal Narayan,* Laeticia Kolly,* Nathalie Bagnoud,* Véronique Chobaz,* Thierry Roger,§ Frédéric Lioté,‡ Alexander So,* and Nathalie Busso*

Basic calcium phosphate (BCP) crystals are associated with severe osteoarthritis and acute periarticular inflammation. Three main forms of BCP crystals have been identified from pathological tissues: octacalcium phosphate, carbonate-substituted apatite, and hydroxyapatite. We investigated the proinflammatory effects of these BCP crystals in vitro with special regard to the involvement of the NLRP3–inflammasome in THP-1 cells, primary human monocytes and macrophages, and mouse bone marrow-derived macrophages (BMDM). THP-1 cells stimulated with BCP crystals produced IL-1β in a dose-dependent manner. Similarly, primary human cells and BMDM from wild-type mice also produced high concentrations of IL-1β after crystal stimulation. THP-1 cells transfected with short hairpin RNA against the components of the NLRP3 inflammasome and mouse BMDM from mice deficient for NLRP3, apoptosis-associated speck-like protein, or caspase-1 did not produce IL-1β after BCP crystal stimulation. BCP crystals induced macrophage apoptosis/necrosis as demonstrated by MTT and flow cytometric analysis. Collectively, these results demonstrate that BCP crystals induce IL-1β secretion through activating the NLRP3 inflammasome. Furthermore, we speculate that IL-1 blockade could be a novel strategy to inhibit BCP-induced inflammation in human disease. The Journal of Immunology, 2011, 186: 2495–2502.

Department of Rheumatology, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland; †Infectious Diseases Service, Department of Medicine, University of Lausanne, 1011 Lausanne, Switzerland; ‡Unité Mixte de Recherche S606, INSERM, Lariboisière Hospital, 75010 Paris, France; and §Institut Claude Bernard-IFR2, Université Paris–Diderot, Faculté de Medicine, 75890 Paris Cedex 18, France

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Address correspondence and reprint requests to Dr. Nathalie Busso, Department of Rheumatology, Centre Hospitalier Universitaire Vaudois, Nestlé 05-5029, 1011 Lausanne, Switzerland. E-mail address: Nathalie.Busso@chuv.ch

Abbreviations used in this article: ASC, apoptosis-associated speck-like protein; BCP, basic calcium phosphate; BMDM, bone marrow-derived macrophage; CA, carbonate-substituted apatite; CAS-1, caspase-1; CPPD, calcium pyrophosphate dihydrate; DAMP, danger-associated molecular pattern; HA, hydroxyapatite; monosodium urate; KCl, potassium chloride; MSU, monosodium urate; NAC, N-acetylcysteine; OCP, octacalcium phosphate; PI, propidium iodide; ROS, reactive oxygen species; sh, short hairpin.

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production (11). However, the mechanism of BCP crystal-induced IL-1β production by macrophages (15) and bovine articular chondrocytes (16) is yet undetermined.

IL-1β is a highly inflammatory cytokine whose production is tightly controlled by at least three distinct steps including expression, processing, or maturation and secretion (17). Thus, pro–IL-1β protein (p35 kDa) is subsequently matured to active IL-1β (p17 kDa) by caspase-1 (CAS-1) or other polymorphonuclear neutrophil proteases, such as proteinase-3, elastase, chymase, and granzyme A (18–23). Active IL-1β is then secreted into the extracellular environment. CAS-1 activation depends on a multi-protein platform called inflammasome (24, 25). The NLRP3 or NALP3 is the most characterized inflammasome and is formed by the adaptor protein apoptosis-associated speck-like protein (ASC), the proinflammatory CAS-1 and NLRP3 (17, 26).

NLRP3 belongs to the nucleotide-binding oligomerization domain-like receptor (NLR) family of pattern recognition receptors. It can be activated by a variety of pathogen and host-derived “danger signals” including whole pathogens, pathogen-associated molecular patterns (PAMP), others pathogen molecules, host-derived signal of cellular damage (the so-called danger-associated molecular patterns or DAMP), and environment irritants (17, 27). The NLRP3 inflammasome is implicated in several human inflammatory diseases. Specific gain-of-function mutations in the NLRP3 protein lead to three related familial autoinflammatory diseases collectively called cryopyrin-associated periodic syndrome, which encompasses the Muckle–Wells syndrome, familial cold autoinflammatory syndrome, chronic infantile neurologic cutaneous, and articular syndrome (28, 29). Similarly, NLRP3 is involved in IL-1β production triggered by MSU and CPPD crystals (7), and recent clinical cases have demonstrated the efficiency of IL-1β blockade in both acute gout and pseudogout attacks (6, 8, 9).

In this study, we investigated the role of NLRP3 in BCP crystal-induced IL-1β production by monocytes and macrophages.

Materials and Methods
Crystal preparation
Sterile, pyrogen-free MSU, CPPD, and BCP crystals were synthesized as described previously (7, 30). Crystals were suspended in sterile PBS and dispersed by brief sonication. All crystals were determined to be endotoxin free (<0.01 EU/10 mg) by Limulus amebocyte cell lysate assay.

THP-1 cells, primary human monocyte, and macrophage preparation and stimulation
The THP-1 monocytic cell line (American Type Culture Collection, Manassas, VA) was maintained in the log phase of growth in RPMI 1640 medium containing 10% FCS (Invitrogen, Basel, Switzerland). THP-1 cells were differentiated by the addition of 0.5 μM PMA for 3 h the day before stimulation. Cells were subsequently washed and plated in 48-well plates at 5 × 10^4 cells/well in complete medium. The following day medium was changed again, and cells were incubated with crystals for 6 h. In some experiments, cells were preincubated 30 min before crystal stimulation with the caspase inhibitor z-YVAD-fmk (50 μM; Sigma-Aldrich, Buchs, Switzerland), cytochalasin-D (2 μM; Calbiochem, Luzern, Switzerland), N-acetylcyesteine (NAC) (25 mM; Sigma-Aldrich), and potassium chloride (KCl) (Sigma-Aldrich). Plasmids for the expression of short hairpin (sh)RNA against labin (sh-Mock), NLRP3 (shNLRP3), ASC (shASC), and CAS-1 (shCAS-1) and THP-1 cells stably expressing these shRNA were generated as described elsewhere (31), and the resulting knocked down THP-1 cells were incubated with crystals in complete medium supplemented with 1 μg/ml puromycin. Human monocytes were purified from peripheral blood from healthy donors with the ficoll-paque gradient and the MACS Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Human macrophages were obtained from monocytes cultured in vitro for 7 d in the presence of 50 ng/ml human M-CSF (eBioscience, CBI, Baar, Switzerland). Human monocytes and macrophages were primed overnight with ultrapure LPS (200 ng/ml InvivoGen, San Diego, CA) to induce pro–IL-1β. Crystal incubation was performed as described for THP-1 cells.

Mouse macrophage preparation
Eight- to 12-wk-old wild-type, NLRP3, ASC, and CAS-1 knockout mice, all in the C57BL/6 background, were sacrificed, and bone marrow cells (bone marrow-derived macrophages [BMDM]) were recovered from tibial and femoral bones. The cells were cultured for 7 d in the presence of 20 ng/ml M-CSF in DMEM supplemented with FCS and 2-ME (50 μM). The day before crystal stimulation, cells were primed with ultrapure LPS (200 ng/ml) overnight to induce the production of pro–IL-1β.

Abs used for Western blotting
Human mature IL-1β p17 was detected with a specific Ab directed against the cleaved epitope (D116) from Cell Signaling Technology (Denver, CO), whereas human IL-1β p35 was detected using a sheep homemade Ab. The Ab against mouse IL-1β was from Cell Signaling Technology. The Ab against human CAS-1 (SC-622) was obtained from Santa Cruz Biotechnology (Nuningen, Switzerland). Murine anti-caspase was from Alexis (Lausen, Switzerland).

ELISA
Human IL-1β and mouse IL-1β ELISA were purchased from eBioscience. Human CAS-1 ELISA was obtained from BenderMedSystems (Vienna, Austria). All results are representative of at least three independent experiments.

Cell viability
Cell viability was assessed using the MTT assay (Sigma-Aldrich) in triplicates. The MIT solution was incubated with the cells after crystal stimulation. The formazan formed by living cells was dissolved with DMSO, which yielded a purple color. Color intensity was measured at 560 nm. To further confirm the MIT viability results, flow cytometric analysis was performed using the FITC Annexin V Apoptosis Detection Kit I (catalog number 556547; BD Pharmingen, San Diego, CA), according to manufacturer’s instructions. Briefly, cells were stained with FITC-conjugated annexin V and propidium iodide (PI). Viable, early apoptotic and late apoptotic and/or necrotic cells were identified as Annexin V-PI-, Annexin V-PI+, and Annexin V+PI+, respectively. The BD FACScalibur (BD Biosciences) flow cytometer was used to acquire data using CellQuest software (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star).

Statistical analysis
Data are reported as mean values ± SEM. Differences between groups were analyzed with one-way ANOVA or unpaired t test when necessary. A level of p < 0.05 was considered as statistically significant.

Results
BCP crystals induce IL-1β maturation and release by monocytic cells
In THP-1 cells, all three types of BCP crystals, namely OCP, HA, and CA crystals, were induced in a dose-dependent fashion, and after 6 h of stimulation, the secretion of IL-1β protein was assessed by ELISA in culture medium (Fig. 1A). All three BCP crystals induced higher amounts of IL-1β than equivalent doses of MSU and CPPD (data not shown) crystals, two well-known crystals inducing IL-1β secretion by THP-1 cells (7). Of the three BCP crystals, OCP crystals were the most potent, inducing significantly increased release of IL-1β at concentrations as low as 10 μg/ml (data not shown). IL-1β secretion was further confirmed by immunoblots of cell supernatants that showed the active p17 IL-1β only in samples from crystal-stimulated cells (Fig. 2B). We also tested the capacity of OCP crystals to stimulate IL-1β secretion from purified human monocytes and macrophages to verify the results from the THP-1 cells. OCP crystal-induced IL-1β production from primary monocytes was also observed and was higher than that induced by an equivalent dose of MSU (Fig. 1C).
When primary human monocyte cultures were differentiated into macrophages, IL-1β secretion was reduced when compared with OCP-stimulated primary monocyte cultures (Fig. 1C). The amount of IL-1β released by macrophages in response to MSU crystals was also reduced when compared with primary monocytes, as has been previously reported (32). This differential IL-1β release upon...
crystal exposure was not due to differential cell viability between monocytes and macrophages as measured by MTT assays (data not shown).

**BCP crystal-induced IL-1β secretion is accompanied by CAS-1 activation**

We next investigated the role of CAS-1 in OCP crystal-induced pro–IL-1β processing. BCP crystal-induced secretion of p17 IL-1β was CAS-1 dependent. All three BCP crystals induced the secretion of CAS-1 into the supernatants in THP1 cells, with OCP crystals being the most potent when compared with others microcrystals (Fig. 1B). Primary human monocytes cultured with OCP also secreted significant amounts of CAS-1 in supernatants (Fig. 1D). In contrast, human macrophages secreted significantly less CAS-1 than monocytes, paralleling the results of IL-1β secretion. The dependency of pro–IL-1β processing on caspase activation was confirmed by addition of zYVAD-fmk, a cell-permeable, irreversible inhibitor of caspase activity, which almost completely blocked MSU and OCP crystal-induced IL-1β secretion as measured by IL-1β ELISA (Fig. 2A) and by immunoblots of active CAS-1 and IL-1β in supernatants (Fig. 2B).

**BCP crystal-induced IL-1β release is dependent on the NLRP3 inflammasome**

To provide direct evidence for the involvement of the NLRP3 inflammasome in BCP crystal-induced IL-1β production, we first used THP-1 cells that were stably transfected with shRNA to knock down the expression of the different components of the NLRP3 inflammasome, namely CAS-1, ASC, and NLRP3. As previously described with MSU and CPPD crystals (7) in vitro, BCP crystal-induced IL-1β release was NLRP3 dependent. Indeed, as shown in Fig. 3A, active IL-1β secretion induced by BCP and MSU crystals were completely abolished in CAS-1, ASC, or NLRP3 knocked-down THP-1 cells (Fig. 3A). Some residual activation was detected in shASC- and shNLRP3-transfected cells owing to incomplete silencing of the corresponding mRNA. As it

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**FIGURE 3.** NLRP3 inflammasome is required for in vitro IL-1β maturation induced by BCP crystals. A, Control THP-1 cells (sh Mock) or THP-1 cells knocked down for ASC (sh ASC), NLRP3 (sh NLRP3), and CAS-1 (sh CAS-1) were incubated with 500 μg/ml CA, HA, MSU, or OCP crystals for 6 h. Western blots of cell extracts (CE) or supernatants (SN) with Abs detecting pro–IL-1β (p35), active IL-1β (p17), and active CAS-1 (p20) were performed. Only cells with intact NLRP3 inflammasome (sh Mock) secreted mature CAS-1 and IL-1β. Note in the lower panel, the presence of intact pro–IL-1β in supernatants of OCP (and to a milder extent also in supernatants of MSU)-stimulated cells, whether or not the cells expressed the inflammasome (B). Mouse macrophages from wild-type (WT), CAS-1– (CAS1−/−), ASC−, and NLRP3-deficient mice were stimulated with 500 μg/ml MSU or OCP crystals for 6 h and cell extracts (CE) or supernatants analyzed by Western blot as described above. The presence of NLRP3 inflammasome was required for the maturation of IL-1β.

**FIGURE 4.** Molecular and cellular mechanisms involved in OCP crystal-induced IL-1β release in THP-1 cells. A, THP-1 cells were preincubated with cytochalasin-D or NAC for 30 min prior to stimulation with 500 μg/ml MSU or OCP crystals for 6 h. Controls were not stimulated with crystals. Supernatants were analyzed by IL-1β ELISA. B, THP-1 cells were stimulated with 500 μg/ml OCP crystals in the presence of increasing doses of KCl for 6 h. Controls were not stimulated with crystals. Supernatants were analyzed by IL-1β ELISA. **p < 0.01, ***p < 0.001.
was presumed, the secretion of the active CAS-1 was also depend-
ent on NLRP3 inflammasome in the THP-1 cells (Fig. 3A). To further confirm our results, we next isolated BMDM from mice deficient in various key proteins of the NLRP3 inflammasome complex. Consistent with our previous findings with THP-1 cells and primary human monocytes, murine BMDM stimulated with BCP crystals secreted active CAS-1 and mature IL-1β. BCP crystal-induced IL-1β maturation was totally abolished in BMDM derived from NLRP3-, ASC-, and CAS-1–deficient mice, confirming the role of NLRP3 inflammasome in this process (Fig. 3B).

**Molecular and cellular mechanisms involved in OCP crystal-induced IL-1β release in THP-1 cells**

To gain insights into the upstream mechanisms involved in NLRP3 activation via OCP crystals, we first investigated whether phagosomes are involved in the OCP-induced IL-1β release by testing the influence of cytochalasin-D using THP-1 cells. As for MSU crystals, phagocytosis of OCP crystals is required for IL-1β activation (Fig. 4A). Reactive oxygen species (ROS) generation is also involved in the process as the broad ROS inhibitor NAC almost completely blocked OCP-induced IL-1β secretion (Fig. 4A).

These marked inhibitory effects of cytochalasin-D and NAC were not due to toxicity because cell viability as determined by MTT, in presence of OCP crystals and inhibitors was comparable to the viability of cells stimulated with OCP alone (data not shown). We finally examined the importance of K⁺ efflux by incubating THP-1 cells with OCP (500 μg/ml) in the presence of increasing extracellular KCl concentrations. OCP-induced IL-1β secretion was inhibited as soon as KCl was added in the cell supernatant, thus indicating that the stimulatory effect of OCP on IL-1β secretion was almost totally dependent on K⁺ efflux (Fig. 4B).

**BCP crystals induce cell death and release of pro–IL-1β**

Besides active p17 IL-1β, we also detected significant amounts of pro–IL-1β (p35) by immunoblotting in the supernatants of OCP crystal-stimulated THP1 and BMDM cells (Fig. 3). We wondered whether this was due to the release of intracellular pro–IL-1β into the supernatant by dying cells as a consequence of contact with OCP crystals. Cell viability was assessed using the MTT assay, as well as by FACS. All three BCP crystals (at 100 and 500 μg/ml) induced a significant decrease in THP-1 cell viability after 6 h, as assessed by the MTT assay (Fig. 5A). OCP crystals induced the highest degree of cell death compared with the other crystals.

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**FIGURE 5.** BCP crystals induce apoptosis of macrophages in vitro. MTT analysis were used to determine cell viability of THP-1 cells stimulated with MSU, OCP, HA, or CA (A) as indicated for 6 h and of mouse macrophages (BMDM) incubated with MSU or OCP as indicated for 6 h (B). MTT viability results were confirmed using FACS of annexin V versus PI staining after incubation with MSU and OCP crystals for 6 h of THP-1 cells (C) and mouse BMDM (D). Viable, early apoptotic and late apoptotic and/or necrotic cells were identified as Annexin V⁻PI⁻, V⁻PI⁺, and V⁺PI⁺, respectively. **p < 0.01, ***p < 0.001.
MSU crystals (at the highest dose of 500 μg/ml) provoked ~20% cell death in the MTT assay (Fig. 5A). We further evaluated the state of apoptosis/necrosis induced by FACS. At the same concentration of crystals, OCP induced a greater degree of cell death than MSU crystals (50 and 20% apoptotic/necrotic cells, respectively) (Fig. 5C). In mouse BMDM, OCP and MSU crystals also induced significant cell death by the MTT assay (~50 and 40%, respectively [Fig. 5B]). This was also confirmed by FACS, where THP-1 apoptosis/necrosis induced by OCP crystals was greater than what was induced by MSU (Fig. 5D). Altogether, these results suggested that part of IL-1β release when human monocytes were differentiated into macrophages, extending previous data that showed a change in the population with MSU and CPPD crystals in our assays confirming our previous findings (30). Interestingly, we did observe a reduction in OCP-induced IL-1β release when human monocytes were differentiated into macrophages, extending previous data that showed a change in the inflammatory state of macrophages to MSU when compared with monocytes (35).

By themselves, BCP, MSU, or CPPD crystals are incapable of directly inducing IL-1β secretion by monocytes (as shown in Fig. 6A). Monocytes and macrophages need to be primed either with PMA (for THP1 cells) or with ultrapure LPS (for primary monocyte/macrophages) or with other inflammatory stimuli, such as TNF-α or TLR2 and TLR9 agonists (PAM3 and CpG, respectively; data not shown), to secrete IL-1β when they are cultured in the presence of microcrystals. This two-signal requirement shows that the process of IL-1β is tightly controlled, with a first signal that induces pro–IL-1β synthesis and a second signal that triggers subsequently pro–IL-1β processing through the CAS-1 complex to form p17 IL-1β. The mechanism underlying BCP crystal-stimulated IL-1β processing is similar to that described for other endogenous and exogenous crystalline molecules, such as MSU and CPPD crystals, cholesterol microcrystals (36), silica, asbestos particles, or alum, namely through activation of the NLRP3 inflammasome and the production of functional CAS-1 (for review, see Ref. 37). As expected, the caspase inhibitor z-YYAD-fmk completely blocked IL-1β release. This requirement was further confirmed using THP1 knockdown cells for CAS-1, ASC, and NLRP3 and further strengthened by results using mice BMDM deficient for the different components of NLRP3 inflammasome. It is interesting to note that in OCP-stimulated THP-1 and BMDM cells, pro–IL-1β was easily detected in the culture supernatant and could theoretically serve as a substrate for non–CAS-1 proteases. In the in vitro setting, we have tested whether the amount of IL-1β released was not modified when serine protease inhibitors were added to the mixture (data not shown), further suggesting that the main pathway of IL-1β maturation in monocytes and macrophages is exclusively via CAS-1.

Although the NLRP3 inflammasome is indispensable in our in vitro setting, there is evidence to suggest that in vivo the situation could be different. It is well known that non–CAS-1 proteases, produced and released by activated neutrophils and mast cells, could also process pro–IL-1β to produce the active form of IL-1β (18, 20, 22), and could thus serve as an “alternative pathway” of IL-1β generation at the inflammatory site. Indeed, CAS-1–independent activation of IL-1β in murine models of arthritis with predominant neutrophilic infiltration has been shown recently (19, 21, 23). We have also observed in neutrophil-predominant inflammatory responses induced by i.p. OCP crystals injection that neutrophil recruitment was IL-1–dependent but mainly NLRP3 inflammasome independent (38). We hypothesize that NLRP3 inflammasome activation may play a dominant role in a macrophage-dependent inflammation model but a limited role in neutrophilic inflammation.

NLRP3 is activated by a list of stimuli (including whole pathogens, pathogen-associated molecular patterns, DAMP, and environmental irritants; for review, see Ref. 37), with OCP crystals now representing an additional DAMP to this growing list. It has been proposed that inflammasome activation by crystals require plasma membrane binding, both via receptor-independent and -dependent mechanisms. Ng et al. (39) observed that MSU crystals could directly bind to cell membrane lipids with subsequent lipid sorting and rearrangement resulting in syk kinase activation, and more recently, it was demonstrated that syk kinase signaling couples to the Nlrp3 inflammasome in the context of antifungal

**FIGURE 6.** BCP crystal-induced IL-1β secretion requires a priming signal. THP-1 cells were unprimed or primed for 3 h with PMA at 0.5 μM, whereas primary monocytes and macrophages were unprimed or primed with ultrapure LPS at 200 ng/ml to induce pro–IL-1β production. Cells were washed and incubated with MSU or OCP (both at 500 μg/ml) or without crystals. Supernatants were analyzed by IL-1β ELISA. A lack of priming results in very low or undetectable levels of IL-1β for all three cell populations. *p < 0.01, **p < 0.001.
host defense (40). The possibility that OCP crystals can be sensed extracellularly by membrane lipid alterations and can transmit cytoplasmonic syk kinase signaling remains to be explored. Another potential interaction between crystals and plasma membrane binding is via TLR2 and TLR4, as demonstrated previously for MSU crystals (41). We also have preliminary data that show a small but significant decrease in the uptake of OCP crystals by TLR2/–/–, TLR4–/– BMDMs in vitro, which is reflected in decreased IL-1β secretion by these cells (data not shown). However, definitive implication of TLR2 and TLR4 in OCP crystal-induced inflammation should be validated in different in vivo models. Indeed, the role for TLR in MSU crystal-induced inflammation in vivo remains controversial. In contrast, the presence of TLR2 and TLR4 exacerbated MSU crystal-induced IL-1β production and polymorphonuclear neutrophil recruitment in the murine air pouch model, but another group found that none of the known TLRs were indispensable in the murine peritonitis model (41, 42).

The exact mechanisms by which the NLRP3 inflammasome senses a variety of chemically and structurally distinct activators remains unclear. It has been proposed that “frustrated” phagocytosis caused by particulates too large to be efficiently cleared induces the production of ROS as well as K+ efflux (although it is still unknown whether ROS induces K+ efflux or vice versa). Both ROS generation and K+ efflux have been shown to be crucial for NLRP3 activation (31, 37). Concerning phagocytosis, we observed in fact that cytochalasin B, a phagocytosis inhibitor, totally prevented OCP crystal-induced IL-1β secretion. In addition, we observed that OCP crystal-induced IL-β secretion involved also ROS formation and K+ efflux, because IL-1β secretion induced by these crystals was completely inhibited by the ROS inhibitor NAC and by higher extracellular concentrations of K+, respectively. Altogether, these results show that different particulates structures (MSU, silica, asbestos, alum, and now OCP) require intracellular K+ depletion and ROS production for NLRP3 activation in vitro. The inflammatory action of these particulates in vivo could be mediated by cell death and release of uric acid, which in turn will crystallize and activate NLRP3 as it has been demonstrated at least for alum (43) and hemozoin (44).

In conclusion, this study shows that BCP crystal-induced IL-1β production is mediated by activation of the NLRP3 inflammasome in a two-step process. However, one has to be cautious to extrapolate these results in vitro findings to the in vivo situation, as non-CAS-1 proteases can contribute to IL-1β production. In clinical terms, the finding that BCP crystals trigger IL-1β release in an analogous manner to MSU crystals in acute gout suggests that IL-1β blockade may be clinically useful. This therapeutic approach may be interesting in treatment of acute calcific tendinitis and in inflammatory phases of osteoarthritis, where these crystals are abundant.

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Disclosures
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