Phagocytosis and inflammation in crystal-induced arthritis: a synovial fluid and *in vitro* study

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Abstract

Objective

The aim of this study was to investigate the relationship between the degree of crystal phagocytosis and the magnitude of the local inflammatory process using fresh synovial fluid (SF) collected from patients with crystal-induced arthritis. In parallel, an in vitro model of crystal-induced inflammation was used to assess the effect of cell priming on crystal phagocytosis and IL-1ß production.

Methods

SF was collected from 20 patients with gout and 20 with pyrophosphate crystal-induced arthritis and examined under ordinary and polarised light microscopy for total and differential white blood cell (WBC) count and crystal search. The total phagocytosis index was determined in SF along with IL-1β, IL-8, IL-10, and TGFβ levels. The in vitro studies were performed using primed or unprimed THP-1 cells stimulated with calcium pyrophosphate (CPP) crystals, monosodium urate (MSU) crystals and/or cytochalasin D.

Results

We demonstrated that the phagocytosis index calculated on the total number of cells was independent from the inflammatory local indices such as WBC and the percentage of polymorphonuclear cells but showed a positive correlation with pro-inflammatory cytokines. By contrast, the local inflammatory indices (WBC and IL-1β) showed a strong positive correlation with the percentage of polymorphonuclear cells with crystals internalised and a negative correlation with the percentage of mononuclear cells with crystals internalised. The in vitro study showed that phagocytosis represents a fundamental step in the induction of the inflammatory response to MSU and CPP crystals, but it also occurs in absence of cell priming.

Conclusion

The results of this study indicate a possible role of non-inflammatory phagocytosis in limiting the acute attack of crystal-induced arthritis.

Key words

crystal arthropathies, inflammation, synovial fluid, phagocytosis, urate, calcium pyrophosphate, interleukin-1

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Received on January 30, 2020; accepted in revised form on May 5, 2020.

Funding: this research was funded by the University of Padova, grant no. DOR1972490/19 to F. Oliviero, DOR1985833/19 to P. Sfriso, and the Italian Ministry of Education, University and Research, grant no. FFABR2017 to F. Oliviero.

Competing interests: none declared.

Introduction

The deposition of monosodium urate (MSU) and calcium pyrophosphate (CPP) crystals in articular and periarticular tissues causes an acute and powerful inflammatory reaction characterised by the recruitment of innate immune cells and massive release of cytokines, chemokines, oxygen reactive species and eicosanoids (1). This process resolves spontaneously in few days but, if not treated, leads to chronic MSU or CPP crystal-induced arthritis (CIA) with pain, articular tissue destruction and limited joint function. Synovial fluid (SF) aspirated from patients during an acute attack shows inflammatory features (2) and different cytokine levels depending on the stage of the acute phase of the disease (3). Among cytokines, IL-1β has been shown to play a central role in CIA and IL-1 inhibitors have demonstrated to be highly effective in patients with CIA refractory or intolerant to standard treatments. IL-1β requires two signals to be produced and released by mononuclear cells. While crystals activate the nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 3 (NLRP3) inflammasome, an additional stimulus, such as TLR2 ligands, proteins or fatty acids (4-6) serves for the initial production of pro-IL-1β (priming). Although this mechanism has been largely characterised, much less is known on the phagocytosis of crystals, the earliest event which allows cells to be activated to generate IL-1 and other mediators. MSU and CPP, like other crystalline materials, are recognised by the immune system as danger signals and after engaging cellular membrane, are internalised by the cells in the form of phagolysosomes (7, 8).

Once inside the cell, crystals cause lysosomial damage with the release of lysosomial proteases and ATP into the cytosol which in turns contribute to affect mitochondrial metabolism and ROS generation (1). Inflammasome activation and the persistence of the stimulus result in sustained inflammation. The main objective of this study was to investigate whether the degree of phagocytosis was related to the magnitude of the inflammatory process in fresh SF. In parallel, we used an *in vitro* model of crystal-induced inflammation to investigate whether cell priming and crystal phagocytosis are two independent processes.

Materials and methods

Study on synovial fluid

- Synovial fluid collection

SF was collected by arthrocentesis from swollen knees of untreated patients with CIA attending the outpatients' clinic of the Rheumatology Unit of the University of Padova. Twenty SFs obtained from patients diagnosed with gout and 20 with pyrophosphate crystal-induced arthritis were tested in the study. The SF was examined as part of routine procedures and examination including ordinary light microscopy for total and differential white blood cell (WBC) count using a Bürker counting chamber and pre-stained slides for cell morphology (Testsimplets®), respectively. Differential white blood cell count provided the percentage of polymorphonuclear (PMN), monocytes (M) in the SF. Crystal search was performed using polarised compensated light microscopy. After examination, SF samples were centrifuged at 1500 rpm for 30 min to remove the cells, particulate material, and debris (9). SF samples were obtained and studied under protocol that included written informed consent and that was approved by the local Institutional Review Board.

- Measurement of synovial fluid phagocytosis index

The total phagocytosis index in SF (P-index) was expressed as percentage of cells with internalised crystals over the total of the cells counted. Monocytes and polymorphonuclear cells (PMN) were also distinguished in percentage of cells with (M-i, PMN-i) or without (M, PMN) internalised crystals as follows:

$$Total\ P-index\ (\%) = \frac{N.\ of\ cells\ with\ \ internalised\ crystals}{\text{Total N. of cells}} \times 100$$

$$M-i\ (\%) = \frac{N.\ of\ monocytes\ with\ internalised\ crystals\ (\%)}{\text{P – index (\%)}} \times 100$$

$$PMN-i\ (\%) = \frac{N.\ of\ polymorphonuclear\ cells\ with\ internalised\ crystals\ \ (\%)}{\text{P – index (\%)}} \times 100$$

- Cytokine measurement in synovial fluid

The following cytokines were measured by ThermoFischer Scientific ELISA kits after appropriate SF dilutions: IL-1β (dilution 1:10, sensitivity 2.34 pg/ml), IL-8 (dilution 1:10, sensitivity 1.20 pg/ml), IL-10 (dilution 1:10, sensitivity 2.34 pg/ml), TGFβ (dilution 1:10, sensitivity 7.81 pg/ml).

In vitro study

- Cell culture and treatment

For the *in vitro* studies on phagocytosis, the immortalised monocyte-like cell line THP-1 (American Type Culture Collection®) was cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS). Cells were primed for 3 hrs with phorbol myristate acetate (PMA) (Merck) at 100 ng/mL and resuspended overnight with fresh medium supplemented with 10% FCS (10). The following day, the cells were stimulated with sterile CPP or MSU crystals (InVivo Gen, final concentration 0.025 mg/mL and 0.05 mg/mL, respectively) and cultured for 3 to 48 h depending on the experimental conditions. Where indicated, cytochalasin D (Merck) was added 30' before the stimulation with crystals. Cells incubated with medium alone served as controls. To exclude any contribution by endotoxin contamination, 10 mg/mL of polymyxin B (Sigma-Aldrich) was included in all the stimulation assays. All the materials were endotoxin free and suitable for cell culture. All the experiments were performed three independent times.

- IL-1β measurement

Cell culture supernatants from different experimental conditions were examined for extracellular IL-1β concentration using ThermoFischer Scientific ELISA kits (detection limit 4 pg/mL). Intracellular IL-1β was determined in lysates obtained after three freeze-thaw cycles and resuspended in phosphate buffer saline (11).

- Evaluation of CPP and MSU crystal phagocytosis

The phagocytosis of MSU and CPP crystals was assessed at different time points evaluating intracellular crystals under ordinary and polarised light microscopy (12). The percentage of cells with internalised crystals was calculated on the total number of examined cells and expressed as phagocytosis index.

$$P-index\ (\%) = \frac{N.\ of\ cells\ with\ internalised\ crystals}{\text{total N. of cells}} \times 100$$

Statistical analysis

Data in Table I are expressed as the mean and standard deviation (SD). Correlations in Table II were calculated using Spearman's correlation test. Experimental results are presented as mean ± SD of 3 independent experiments. Differences between stimulation and control were calculated using the non-parametric Mann-Whitney test. Anova for repeated measures with Tukey's Multiple Comparison Test was used to analyse variables variation over time. A p-value <0.05 was considered as significant.

Results

Synovial fluid characteristics

SF features and cytokine levels are outlined in Table I. All SFs were collected during an acute phase of the disease and all WBC were >2,000 cell/mm^3. As expected, no differences in inflammatory local indices were observed between patients with MSU or CPP in their SFs. Patients with acute CPP crystal arthritis were significantly older with respect to those with gout.

Relationship between SF phagocytosis index and local inflammatory indices

The 40 SFs analysed had various P-index values which were independent from the local inflammatory indices WBC and PMN percentage (Table II). By contrast, a strong positive correlation between SF-WBC and the percentage of PMN with crystals internalised (PMN-i) and a strong negative correlation between total SF-WBC and the percentage of M with crystals internalised (M-i) were observed (Table II; Fig. 1). The P-index showed a good positive correlation with IL-1ß and IL-8. Interestingly, IL-1β was the only cytokine, among those measured, showing a

Table I. Characteristics of the patients included in the study and their synovial fluid.

	TOT	MSU	CPP
Patients, n.	40	20	20
Age, years	74.5 10.9)	68.6 (9.9)	80 (8.8)*
WBC, n. cell/mm^3	17800 (14800)	13900 (12300)	21900 (15900)
PMN, %	76.9 (19.5)	77 (20.3)	79.5 (15.2)
M, %	23 (20.5)	24.1 (21.6)	19.4 (15.6)
P-index, %	20.8 (17.6)	21.1 (20.1)	19.5 (15.2)
PMN-i, %	73.9 (26.8)	71.2 (30.5)	79.1 (19.5)
M-i, %	26 (26.7)	28.6 (30.5)	20.8 (19.3)
IL-1ß, pg/ml	27.7 (28.9)	26.81 (26.4)	30.15 (32.27)
IL-8, pg/ml	652 (761.8)	818.8 (1006)	523.6 (538.1)
IL-10, pg/ml	31.9 (20.4)	37.88 (21.5)	27.24 (19.10)
TGFβ, pg/ml	574.8 (595.1)	659.8 (699.4)	518.1 (511.9)

M: monocytes; P-index: phagocytosis index; PMN: polymorphonuclear cells; WBC: white blood cell. *p<0.05 Mann-Whitney test.

Table II. Correlations between the P-index, PMN-i and M-i with local inflammatory indices.

	P-index	PMN-i	M-i
WBC	NS	<0.001 (+)	<0.001 (-)
PMN	NS	<0.001 (+)	<0.001 (-)
M	NS	<0.001 (-)	<0.001 (+)
P-index	-	NS	NS
IL-1ß	0.02 (+)	0.02 (+)	0.02 (-)
IL-8	0.013 (+)	NS	NS
TGFß	NS	NS	NS
IL-10	NS	NS	NS

M: monocytes; P-index: phagocytosis index; PMN: polymorphonuclear cells; WBC: white blood cell. Correlations calculated using Spearman's correlation test; (+) positive correlation; (-) negative correlation.

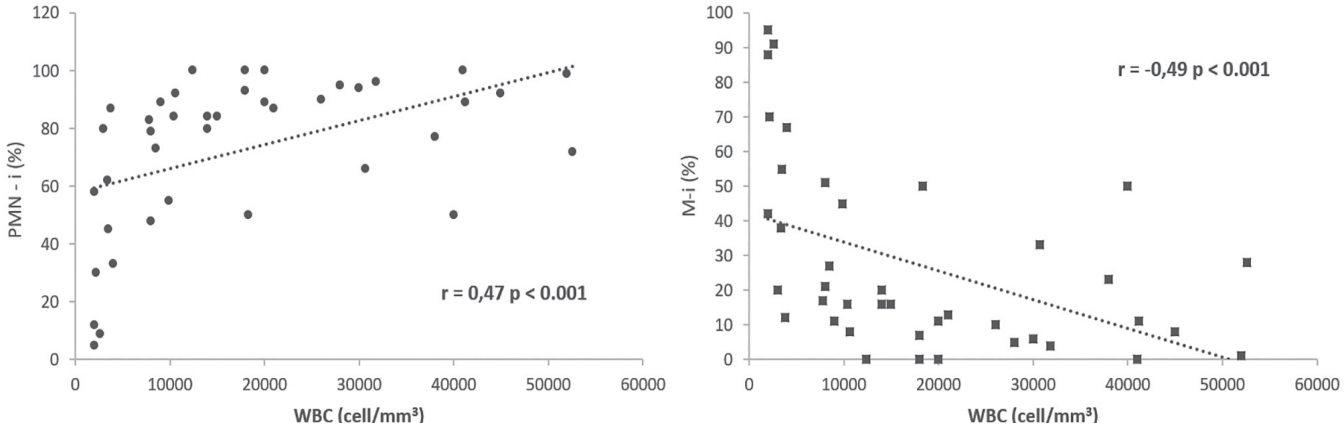


Fig. 1. Correlations between SF total white cell count and the percentage of cells with internalised crystals.
A positive correlation between SF-WBC and the percentage of PMN with crystals internalised (PMN-i) and a negative correlation between total SF-WBC and the percentage of M with crystals internalised (M-i) are shown. Significances calculated using Spearman's correlation test.

positive correlation with PMN-i and a negative one with M-i.

Cell viability

Preliminary experiments were conducted to assess the effect of crystals, PMA and cytochalasin D on cell viability using the Trypan Blue exclusion method. Cell stimulation with 0.025 mg/ml CPP and 0.05 mg/ml MSU crystals caused a slight decrease in cell death which was inferior to 20% (data not shown).

Effect of cell priming on crystal phagocytosis and IL-1ß production

After cell priming, CPP crystals induced a sustained intra- and extra-cellular release of IL-1ß which increased over time (Fig. 2A). The production of IL-1β under MSU crystal stimulation was reduced with respect to CPP and showed a peak after 6h (Fig. 2B).

Figure 3 A-B shows the time course of the phagocytosis index. The uptake of MSU crystals showed a maximum at 6h while after 48h most of the cells still had detectable CPP crystals (Fig. 3B).

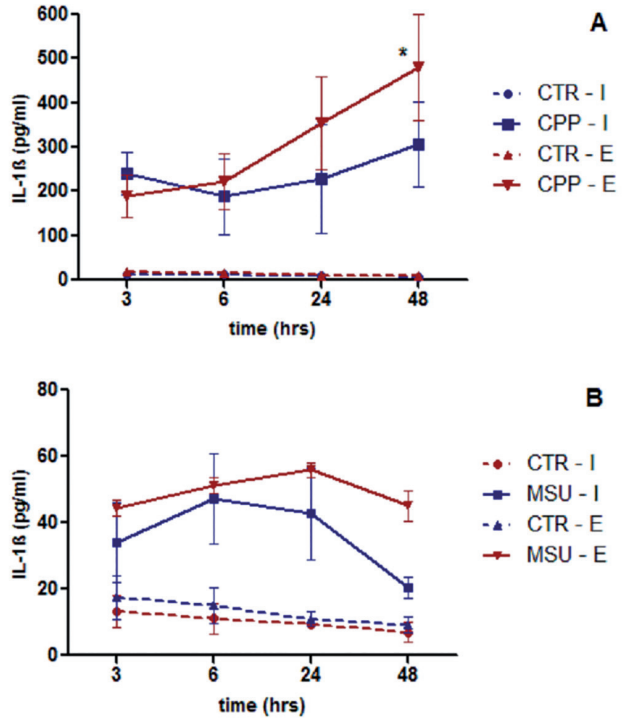


Fig. 2. Effect of priming on extracellular and intracellular IL-1ß induced by crystals.
Cells were primed with PMA 100 ng/mL for 3 h and left overnight in fresh medium. The cells were then treated with CPP 0.025 mg/ml (**A**) or MSU 0.05 mg/ml (**B**) for the indicated time. Extracellular (-E) and intracellular (-I) levels of IL-1β induced by crystals were evaluated using ELISA assays at the indicated time points. CPP-E: Anova for repeated measures 0.0042 and post hoc test **p*<0.05 48h *vs*. 3–6h.

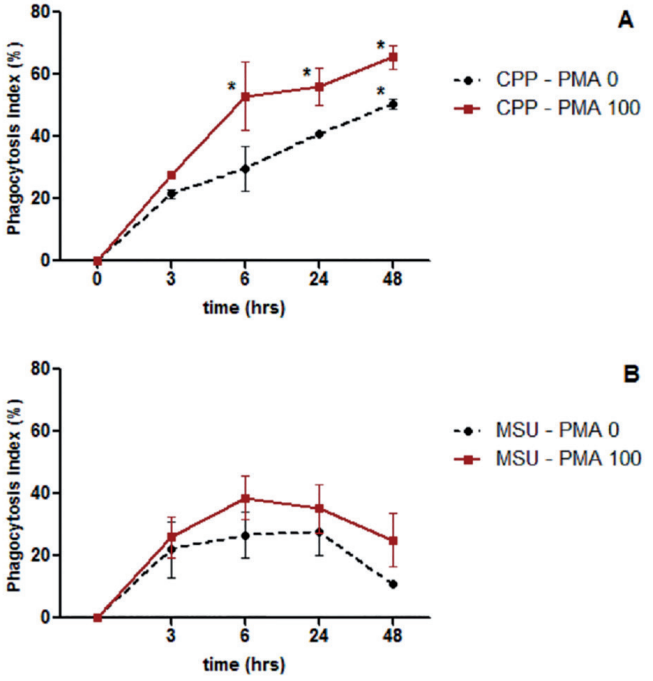


Fig. 3. Variation of the phagocytosis index over time.
Cells were stimulated with 0.025 mg/ml CPP crystals (**A**) or 0.05 mg/ml MSU crystal (**B**) after or in the absence of priming. Phagocytosis was evaluated using ordinary/polarised light microscopy assessing the presence of intracellular crystals at the indicated time points. Solid line: PMA 100ng/ml, dotted lines PMA 0 ng/ml.
CPP - PMA 100: Anova for repeated measures 0.002 and *post hoc* test **p*<0.05 48h, 24h and 6h *vs*. 3h. CPP-PMA 0: Anova for repeated measures 0.02 and post hoc test * *p*<0.05 48h *vs*. 3h.

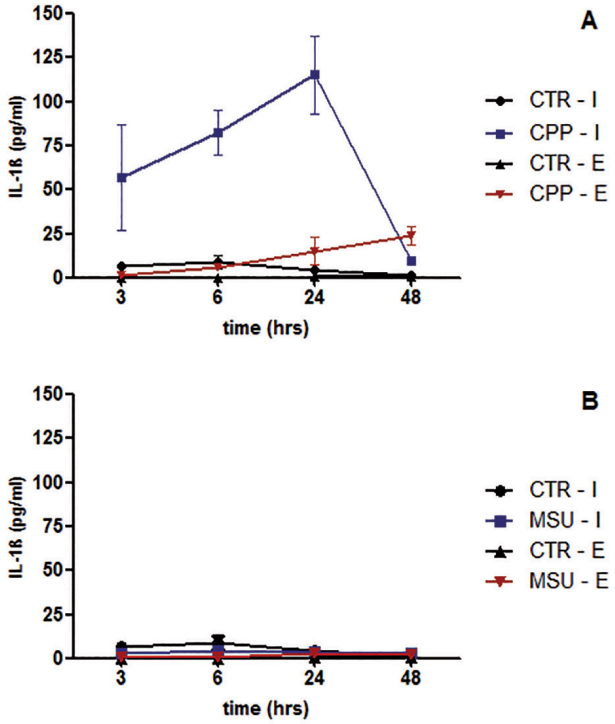


Fig. 4. IL-1β production induced by crystals in the absence of priming. The THP-1 cells were treated with CPP 0.025 mg/ml (**A**) or MSU 0.05 mg/ml (**B**) without previous activation with PMA for the indicated time. Extracellular (-E) and intracellular (-I) levels of IL-1β induced by crystals was evaluated at the indicated time points.

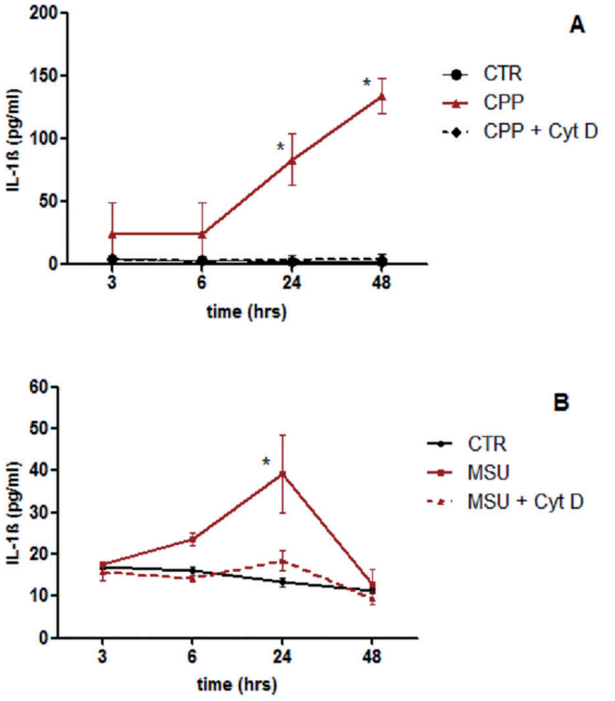


Fig. 5. Effect of cytochalasin D on IL-1β production induced by crystals. Cells were primed with PMA 100 ng/mL for 3 h and left overnight in fresh medium. The cells were then treated with cytochalasin D 1 μM for 30min and stimulated with 0.025 mg/ml CPP (**A**) or 0.05 mg/ml MSU (**B**) for the indicated time. Mann-Whitney test, **p*<0.05: CPP *vs*. CPP + CytD or MSU *vs*. MSU + CytD.

Crystal phagocytosis process in absence of cell priming

We then performed the same experiments described above without priming cells with PMA.

In the absence of cell priming, no IL-1β was observed in culture supernatants at the different time points (Fig. 4A-B). However, a consistent amount of IL-1ß was found intracellularly after cell stimulation with CPP (Fig. 4A).

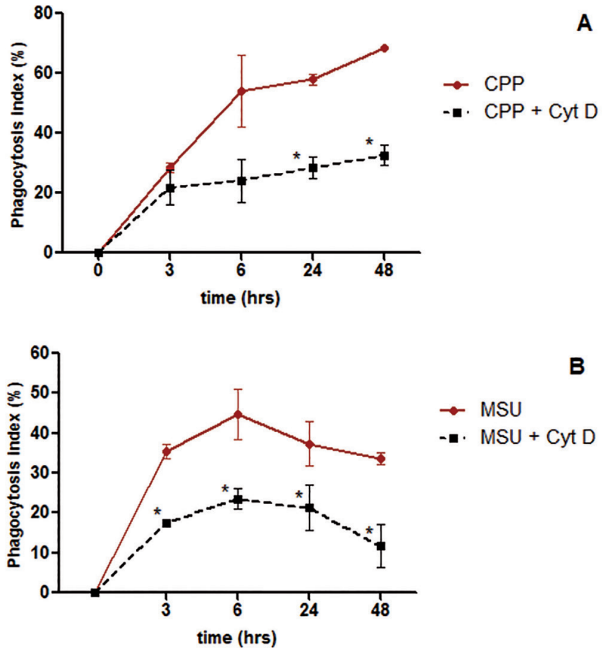
Although to a lesser extent than primed cells, unprimed cells showed a high degree of crystal phagocytosis (Fig. 3A-B).

Cytochalasin D inhibits crystal phagocytosis and IL-1 production

To assess if phagocytosis of crystals was required to trigger inflammation in the presence of priming, we pretreated cells with cytochalasin D, a potent inhibitor of actin polymerisation, 30 min before each experiment.

Cytochalasin D significantly inhibited both crystal phagocytosis and IL-1β extracellular release over time ($p<0.05$) (Fig. 5-6).

Fig. 6. Effect of cytochalasin D on the phagocytosis index.
Cells were primed with 100 ng/mL PMA for 3 h and left overnight in fresh medium. The cells were then treated with cytochalasin D 1 μM for 30 min and then stimulated with 0.025 mg/ml CPP (**A**) or 0.05 mg/ml MSU (**B**) for the indicated time.
The phagocytosis index was calculated for each time point as described in *Methods*.
Mann-Whitney test, **p*<0.05: CPP *vs*. CPP + CytD or MSU *vs*. MSU + CytD



Discussion

In this study we used fresh SFs obtained from patients with CIA to investigate the relationship between crystal phagocytosis and the local inflammatory indexes and a monocytic cell line to assess the effect of cell priming on crystal phagocytosis and IL-1β production. It is well known that SF collected from patients with CIA during an acute attack present inflammatory features such as high WBC number and PMN

percentage (2). The acute attack is characterised by a triggering phase, during which crystals are actively phagocytised by immune cells, and a resolution phase which occurs spontaneously thanks to the intervention of different endogenous factors. Among them, anti-inflammatory cytokines, lipoproteins, neutrophil extracellular traps and alpha-1-anti trypsin have demonstrated a crucial role in the self-limiting course of the disease (13). Investigating the levels of various cytokines during the different stages of acute gout, we previously showed a decreased SF WBC count, IL-1β, TNF-α, IL-6, and IL-8 and increased TGFß levels in the late stage of the acute attack (3). It has been observed that crystal phagocytosis precedes IL-1β in different cellular models of MSU crystal-induced inflammation (14-15) but no studies have so far investigated the relationship between crystal phagocytosis, the degree of inflammation and the resolution of the inflammatory process in fresh SF. Our study included 20 SFs of patients with gout and 20 with acute CPP arthritis at different stages of the acute attack as shown by the wide dispersion of WBC count and PMN percentage standard deviation reported in Table I. In these fluids we calculated the P-index using polarised light microscopy (12). Given the weak birefringence exhibited by most CPP crystals, the search for those crystals was simultaneously performed under ordinary light (16). We observed that the P-index calculated on the total cell count was independent from the inflammatory local indices such as WBC and the percentage of PMN cells. Highly inflammatory SFs could in fact show both low and high P-index. By contrast, the percentage of PMN cells with internalised crystals (PMNi) calculated on the total P-index, showed a significant positive correlation with cellular inflammatory indices (WBC and PMN) and IL-1β and a negative correlation with the percentage of monocytes. In parallel, the percentage of monocytes with internalised crystals (Mi), calculated on the total P-index, showed a negative significant correlation with cellular inflammatory indices (WBC and PMN) and IL-1β levels and a positive correlation with the percentage of M. These results confirm the role of PMN and M in the early and resolution phases of crystal-induced inflammation, respectively, through a direct observation on SF. Given the lack of correlation between the P-index and the local classical inflammatory indices (WBC and PMN) in SF, we carried out an *in vitro* study to investigate whether the phagocytosis process could depend on cell priming which, both in the *in vivo* and *in vitro* setting, triggers inflammation. We observed that, under priming condition, cells phagocyte crystals inducing IL-1β in a time-dependent manner and that the levels of IL-1β released in the extracellular space varied importantly according to the crystal type and its concentration, with CPP crystals inducing a more sustained degree of inflammation over time. The *in vitro* P-index also differed according to the nature of the crystals and was higher for CPP crystals. Of interest, we noticed that in absence of priming, cells were still capable to phagocytise crystals but without inducing relevant levels of IL-1β. As opposite to MSU, CPP crystals induced an intracellular production of IL-1ß in those settings. Although the underlying mechanism has to be investigated more in depth, the different results obtained by MSU and CPP might be explained by the different chemical nature of the crystals which lead to specific inflammatory potentials (17).

Cell priming is a necessary condition for pathogenetic crystals to induce IL-1β and the inflammatory process. *In vivo*, free fatty acids (18), SF proteins (4), complement (19) and uric acid (20) have been described to act as first or co-stimulatory signals in crystal-induced inflammation. Once activated, cells produce IL-1β through the assembly of NLRP3 and caspase-1 activation (21). In the absence of priming, cells are not able to induce IL-1β release but, as demonstrated here, they retain their phagocytic capacity. We therefore assessed if the process of phagocytosis was indispensable for IL-1β production. To address this question cytochalasin D, an inhibitor of phagocytosis (22), was added to the cultures and IL-1β levels and P-index was evaluated. At the non-toxic concentration of 1 μM, cytochalasin blocked phagocytosis and IL-1ß production. It has been recently observed that a glycoprotein produced by synovial fibroblasts, proteoglycan-4, is capable to inhibit MSU crystal uptake by macrophages resulting in a significantly reduced expression and production of IL-1β, and other pro-inflammatory cytokines (15).

Consequently, if phagocytosis is an indispensable condition for inducing the initial steps of inflammation, it proves fundamental also in the resolution of the acute attack. Whether this process is due to the involvement of natural locally produced phagocytosis-inhibiting factors or to a shift in the capacity of macrophage to respond to crystals, represents a matter of future investigation. Certainly, the non-inflammatory phagocytosis of pathogenic crystals could represent a mechanism which clear the crystals from the environment and limit the ongoing inflammatory process. The lack of correlation between the total P-index and the degree of inflammation observed in SF of the patients supports this hypothesis and reinforces the crucial role of crystal non-inflammatory phagocytosis. Furthermore, this mechanism might also account for the autoinflammatory features of crystal diseases. In conclusion, phagocytosis is an indispensable condition in triggering the inflammatory response to crystals but, according to the results of this study, it demonstrates also a crucial role in limiting inflammation and the acute attack.

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