The deposition of crystals into the joints, where it can cause inflammation, is a relatively common phenomenon. Gout is the most prevalent crystal-induced arthritis and is characterized by the presence of monosodium urate (MSU) crystals in the synovial fluid (SF). The deposition of these crystals in articular and periarticular tissues triggers a series of cellular events that lead to a massive release of various inflammatory mediators, in particular interleukin (IL)-1β. Although many studies have been conducted on this type of inflammation, the molecular mechanisms involved remain unclear. It has been shown that innate immunity plays an important role in these processes. Indeed, MSU crystals are potent inducers of the NALP3 inflammasome, a cytoplasmic protein complex that mediates the caspase-1-dependent activation of IL-1β (3). However, it has emerged that MSU crystals alone cannot induce IL-1β production and that a second trigger is needed for the expression of pro-IL-1β (4). Indeed, IL-1β production is controlled by four steps: transcript expression, pro-IL-1β production, maturation to active IL-1β, and secretion. It has been proposed that, in crystal-induced inflammation, the first step is triggered by stimuli inducing nuclear factor kappa B (NFκB) activation, while maturation and secretion are due to NLRP3 inflammasome activation mediated by MSU crystals.

As the SF is the medium in which crystals mainly exert their inflammatory potential, many studies have been conducted to identify substances in SF that may modulate crystal-induced inflammation. For instance, apolipoprotein (apo)AI, apoB, apoE, and high-density lipoprotein (HDL) contribute to the resolution of acute inflammatory attacks induced by MSU crystals in vitro and in vivo models (5–9), while complement fractions, such as C1q, C5, and C6, as well as immunoglobulin (Ig)G, IgM, and Ig Fe fragments favour crystal phagocytosis and cell activation in vitro (10–14). More recently, various endogenous priming signals have been suggested for the initiation of MSU-induced IL-1β production, such as free fatty acids, myeloid-related proteins...
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(MRP)-8 and MRP-14, and complement C5a (15–17). All these compounds are likely to be present in SF. However, the direct effects of SF on the production of IL-1β in MSU crystal-activated macrophages have not been assessed. Here we describe for the first time the effects of whole and fractionated SF on MSU crystal-induced cytokine production.

Method

Human materials

Human SF from patients and blood from healthy volunteers were obtained with the approval of the Institutional Review Board of the University of Padova, which approved the study. An informed consent form was signed by the patients and the volunteers.

Preparation of crystals

MSU crystals were prepared as described previously (8), according to Denko and Whitehouse methods (18). Crystals were sterilized by heating at 180°C for 2 h before each experiment and were determined to be free from endotoxin by the Limulus amebocyte lysate assay. (E-toxate kit, Sigma-Aldrich, St. Louis, MO, USA).

Collection and analysis of SF

SF was collected by arthrocentesis from the knees of 16 untreated patients: four with non-inflammatory arthritis and 12 with inflammatory arthritis. The SF was examined under optical light microscopy as described previously (2). In brief, the total white cell count (WBC) was measured using a standard haematological counting chamber and a differential cell count was determined by May–Grünwald–Giemsa staining. MSU crystals were identified by ordinary and polarized light microscopy. After examination, SF samples were centrifuged at 1500 rpm for 30 min (ALC PK 130 centrifuge, rotor No. T535) to remove the cells, particulate material, and debris.

SF delipidation and subfractionation

SF free of cells, particulate material, and debris were diluted 1:1 in phosphate-buffered saline (PBS) and adjusted to d = 1.23 g/mL by the addition of solid NaBr and centrifuged for 45 h at 50 000 rpm (19). Two fractions were obtained: the upper fraction containing lipids and lipoproteins (LF) and the lower fraction containing proteins (PF). Ultracentrifugation was carried out at 4°C using a Beckman MLA-55 rotor (Beckman Instruments), and the recovered lipid and protein fractions were then dialysed against PBS. Thereafter, PF was subfractionated by ultrafiltration through microconcentrators (Millipore, Darmstadt, Germany) according to different molecular weight (MW) cut-offs: PF1 > 50 kDa; PF2 30–50 kDa; and PF3 < 30 kDa. PFs were tested for their protein concentrations by the method of Bradford (20).

Cells

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Monocytes/macrophages were isolated from the peripheral blood of three healthy volunteers by density gradient centrifugation with Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) and differential adhesion (21). Neutrophils were isolated from peripheral blood from healthy volunteers by density gradient centrifugation (sedimentation) with Histopaque 1077 and hypotonic lysis of red blood cells.

Cytokine production

THP-1 cells or monocytes/macrophages (5 × 10⁴ cells/well/200 µL) were stimulated for the indicated time periods by MSU crystals (0.5 mg/mL) or nigericin (2.5 µM) (Sigma-Aldrich, St.Louis, MO, USA) in RPMI 1640 supplemented with 50 µg/mL streptomycin, 50 U/mL penicillin, 2 mmol/L glutamine, and 5 µg/mL polymyxin (medium). SF was added together with or 5 h before stimulation by MSU crystals. Alternatively, LF (5%) or PFs at the same total protein concentration as the corresponding SF (1.47–3.07 mg/mL, final concentration) were used instead of SF. In some experimental sets, THP-1 cells or MSU crystals were pretreated for 1 h with PF, and then the medium was replaced by a medium without PF. In additional experiments, albumin (0.1 mg/mL), haptoglobin (0.1 mg/mL), or fibrinogen (1 mg/mL) was used as the stimulus. Culture supernatants were tested by an enzyme-linked immunosorbent assay (ELISA) for the production of IL-1β and IL-8 (eBioscience, San Diego, CA, USA). Cell viability was assessed by trypan blue exclusion staining and the MTT assay (Millipore, Darmstadt, Germany) (8).

Chemotaxis assay

The chemotactic activity of supernatants of THP-1 cells activated by MSU crystals and SF on neutrophils was assessed by using a 48-well modified Boyden chamber (AC48; Neuroprobe, Gaithersburg, MD, USA) (8). RPMI was used as a negative control and 10 ng/mL IL-8 (PeproTech, Ricky Hill, NJ, USA) was used as a positive control. A polyvinylpyrrolidone-free polycarbonate 8-mm membrane with 3-µm pores, pretreated with 10 µg/mL fibronectin, was placed between the chambers. In brief, 28-µL aliquots of cell culture supernatants were dispensed into the bottom wells of the chamber. Fifty microlitres of neutrophils resuspended in medium (3 × 10⁴ cells/mL) were added to the top wells. The chambers were incubated at 37°C with 5% CO₂ for 45 min. The membrane
was then removed, washed with PBS on the upper side, fixed, and stained with DiffQuik (Baxter Scientific, Round Lake, IL, USA). Cells were counted microscopically at 1000× magnification in four fields per membrane and the results are expressed as the number of migrated neutrophils. All assays were performed in duplicate.

Quantitative real-time polymerase chain reaction (qPCR)
THP-1 cells (1.5 × 10⁶ cells/well/3 mL) were cultured in six-well plates with the indicated stimulus for the indicated time periods. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and subjected to quantitative real-time duplex PCR analysis (TaqMan 7300 quantitative real-time PCR system, Applied Biosystems), which was conducted after reverse transcription by SuperScript II (Invitrogen, Carlsbad, CA, USA), as described previously (22). The levels of mRNA expression were normalized with the expression of a housekeeping gene (18S) analysed simultaneously with the IL-1β transcript. IL-1β and 18S labelled probes were purchased from Applied Biosystems. All measurements were conducted in triplicate.

Statistics
When required, data significance was assessed by a one-way analysis of variance (ANOVA) followed by Dunnett’s test; p < 0.05 was considered significant.

Results

SF characteristics and analysis
As expected, in SF from patients with non-inflammatory arthritis, that is osteoarthritis (OA), the WBC was < 500 cells/mm³ and comprised < 5% neutrophils (Table 1). By contrast, in SF from patients with inflammatory arthritis (SFi), the WBC was > 5000 cells/mm³ with > 40% neutrophils. These results confirmed the patients’ diagnoses.

SFi synergizes with MSU crystals to induce inflammation
MSU crystals did not induce the release of IL-1β in THP-1 cells whereas they did induce the secretion of IL-8 in a dose-dependent manner (Figure 1A). When THP-1 cells were treated with SFi, IL-1β production was not observed whereas that of IL-8 remained low (Figure 1B). In the presence of both MSU crystals and SFi, IL-1β production was induced and IL-8 secretion enhanced (Figure 1B). MSU crystals alone or in the presence of SF did not significantly affect cell viability (see Supplementary Figure S1). These results suggest that the presence of both MSU crystals and SF was required to induce large amounts of cytokine production. SF from OA patients per se did not induce the production of IL-1β or IL-8, the concentrations of the latter in culture supernatants remaining under the detection limit (Figures 2A and 2B). When added together, SF from OA patients and MSU crystals induced the production of IL-8 in THP-1 cells, but not the production of IL-1β, which required the presence of SFi, that is SF from patients with inflammatory arthritis (Figures 2A and 2B). Of note, the production of IL-8 was enhanced to higher levels in the presence of SFi than with SF from OA patients. These results suggest the presence in SFi of factor(s) able to synergize with MSU crystals to induce IL-1β production and enhance that of IL-8.
There was no relationship between SFi cell counts, that is either WBC, neutrophils, macrophages, or lymphocytes (Table 1), and the ability of SF to synergize with MSU crystals to trigger IL-1β and IL-8 production in THP-1 cells (Figures 2A and 2B), suggesting that the canonical way to characterize the degree of inflammation of arthritic joints was not related to the ability of SF to synergize with MSU crystals. By contrast, supernatants of THP-1 cells
treated with both SF and MSU crystals induced the migration of neutrophils (Figure 2C), which corroborated with the production of IL-8 (Figure 2D).

Usually, in in-vitro studies, THP-1 cells are pre-activated for different time periods with either the Toll-like receptor (TLR)-4 agonist lipopolysaccharide (LPS) or phorbol myristate acetate (PMA) before treatment with MSU crystals to trigger substantial IL-1β release (3, 17, 23). To evaluate whether pretreatment of cells with SF induced a higher release of cytokines than co-stimulation, THP-1 cells were incubated with SF for 5 h before the addition of MSU crystals. As shown in Figure 2E, the production of IL-1β induced by MSU crystals was not affected by SF pretreatment, while IL-8 release tended to be more pronounced when cells were pretreated with SF (Figure 2F), in accordance with the premise that SF alone induced IL-8 secretion (Figure 1B). Of interest, the latter effect was not observed upon OA SF pretreatment, which did not change the levels of IL-8 production induced by simultaneous addition of both SF and MSU crystals.

Because THP-1 cells may respond differently over time, these results were confirmed using primary human monocytes/macrophages to demonstrate the synergism between SF and MSU crystals. Exposure of primary monocytes/macrophages to different crystal concentrations induced low production of IL-1β at high MSU crystal concentrations, but clearly triggered IL-8 secretion. The concentrations of both cytokines reached peak levels at 0.5 mg/mL MSU crystals (Figures 3A and 3B). At these MSU crystal concentration, cell viability was not affected (see Supplementary Figure S2). When monocytes/macrophages were activated by MSU crystals in the presence of SF, similarly to THP-1 cells, SF but not OA SF increased the release of IL-1β and IL-8 (Figures 3C and 3D), thus confirming that THP-1 cells behaved similarly to primary human cells.

To determine whether the synergistic effects of SF were directed only to crystals or may synergize with other NLRP3 activators, we used nigericin, a soluble NLRP3 inflammasome activator (24), to activate THP-1 cells together with SF. As expected, IL-1β release was induced by co-stimulation of THP-1 cells with both nigericin and SF (Figure 4A). Of note, OA SF did not affect IL-1β production in the presence of nigericin. By contrast, nigericin increased the production of IL-8 in the presence of all SF (Figure 4B). These data suggest that SF alone was able to prime THP-1 cells for the production of IL-1β and contributed to enhancing the secretion of IL-8.

SFI contains proteins that synergize with MSU crystals to induce IL-1β and IL-8 release

As free fatty acids were shown to cooperate with MSU crystals to induce IL-1β (15), SF was subjected to fractionation into the lipid and protein fractions. Surprisingly, PF but not LF of SF from patients with inflammatory arthritis synergized with MSU crystals to induce IL-1β and IL-8 release to levels comparable to whole SF (Figure 5A). As with the whole SF, PF or LF alone induced a low release of IL-8 but did not stimulate IL-1β production (Figure 5A).
Figure 2. Effects of MSU crystals and different SF samples on the production of IL-1β and IL-8 in THP-1 cells. THP-1 cells were treated for 24 h with or without (−) SF from patients described in Table 1 in the presence (grey columns) or absence of MSU (white columns). Supernatants were measured for (A) IL-1β and (B) IL-8 production and (C) tested for their ability to induce neutrophil migration; in the positive control (10 ng/mL IL-8), 628 ± 14.42 migrating neutrophils were counted. (D) Correlation between IL-8 production and neutrophil migration; SF was from OA patients (open circles) and SFi (closed circles). Alternatively, THP-1 cells were pretreated (Ptt) for 5 h with SF, and then stimulated for 24 h with MSU crystals. Supernatants were measured for (E) IL-1β and (F) IL-8 production. Experiments were carried out in triplicate and expressed as mean ± standard deviation.
To determine whether cytokine induction was due to the formation of complexes between SF proteins and MSU crystals or to a direct effect on THP-1 cells, as suggested by the results of Figure 4, cells or crystals were pretreated with PF for 1 h before stimulation by MSU crystals. As shown in Figure 5B, the pretreatment of THP-1 cells but not that of MSU crystals with proteins (PF) of SFi induced the release of IL-1β and increased that of IL-8. These results demonstrate that the PF of SFi contained activating factors that acted directly on macrophages and synergized with MSU crystals to induce pro-inflammatory cytokines.

Co-stimulation of THP-1 cells by SFi and MSU crystals induces the expression of IL-1β mRNA

Because IL-1β production requires two signals, one to induce transcript expression and another to trigger IL-1β maturation and secretion (25), we then determined whether the triggering of IL-1β release in co-stimulated cells would be accompanied by changes in IL-1β mRNA levels. As shown in Figure 6A, IL-1β transcript levels were maximal after 8 h of activation by SFi and MSU crystals. Unexpectedly, neither SFi nor MSU crystals were able to induce the expression of IL-1β mRNA, which required the presence of both activators (Figure 6B). Similar levels of IL-1β transcript were induced when THP-1 cells were co-activated by MSU crystals and either SFi or their PF (Figure 6B). Of note, PF isolated from the SF of OA patient no. 4 was not able to synergize with MSU crystals to induce IL-1β mRNA expression (Figure 6B). These results suggest that MSU crystals and SFi proteins were both required to induce IL-1β transcript expression.

SFi proteins with MW > 50 kDa synergize with MSU crystals to trigger IL-1β production

To better characterize the activating factor(s), the PFs of SFi were subfractionated according to their MW by serial ultrafiltration. Stimulation of THP-1 cells with subfractions of PFs in the presence of MSU crystals revealed that the activating activity was contained in fraction PF1, that is in the fraction containing proteins with MW > 50 kDa (Figure 7A). PF2 and PF3 did not display activating activity towards either IL-1β or IL-8 (Figure 7A). These data demonstrate that the factor that synergized with MSU crystals was likely to be a protein with MW > 50 kDa.

As the amount of SF did not allow further fractionation, we assessed the activity of three of the highest abundant proteins in SF displaying MW > 50 kDa, namely albumin (MW = 66.5 kDa), haptoglobin (MW = 100 kDa) (26), and fibrinogen (MW = 340 kDa) (27, 28). Co-stimulation of THP-1 cells with MSU crystals...
and IL-8 in THP-1 cells. THP-1 cells were treated for and (B) IL-8 production.

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in the production (β) of C5a receptor, induces pro-IL-1β production (16), although long-chain fatty acids may display inhibitory activity (33). Lipoproteins that are contained in the LF may also play a modulatory role. For instance, HDLs might play a part in modulating the inflammatory potential of MSU crystals. Indeed, SF contains HDLs (34), which decrease the inflammatory response induced by MSU crystals by reducing the production and expression of inflammatory mediators, such as IL-1β (9).

Our study also demonstrates that SFi proteins act directly on macrophages and do not act through adsorption on the crystal surfaces, as documented previously for various plasma proteins (10–14). Indeed, co-stimulation of cells with SF and nigericin increased the release of both IL-1β and IL-8, and pretreatment of MSU crystals with PFs did not affect MSU crystal activity. More recently, different studies have demonstrated that endogenous priming signals may synergize with MSU crystals to activate inflammatory pathways through interaction with specific receptors expressed on cells. MRP-8 and MRP-14, through their binding with TLR4, enhance MSU crystal-induced IL-1β secretion by triggering pro-IL-1β expression. Nevertheless, in-vivo experiments revealed that the absence of MRPs causes only a partial decrease in IL-1β levels, indicating that additional factors are involved in these inflammatory processes (16). In addition, the complement component C5a, by interacting with the C5a receptor, induces pro-IL-1β through a pathway independent of NLRP3, priming cells for MSU crystal-induced inflammasome activation (17). Because all these molecules are able to induce low but significant IL-1β levels even without MSU crystals (16, 17), they are probably not involved in the SFi effects observed here. They might, however, cooperate with fibrinogen or fibrinogen degradation products that synergize with MSU crystals to induce IL-1β production. Fibrinogen also cooperates with calcium pyrophosphate crystals to induce IL-1β.

Discussion

This is the first study to evaluate the direct effect of SF on crystal-induced inflammation. SFi, but not OA SF, synergized with MSU crystals to induce IL-1β production. The main finding of the study is that SFi proteins and not lipids or lipoproteins synergize with MSU crystals to induce IL-1β production in macrophages and that these SFi proteins display a MW > 50 kDa. Finally, fibrinogen might be part of the SFi proteins that synergize with MSU crystals.

Consistent with previous studies, our results demonstrate that exposure of THP-1 cells or primary monocytes/macrophages to MSU crystals alone did not induce significant production of IL-1β. Indeed, priming of THP-1 cells with LPS or PMA before crystal stimulation was shown to be required in IL-1β production by MSU crystals (3, 4, 23, 29–32). Notably, SFi does not ‘prime’ macrophages because it does not per se induce IL-1β mRNA expression, and pretreatment of cells with SFi did not enhanced IL-1β production induced by MSU crystals and SFi when added to the cells simultaneously. In addition to the release of IL-1β, co-stimulation with SFi and MSU crystals enhances the production of the chemokine IL-8, which increases the migration of neutrophils observed in vitro in the chemotaxis assay. However, there was no correlation between the synergizing activity of SFi and their content in WBC or neutrophils, that is their inflammatory degree, suggesting that regulatory mechanisms in the inflamed joints may modulate IL-8 production and neutrophil migration.

We identified the presence of activating factors in PFs of SFi. Indeed, proteins isolated by delipidation of SFi display, in combination with MSU crystals, a high pro-inflammatory activity, that was not observed with LFs derived from the same SFi. This contrasts with previous studies that suggest, based on in-vitro experiments, that long-chain free fatty acids might synergize with MSU crystals to induce IL-1β production (15), although short-chain fatty acids may display inhibitory activity (33). Lipoproteins that are contained in the LF may also play a modulatory role. For instance, HDLs might play a part in modulating the inflammatory potential of MSU crystals. Indeed, SF contains HDLs (34), which decrease the inflammatory response induced by MSU crystals by reducing the production and expression of inflammatory mediators, such as IL-1β (9).

Our study also demonstrates that SFi proteins act directly on macrophages and do not act through adsorption on the crystal surfaces, as documented previously for various plasma proteins (10–14). Indeed, co-stimulation of cells with SF and nigericin increased the release of both IL-1β and IL-8, and pretreatment of MSU crystals with PFs did not affect MSU crystal activity. More recently, different studies have demonstrated that endogenous priming signals may synergize with MSU crystals to activate inflammatory pathways through interaction with specific receptors expressed on cells. MRP-8 and MRP-14, through their binding with TLR4, enhance MSU crystal-induced IL-1β secretion by triggering pro-IL-1β expression. Nevertheless, in-vivo experiments revealed that the absence of MRPs causes only a partial decrease in IL-1β levels, indicating that additional factors are involved in these inflammatory processes (16). In addition, the complement component C5a, by interacting with the C5a receptor, induces pro-IL-1β through a pathway independent of NLRP3, priming cells for MSU crystal-induced inflammasome activation (17). Because all these molecules are able to induce low but significant IL-1β levels even without MSU crystals (16, 17), they are probably not involved in the SFi effects observed here. They might, however, cooperate with fibrinogen or fibrinogen degradation products that synergize with MSU crystals to induce IL-1β production. Fibrinogen also cooperates with calcium pyrophosphate crystals to induce IL-1β.

Figure 4. Effects of nigericin and different SF samples on the production of IL-1β and IL-8 in THP-1 cells. THP-1 cells were treated for 24 h with or without (~) SF from patients described in Table 1 in the presence (grey columns) or absence of nigericin (white columns). Supernatants were measured for (A) IL-1β and (B) IL-8 production. Experiments were carried out in triplicate and the results expressed as mean ± standard deviation.

Figure 7B
Indeed, fibrinogen enhances the inflammatory action of calcium pyrophosphate crystals in THP-1 cells primed with low doses of PMA (35). Here we show that priming of THP-1 cells is not required for the induction of IL-1β by SFi and MSU crystals. In our study, although the identity of the SF activating factor(s) is still elusive, it is likely that fibrinogen is involved in macrophage activation. Indeed, fibrinogen and its degradation products, all displaying MW > 50 kDa, are highly abundant in SFi proteins (27, 28, 36–38). We thus hypothesized that fibrinogen is a prominent candidate for driving crystal-induced inflammation. Fibrinogen is a plasma glycoprotein that plays a key role in blood clotting. It is an acute phase protein and its concentration in SF seems to correlate with the degree of inflammation present.

Figure 5. The protein fractions of SF contain the activating factor(s). (A) THP-1 cells were activated for 24 h with SF, lipid fraction (LF), or protein fraction (PF) prepared from SF in the presence or absence of MSU crystals. (B) THP-1 cells were pretreated for 1 h with PF (PF-THP-1) from SF of patients 8, 10, and 15 (Table 1), washed, and then cultured for 24 h. Alternatively, MSU crystals were incubated for 1 h with PF (PF-MSU) from the same SF, and then used to stimulate THP-1 cells for 24 h. IL-1β and IL-8 were measured in harvested supernatants. Experiments were carried out in triplicate and the results expressed as mean ± standard deviation. The production of IL-8 was 35 ± 13 pg/mL and 212 ± 32 pg/mL in unstimulated and MSU crystal-stimulated THP-1 cells, respectively. Both basal IL-1β production and MSU crystal-induced IL-1β production were below the detection limit.

Figure 6. Both MSU crystals and SF are required to induce IL-1β mRNA expression. (A) THP-1 cells were activated with MSU crystals and SFi from patient 15 (Table 1) for the indicated time. (B) THP-1 cells were treated for 8 h with SF or PF from the indicated patient in the presence (+ MSU) or absence (− MSU) of MSU crystals. Total RNA was isolated and the IL-1β transcript was measured by qPCR. Experiments were carried out in triplicate and the results expressed as mean ± standard deviation.
It is known that fibrinogen can display immunoregulatory functions at sites of inflammation and can stimulate cytokine production in macrophages through TLR4 ligation (40). The present study shows that co-stimulation of THP-1 cells with MSU crystals and fibrinogen induced the production of IL-1β and increased that of IL-8 in the absence of PMA. Under the same experimental conditions, fibrinogen alone induced the release of IL-8, but not of IL-1β, similar to SF and PF although to a smaller extent.

In conclusion, this study demonstrates that SFi proteins with MW > 50 kDa contribute to MSU crystal-induced inflammation in gout by acting directly on macrophages and that fibrinogen may contribute to initiate gouty inflammation. However, further studies are needed to better identify which SF proteins are specifically involved in MSU crystal-induced inflammation and which signal transduction pathways they activate.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: MSU crystals alone or in the presence of SF did not significantly affect THP-1 cell viability.

Figure S2: Increasing concentrations of MSU crystals did not significantly affect the viability of primary monocytes/macrophages.

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