Safe Disposal of Inflammatory Monosodium Urate Monohydrate Crystals by Differentiated Macrophages

R. Clive Landis, Darshna R. Yagnik, Oliver Florey, Pandelis Philippidis, Vivien Emons, Justin C. Mason, and Dorian O. Haskard

Objective. Although monosodium urate monohydrate (MSU) crystals have been recognized since the 18th century as the etiologic agent of gout, it is still unknown why certain hyperuricemic individuals remain asymptomatic, and how an acute attack of gout spontaneously resolves. We hypothesized that mononuclear phagocytes hold the key to these questions, and that the state of monocyte/macrophage differentiation is critical.

Methods. Human peripheral blood monocytes were differentiated for 1–7 days in vitro and examined with respect to 1) uptake of MSU crystals, 2) expression of macrophage, dendritic cell, and activation markers, 3) secretion of tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), IL-6, and IL-10, 4) activation of endothelial E-selectin expression, and 5) enhancement of secondary neutrophil recruitment by endothelial cells.

Results. MSU crystals induced TNFα, IL-1β, and IL-6 (but not IL-10) secretion in undifferentiated monocytes, which in turn promoted endothelial cell E-selectin expression and secondary neutrophil capture under shear flow. In contrast, differentiation over 3–5 days led to development of a noninflammatory phenotype characterized by a lack of proinflammatory cytokine secretion, lack of endothelial cell activation, and lack of secondary neutrophil recruitment. Acquisition of the noninflammatory phenotype correlated with expression of macrophage antigen but not with expression of dendritic cell marker or activation marker. Monocytes and macrophages were similarly phagocytic, and a control particle, zymosan, elicited secretion of the full panel of cytokines in both cell types. However, coincubation with MSU led to a significant suppression of zymosan-induced TNFα secretion (P = 0.009) from macrophages but not monocytes.

Conclusion. These findings imply that differentiated macrophages provide a safe-disposal mechanism for the removal of inflammatory urate crystals. This may be of clinical relevance to the maintenance of asymptomatic hyperuricemia and the resolution of acute gout.

It has long been recognized that hyperuricemia does not inevitably lead to acute gout (1). In patients with gout, monosodium urate monohydrate (MSU) crystals are readily detectable in asymptomatic joints (2) and during the intercritical phase (3). In contrast with joints of patients with acute gout, which have intense neutrophilic infiltration of the synovium, asymptomatic joints of patients with gout have a predominantly mononuclear cell infiltrate (4). Moreover, crystals can be detected within the cytoplasm of mononuclear cells in the absence of symptomatic gout (5). These observations suggest that cells of the monocyte/macrophage lineage might play a beneficial role in maintaining asymptomatic hyperuricemia.

A beneficial role of monocyte/macrophages, however, is difficult to reconcile with the fact that monocytes, when challenged with urate crystals, secrete tumor necrosis factor α (TNFα) (6,7), interleukin 1β (IL-1β) (8), IL-6 (9), and IL-8 (10). This in turn activates expression of endothelial cell adhesion molecules (E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1), which leads to secondary neutrophil recruitment to sites of crystal deposition (11–13). In order to resolve these apparently disparate
observations, we hypothesized that the state of differentiation of mononuclear phagocytes may determine how they respond to urate crystal uptake. Evidence in support of this idea has been demonstrated in a study of mouse monocyte/macrophage cell lines, which showed that incompletely differentiated monocytic cell lines synthesized TNFα and activated endothelial cells upon challenge with urate crystals, whereas well-differentiated macrophagic lines did not (14).

Here, we extend this hypothesis to humans and demonstrate that, in contrast to freshly isolated monocytes, in vitro–differentiated macrophages can ingest MSU crystals in the absence of concomitant proinflammatory cytokine synthesis, endothelial cell activation, or secondary neutrophil recruitment. This suggests that differentiated macrophages may be involved in maintaining asymptomatic hyperuricemia and in resolving acute attacks of gout.

MATERIALS AND METHODS

Reagents and antibodies. Uric acid and unopsonized zymosan were purchased from Sigma-Aldrich (Poole, UK). Anti-CD1a, anti-CD14, anti-CD16, anti-CD33, anti-CD68, anti-CD71, anti-CD83, and anti-CD163 primary antibodies were purchased from BD PharMingen (Oxford, UK). Anti-CD36 (monoclonal antibody [mAb] SM4), anti-CD64 (mAb 10.1), and anti-CD11b (mAb 44) were generously provided by Dr. Nancy Hogg (Cancer Research UK, London, UK). Anti–E-selectin mAb 1.2B6 was generated within our group as previously described (15). Antibody RFD7 (macrophage antigen) and fluorescein isothiocyanate–conjugated F(ab’)2 goat antimouse IgG secondary antibody were purchased from Serotec (Oxford, UK).

MSU crystal preparation. MSU crystals were prepared by recrystallization from uric acid, as previously described (13). All glassware used for crystal preparation was washed with E-Toxa-Clean concentrate (Sigma-Aldrich) prior to steam sterilization and dry heating. Endotoxin levels, as assessed by the limulus lysate chromogenic substrate method, were <5 pg/ml (<0.0625 endotoxin IU/mg). Crystal size varied between 2–40 μm, with 31% of crystals between 2–3 μm and 52% <10 μm in length, resulting in a mean ± SD crystal size of 11.82 ± 8.07 μm. The functional effect of crystal size was examined by sonicating crystals for 5, 10, or 20 minutes, which reduced the mean crystal size to 5.64, 4.66, and 2.00 μm, respectively.

Monocyte/macrophage cultures. Human monocyte–derived macrophages were isolated from human venous blood as previously described (16). Briefly, heparinized venous blood was sedimented for 30 minutes in the presence of 6% high molecular weight dextran T500 (Amersham Pharmacia Biotech, Amersham, UK) and the leukocyte-rich plasma layered onto a discontinuous 70% Percoll gradient (Amersham Pharmacia Biotech). The monocyte-enriched fraction was collected from the 70% interface, washed 3 times in Hank’s balanced salt solution (HBSS) without calcium and magnesium, and adhered to 24-well plates at 5 × 10⁶ cells/well in RPMI 1640 medium supplemented with 1-glutamine and 10% fetal calf serum (FCS; Life Technologies, Gaithersburg, MD). After incubation for 2 hours at 37°C, nonadherent cells were gently removed by aspiration, and medium was replaced with Iscove’s modified Dulbecco’s medium supplemented with 10% autologous serum. Cells were cultured for 1, 3, 5, or 7 days prior to phenotypic analysis or stimulation in the presence of MSU crystals (0.5 mg/ml), zymosan (0.4 mg/ml), or control medium, prior to collection of supernatants and determination of TNFα, IL-1β, IL-6, and IL-10 levels by enzyme-linked immunosorbent assay. Results are expressed as the mean ± SEM of at least 3 experiments.

Figure 1. Tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), IL-6, and IL-10 synthesis by macrophage versus macrophage in response to monosodium urate monohydrate (MSU) crystal stimulation. Monocytes (A) and macrophages (B) were prepared from peripheral blood by in vitro differentiation of the adherent mononuclear cell fraction for 1 and 7 days, respectively, in the presence of 10% autologous serum. Monocyte and macrophage cultures were stimulated for 16 hours with MSU crystals (0.5 mg/ml), zymosan (0.4 mg/ml), or control medium, prior to collection of supernatants and determination of TNFα, IL-1β, IL-6, and IL-10 levels by enzyme-linked immunosorbent assay. Results are expressed as the mean ± SEM of at least 3 experiments.

Electron microscopy. For electron microscopy, monocyte/macrophage cultures were established as described above, except that they were cultured in 9-cm² tissue-culture-grade slide flasks (Nalge Nunc International, Roskilde, Denmark). One-day and 7-day cultures were fixed in 2% glutaraldehyde and dehydrated through graded alcohols to absolute alcohol. Alcohol/TAAB resin (TAAB Laboratories, Reading, UK) was added and allowed to evaporate for 2 minutes before inverting slides onto TAAB resin in embedding molds for polymerization overnight at 60°C. Sections (80 μm) were collected on nickel 300 mesh grids and stained with uranyl acetate/lead citrate prior to examination with a Philips CM10 electron microscope (Philips, Mahwah, NJ) at 80 kV.
Enzyme-linked immunosorbent assays (ELISAs). TNFα, IL-1β, IL-6, and IL-10 levels in culture supernatants were determined by capture ELISA, using matched antibodies (DuoSet; R&D Systems, Abingdon, UK) according to the manufacturer’s recommendations. All samples were measured in triplicate, with results expressed as the mean ± SEM cytokine concentration (ng/ml) from at least 3 experiments.

Parallel-plate flow chamber. Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cords by collagenase type II (Boehringer Mannheim, Lewes, UK) digestion, as previously described (13). HUVECs (passages 2–5) were grown to confluence in slide flaskettes (Nalge Nunc International) 2 days prior to use and mounted in a parallel-plate flow chamber (channel height 0.15 cm). Human neutrophils were isolated from citrated venous blood by Percoll density sedimentation over a 2-step (74% and 50%) gradient, as previously described (17). Neutrophils collected from the 74% interface were washed twice and resuspended in RPMI 1640 medium containing 2% FCS for labeling with calcein-AM (1 μg/ml; Molecular Probes, Eugene, OR). Fluorescently labeled neutrophils were resuspended at 0.3 × 10⁶ cells/ml in HBSS containing 2% FCS (viscosity 0.007 poise) prior to perfusion at 37°C over HUVEC monolayers at a wall shear stress of 1.5 dynes/cm². Experiments were visualized at 100× magnification using an inverted Nikon Diaphot 300 fluorescence microscope connected to a JVC TK-C1360B color video camera (Microscope Service & Sales, Egham, UK). S-VHS video clips from 10 random fields were analyzed using EML Motion Analysis software (Ed Marcus Laboratories, Brighton, MA) to determine the number of rolling and adherent neutrophils per field. Rolling cells were defined as those with a mean rolling velocity <20 μm/second, and arrested cells were defined as those moving <5 μm in 10 seconds (17). Results are expressed as the mean ± SD number of rolling or adherent cells.

Flow cytometric analysis. Indirect flow cytometric analysis was performed as previously described (14), using primary antibodies at 10 μg/ml and secondary antibodies at 1:64 dilution. Endothelial cells were detached by trypsin/EDTA, and the trypsin activity was quenched in excess growth medium supplemented with serum. Monocyte/macrophage cultures were detached by 15-minute incubation in ice-cold phosphate buffered saline (PBS) containing 2.5 mM EDTA, followed by scraping and washing in growth medium containing serum.
Flow cytometric analysis was performed using an EPICS XL flow cytometer (Coulter Electronics, Luton, UK), and histogram overlays were created using the public domain WinMDI software, version 2.5 (available at http://facs.scripps.edu). Expression of monocyte/macrophage markers on days 1, 3, 5, and 7 of culture was calculated by dividing the staining intensity (mean fluorescent intensity [MFI]) of test antibody on any given day by the staining intensity of class-matched control antibody on the same day, thus resulting in a relative fluorescent intensity (RFI) of expression (where an RFI of 1.00 is equivalent to no antigen expression). Results were expressed as the mean ± SD RFI from 5 separate experiments.

Statistical analysis. The effect of MSU crystals on zymosan-induced TNFα secretion was analyzed using an unpaired t-test, and the effect of differentiation for 3, 5, and 7 days on proinflammatory cytokine secretion relative to day 1 cells was analyzed using 1-way analysis of variance (ANOVA) with Dunnett’s correction (GraphPad Prism software, San Diego, CA).

RESULTS

Effect of MSU crystals on TNFα, IL-1β, IL-6, and IL-10 synthesis by monocyte/macrophages. Monocyte or macrophage isolates from the same donor were prepared by in vitro culture of the adherent mononuclear cell fraction for 1 or 7 days, respectively. Cytokine levels in supernatants were measured by ELISA 16 hours after stimulation in the presence of MSU (0.5 mg/ml), zymosan (400 μg/ml), or medium. These studies revealed that whereas MSU crystals stimulated TNFα, IL-1β, and IL-6 secretion by monocytes (Figure 1A), no comparable proinflammatory cytokine secretion was observed in macrophage cultures (Figure 1B). However, macrophages were not inherently deficient in their capacity to synthesize inflammatory cytokines, because treatment with the control particulate stimulus, zymosan, triggered secretion of all 4 cytokines (TNFα, IL-1β, IL-6, and IL-10) in both monocyte and macrophage cultures. To confirm that both cell types had internalized urate crystals, polarized light microscopy and electron microscopy were performed. No significant difference in phagocytic uptake of MSU crystals was detected between cultures containing urate crystals (Figure 2). Varying the mean crystal size between 2.0 μm and 11.8 μm did not affect TNFα production by day 1 or day 7 cells (Figure 3A).

Development of the noninflammatory phenotype over time. In order to better understand the noninflammatory phenotype described above, a variety of phenotypic markers were assessed on days 1, 3, 5, and 7 of in vitro culture. These studies showed, first, that TNFα and...
uptake on zymosan-induced TNF-α secretion in response to MSU crystals was significantly reduced after 3 days ($P < 0.01$) and completely absent after 5 days of differentiation (Figure 3B). Second, acquisition of the noninflammatory phenotype correlated well with expression of the macrophage markers RFD7, CD16, CD64, and CD163 (Table 1), but not with dendritic cell markers (CD1a, CD33, CD83), activation markers (CD11b, HLA-DR), or scavenger receptors (CD36, CD68).

**Antiinflammatory effects of MSU crystal uptake by macrophages.** In addition to better understanding the noninflammatory phenotype described above, we were interested in determining whether macrophage differentiation could elicit an antiinflammatory phenotype. In these experiments, we studied the effect of MSU crystal uptake on zymosan-induced TNF-α secretion. Figure 4 demonstrates that whereas MSU crystals exerted no effect on TNF-α secretion in monocytes, they significantly inhibited zymosan-induced TNF-α levels in macrophages ($P = 0.009$). This was independent of possible steric hindrance between zymosan and MSU particles, because uptake of fluorescently conjugated BODIPY-zymosan was not inhibited by coincubation with MSU crystals (staining intensity of zymosan alone, 1,794.61; staining intensity of zymosan plus MSU, 1,982.96).

**Induction of E-selectin expression and secondary neutrophil recruitment by endothelial cells.** In order to determine whether macrophage supernatants contained any other factors (besides TNF-α, IL-1β, and IL-6) capable of activating endothelial cells, conditioned supernatants from day 1 and day 7 cells were added to HUVEC cultures for 3 hours, followed by examination of E-selectin expression, using flow cytometry. These experiments demonstrated that whereas macrophage supernatants from MSU-treated cultures failed to stimulate E-selectin expression, corresponding monocyte supernatants induced high levels of E-selectin (Figure 5A). To link these observations with secondary neutrophil recruitment, endothelial lawns were established in parallel-plate flow chambers and were treated as de-
scribed above with monocyte/macrophage supernatants prior to perfusion of neutrophils at physiologic shear flow rates (1.5 dynes/cm²). These experiments revealed that whereas macrophage supernatants from MSU-treated cultures failed to promote neutrophil capture, corresponding monocyte supernatants promoted both rolling and firm adhesion (Figure 5B). Similar results were obtained using whole blood in the perfusate, thus confirming that macrophages did not secrete factors upon engulfment of urate crystals that might lead to recruitment of cell types other than neutrophils (results not shown).

**DISCUSSION**

Using an in vitro model of acute gout, we demonstrated that human macrophages, obtained by differentiation of peripheral blood monocytes for a minimum of 3–5 days in the presence of autologous serum, did not secrete TNFα, IL-1β, IL-6, or any other factors capable of inducing endothelial cell E-selectin expression or promoting secondary neutrophil recruitment under hydrodynamic shear flow. In contrast, freshly isolated monocytes from the same donor challenged with MSU crystals secreted these proinflammatory cytokines, induced E-selectin expression, and promoted rolling and adhesion of neutrophils on HUVECs. These observations support and extend our previous observations made with a panel of mouse monocytic cell lines, which showed divergent responses to MSU crystals according to the state of differentiation (14). Our results suggest that the process of macrophage differentiation may break the cycle of inflammation triggered by urate crystals, preventing proinflammatory cytokine synthesis, endothelial cell activation, and secondary neutrophil recruitment.

Several factors control the incidence, duration, and severity of gout in hyperuricemia. The capacity to nucleate and grow crystals is clearly an important variable. However, other factors besides the presence of crystals must be involved, because crystals can be detected in asymptomatic joints (2–5) and at sites of experimental intracutaneous crystal injection after erythema has resolved (12). One possibility is that crystals are coated with protective proteins, such as apolipoproteins, that reduce the inflammatory potential of crystals following uptake by polymorphonuclear cells (18–20). Dyslipidemia may therefore be another variable affecting the inflammatory response of given individuals to urate crystals. Another possibility is that products of the hypothalamic–pituitary axis may exert direct antiinflammatory effects on monocytes involved in MSU crystal clearance (21). The in vitro evidence presented here suggests that proper differentiation into macrophages should be considered as another variable.

Very little is known about the state of monocyte/macrophage differentiation in joints, either in health or disease. Histologic studies in mice suggest that synovial lining macrophages exist in a mature state of differentiation (22). The presence of end-stage macrophages in any given tissue is controlled by the availability of growth factors and cytokines, such as macrophage colony-stimulating factor and IL-6 (23,24), that promote differentiation into the macrophagic end point. It is possible
that polymorphisms in genes that control monocyte differentiation, such as the G174C polymorphism in IL-6 that reduces IL-6 levels (25), may prevent adequate differentiation of monocytes into macrophages, thus predisposing such individuals toward a more proinflammatory response to MSU crystals. Because the noninflammatory phenotype we have described here is closely linked with a macrophagic but not dendritic cell phenotype, a prediction arising from the present work is that mononuclear cells recovered from synovial fluid aspirates during the intercritical phase would be expected to express markers characteristic of differentiated macrophages, such as CD64, CD163, and RFD7 antigen.

Besides failing to induce TNFα release in macrophages, MSU crystals also actively suppressed secretion of TNFα in response to zymosan. This was not attributable to steric hindrance between MSU and zymosan particles, which suggested that the 2 stimuli were taken up by distinct phagocytic receptor pathways. Although zymosan is thought to be taken up via the glucan binding site on Mac-1 (CR3) (26), at present it is unknown how monocyte/macrophages phagocytose MSU crystals. Our present hypothesis is that phagocytosis of MSU and zymosan occurs through distinct receptor pathways, leading to separate but potentially interactive signaling events. It also remains possible, based on studies in neutrophils, that contact with the cell membrane alone, before phagocytosis has taken place, may be sufficient to trigger signaling events resulting in the eventual suppression of the zymosan response (27,28).

The resolution phase of gout has previously been recognized to involve a contribution from macrophages based on their engulfment of apoptotic neutrophils, thus giving rise to Reiter’s cells in synovial fluid (29,30). Here, we have provided evidence that MSU uptake can initiate antiinflammatory responses in macrophages ab initio. This does not involve elaboration of IL-10 (or soluble p55 TNF receptor or IL-1 receptor antagonist [data not shown]) but may involve activation of antiinflammatory signaling pathways and/or elaboration of other antiinflammatory cytokines.

Although these possibilities have yet to be tested, the study of apoptotic cell removal, the only other phagocytic process thus far described to be uncoupled from inflammatory cytokine synthesis, has confirmed the existence of such antiinflammatory mechanisms in macrophages. Apoptotic cell removal is linked to crkII/Dock180/Rac 1 signaling (31) and antiinflammatory cytokine synthesis, including transforming growth factor β, prostaglandin E2, and platelet-activating factor (16). Regardless of whether similar antiinflammatory mechanisms are invoked following MSU crystal uptake, it remains likely that apoptotic neutrophil removal and MSU crystal phagocytosis will proceed hand-in-hand during the resolution phase of an acute gout attack.

Another relevant observation from the apoptotic neutrophil field is that the normal noninflammatory clearance mechanisms of macrophages can be subverted by concomitant engagement of Fc receptors (32). Coating of MSU crystals with IgG may thus predispose certain individuals to a more proinflammatory reaction to crystal deposition in their joints. This is consistent with other research showing that crystal-specific antibodies can help nuclease MSU crystallization (33), and that IgG coating of crystals can enhance the reactive oxygen burst elicited from polymorphonuclear phagocytes (34).

We have postulated a possible mechanism whereby monocyte/macrophage differentiation may influence the inflammatory response to MSU crystal precipitation in joint fluids (35). In this scenario, resident synovial macrophages would be expected to provide joint protection in hyperuricemia through the continual noninflammatory removal of insoluble MSU crystals. However, any of the known precipitants of gout, such as mechanical trauma, infection, or surgical stress, could tip the balance toward inflammation by triggering a fresh wave of monocyte and neutrophil recruitment from the bloodstream into the affected joint. Spontaneous resolution of an acute attack may be reinforced by differentiation of recently infiltrated monocytes into macrophages, which safely dispose of apoptotic neutrophils and MSU crystals through noninflammatory and antiinflammatory clearance mechanisms.

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