Differences in MSU-induced Superoxide Responses by Neutrophils from Gout Subjects Compared to Healthy Controls and a Role for Environmental Inflammatory Cytokines and Hyperuricemia in Neutrophil Function and Survival

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ABSTRACT. Objective. To determine whether monosodium urate (MSU) crystal-induced superoxide production is greater for neutrophils from patients with gout compared to asymptomatic hyperuricemic and healthy controls, and whether neutrophil functions are altered by an MSU crystal-induced inflammatory environment.

Methods. Neutrophils were purified from the whole blood of study participants, restimulated with 500 mg MSU crystals \textit{ex vivo}, and superoxide production measured using the colorimetric dye WST-1. Purified neutrophils were exposed to conditioned media from MSU crystal-activated blood monocyte cultures with and without neutralizing antibodies for interleukin 1ß (IL-1ß), IL-8 (CXCL8), IL-6, and tumor necrosis factor-α (TNF-α). Neutrophil superoxide production was measured and neutrophil apoptosis and IL-8 production were determined by flow cytometry. Serum samples were collected from participants and analyzed by Lincoplex bead array for IL-1ß, IL-8, IL-6, and TNF-α.

Results. Neutrophils from gout and asymptomatic hyperuricemic subjects produced higher levels of MSU crystal-induced superoxide, and a weak trend toward elevated serum cytokines was observed compared to healthy controls. A correlation between serum uric acid and elevated neutrophil superoxide production was also observed. Neutrophils exposed to media from MSU crystal-activated monocytes exhibited enhanced superoxide production to MSU-crystal stimulation, increased IL-8 production, and extended cell survival that was predominantly dependent on IL-8, TNF-α and IL-6, respectively.

Conclusion. Neutrophils from gout and asymptomatic hyperuricemic individuals are primed for enhanced MSU crystal-induced superoxide production that may be driven by a subclinical inflammatory cytokine environment combined with hyperuricemia. This inflammatory environment likely contributes to elevated neutrophil IL-8 production and survival in the absence of direct crystal stimulation. Asymptomatic hyperuricemia is not associated with suppressed neutrophil function.

Key Indexing Terms: GOUT NEUTROPHILS MONOSODIUM URATE CRYSTAL SUPEROXIDE

Gout is an inflammatory arthritis characterized by intensely painful self-limiting episodes associated with hyperuricemia and the formation of monosodium urate (MSU) crystals in the joints. A characteristic feature of gout is the infiltration of neutrophils into the joint, indicating that these cells play a major role in inflammatory response to MSU crystals.

Neutrophils exposed to MSU crystals \textit{in vitro} generate and release damage-causing superoxide, produce the neutrophil chemokine interleukin 8 (IL-8; CXCL8), and exhibit a delay of homeostatic apoptosis. In addition to direct interaction with inflammatory stimuli, the local inflammatory environment has been shown to influence neutrophil responses. During a gout attack, MSU crystal deposition triggers the local production of classical proinflammatory cytokines including IL-1ß, IL-8, IL-6, and tumor necrosis factor-α (TNF-α). Both TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) have
been shown to enhance MSU crystal-induced neutrophil superoxide production in vitro. It therefore appears that neutrophil activity in MSU crystal-induced inflammation may be influenced indirectly by soluble mediators produced by other immune cells at the site of inflammation in vivo as well as direct MSU crystal interaction.

The possibility and potential significance of biological variation in neutrophil responses in gout have not been investigated. Differences in neutrophil function in other forms of inflammatory arthritis have been reported, including elevated superoxide production by neutrophils from patients with rheumatoid arthritis (RA). Increased neutrophil superoxide and IL-8 production have also been reported in subjects with psoriatic arthritis (PsA). These data provide the rationale to compare the MSU crystal-induced inflammatory responses of neutrophils from gouty and healthy individuals.

Our aim was to determine whether neutrophils from patients with gout exhibit enhanced immune responses to MSU crystal-induced stimulation compared to normouricemic and hyperuricemic healthy controls, and whether proinflammatory mediators produced by MSU crystal-activated monocytes alter neutrophil function.

**MATERIALS AND METHODS**

Reagents. Uric acid, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, saponin, 10% buffered formalin, and sodium azide were obtained from Sigma-Aldrich, Auckland, NZ. NaCl and CaCl₂ were obtained from BDH Chemicals NZ Ltd., Auckland, NZ. Polymorphrep (Axis-Shield, Oslo, Norway) was obtained through Medica Pacifica Ltd., Auckland, NZ. Low cell binding plates (Nunc, Rochester, NY, USA) were obtained through In Vitro Technologies, Auckland, NZ. Red blood cell (RBC) lysis buffer was obtained from ProGENZ, Auckland, NZ. We obtained 2-(4-iophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfophenyl)-2'H-tetrazolium monosodium salt from Dojindo, Kumamoto, Japan. Limulus amebocyte lysate kit (LAL) was obtained from Associates of Cape Cod Inc., East Falmouth, MA, USA. Cell culture reagents including media, phosphate buffered saline (PBS), and Hank's balanced salts solution (HBSS), penicillin-streptomycin, glutamax, and bovine serum albumin (BSA) were obtained from Invitrogen, Auckland, NZ. Mouse anti-human IL-18, rat anti-human IL-6, and mouse anti-human TNF-α were obtained from BioLegend, San Diego, CA, USA. Lincoplex multiplex kits were obtained from Abacus ALS, Auckland, NZ. Sodium-heparin 10 ml vacutainers, r-phycocerythrin (R-PE) and unlabeled mouse anti-human IL-6, rat anti-human IL-8, allopurinol, and WST-1 (250 µg/ml) were purchased from Boehringer Ingelheim, Germany. Lactated Ringer’s solution was then cooled and filtered through a 0.2 µM filter. That solution was then cooled and filtered through a 0.2 µM filter. That solution was then cooled and filtered through a 0.2 µM filter.

Preparation of MSU crystals. MSU monohydrate crystals were prepared as described. Uric acid (250 mg) was added to a 45 ml solution of 30 mM NaOH/diH₂O and boiled until the uric acid was completely dissolved. The solution was then cooled and filtered through a 0.2 µM filter. That solution was boiled and 1 ml of 5 M NaCl was added. The solution was left to cool to room temperature and was then stored at 26°C for 7 days to allow crystal formation. The resulting MSU crystals were washed with ethanol and acetone and air-dried under sterile conditions. Triclinic MSU crystals were needle-shaped, between 5 and 20 µm in length, and showed appropriate optical birefringence. MSU crystals were confirmed as endotoxin-free by LAL assay (< 0.01 EU/10 mg).

Isolation of cells. Blood was collected from healthy volunteers into heparinized vacutainer tubes. To harvest whole white blood cells (WBC), blood was centrifuged at 500 × g for 10 min and the top layer containing WBC (buffy coat) collected by aspiration. Neutrophils and peripheral blood mononuclear cells (PBMC) were purified from peripheral blood by sedimentation using Polymorphrep. Blood was diluted in an equal volume of HBSS, then 10 ml of diluted blood was layered over 4 ml of Polymorphprep and centrifuged 500 × g for 30 min at 19°C. This treatment resulted in 2 distinct white bands: an upper band containing PBMC, and a lower band containing polymorphonuclear cells. Each layer was removed separately by aspiration, washed twice with PBS (pH 7.4), and resuspended in phenol red-free RPMI-1640 supplemented with 100 U/ml penicillin-streptomycin, 2 mM glutamax, and 10% human serum (complete RPMI-1640). Contaminating RBC were removed by treatment with RBC lysis buffer. This procedure yielded > 90% pure neutrophils and > 95% pure PBMC as determined by Diff-Quik staining of cytocentrifuged cell samples.

Preparation of PBMC-conditioned media. Purified human PBMC were suspended at 5 × 10⁵ cells/ml in complete RPMI-1640 and cultured in 75 cm² cell culture flasks. Conditioned media and MSU crystal-conditioned media were prepared by culturing PBMC for 16 hours (37°C, 5% CO₂). 5 × 10⁶ cells/ml in the presence of PBS (0.5 ml) or 200 µg/ml MSU crystals, respectively. Complete RPMI-1640 (nonconditioned media) was incubated for 16 hours as a control. Cell cultures were then centrifuged and the supernatants collected and stored at −20°C for subsequent experiments and cytokine analysis.

Neutrophil viability assay. Purified human neutrophils or WBC were suspended in either complete RPMI-1640, with different conditioned media, or with nonconditioned media containing IL-6, in 24-well low cell-binding plates (1 × 10⁶ cells/1 ml). Cells were then cultured with 200 µg/ml MSU crystals, or with 20 µl of PBS. At different times, cells were harvested, stained with propidium iodide (PI) and fluorescently labeled annexin-V, and analyzed by flow cytometry. WBC cultures were also stained with APC mouse anti-human CD15 to identify neutrophils.

Superoxide assay. The generation of superoxide was measured using the reduction of the formazan colorimetric dye WST-1, as described. Purified neutrophils (1 × 10⁶/ml) were suspended in HBSS containing WST-1 (250 µg/ml) and incubated with different concentrations of MSU crystals (1 h, 37°C). Crystal-free supernatants were harvested by centrifugation and transferred to a 96-well plate. The absorbance at wavelength 450 nm was then measured using a Versamax spectrophotometer. For conditioned media experiments, neutrophils were incubated in the different phenol red-free conditioned media, or in nonconditioned media containing treated hypertension, diabetes mellitus, and cardiovascular disease including myocardial infarction, angina with positive treadmill test, or angiographic evidence of coronary artery disease, peripheral vascular disease, stroke or transient ischemic attack.

Serum was separated by centrifugation and stored at −70°C for subsequent analysis. Neutrophils were purified and neutrophil superoxide production measured after stimulation with different concentrations of MSU crystals as described below. Serum samples were analyzed by Lincoplex bead array for IL-1β, IL-8, IL-6, and TNF-α.

This study was approved by the Central Regional Ethics Committee (NZ) and all participants provided written informed consent.
IL-8, for 2 h. Neutrophil superoxide production in the presence or absence of MSU crystals was then determined as described.

Cytokine assays. Neutrophils (1 x 10^6/ml) or PBMC (5 x 10^5/ml) were suspended in complete RPMI-1640 and incubated in the presence or absence of MSU crystals (200 µg/ml). At different times the cell cultures were centrifuged and the supernatants collected. Supernatants were analyzed for cytokines with a Lincoplex multiplex kit.

Intracellular IL-8 staining of neutrophils. Neutrophils were cultured in the different conditioned media, or in nonconditioned media containing TNF-α, in 24-well low cell-binding plates (1 x 10^6 cells/ml media, 1,500 GolgiStop), in the presence or absence of MSU crystals (200 µg/ml). After 4 hours, neutrophils were washed 3 times in PBS, fixed in 10% buffered formalin, permeabilized with saponin buffer (PBS containing 0.1% saponin, 0.1% BSA, 0.1% sodium azide), and then stained with R-PE mouse anti-human IL-8 or with mouse IgG2b isotype control. The neutrophils were washed and resuspended in FACS buffer (PBS, 0.1% BSA, 0.1% sodium azide) and analyzed by flow cytometry.

Neutralizing antibody experiments. Neutrophils were suspended in the MSU crystal-conditioned media containing different combinations of mouse anti-human IL-1β, rat anti-human IL-6, mouse anti-human IL-8, or mouse anti-human TNF-α (final concentration of each antibody, 40 µg/ml), and the experiments for viability, IL-8 production, or superoxide generation repeated as described.

Statistical analysis was carried out using the Mann-Whitney U test, the Wilcoxon rank-sum test, the Student T test, or the Tukey multiple comparisons test.

RESULTS

We hypothesized that subjects with gout may have enhanced neutrophil production of superoxide after exposure to MSU crystals, as a potential trigger to gouty inflammation. We compared superoxide production of purified peripheral blood neutrophils from subjects with gout and healthy individuals after in vivo exposure to varying concentrations of MSU crystals. Demographic data of subject participants is presented in Table 1. Subjects had a mean age of 60.2 ± 12.1 years with no difference between gout and healthy control groups (p = 0.8803) and were predominantly men (96%). Neutrophils from subjects with gout, asymptomatic hyperuricemia, and healthy controls produced similar background levels of superoxide, but neutrophils from subjects with gout and asymptomatic hyperuricemia produced higher levels of superoxide following stimulation with MSU crystals (Figure 1A). To determine whether an increased neutrophil responsiveness correlated with heightened systemic inflammatory markers, levels of relevant inflammatory mediators were measured in patient sera. Although not statistically significant, IL-6, IL-8, and TNF-α levels showed a trend toward elevation in patients with gout and asymptomatic hyperuricemia (Figure 1B). These trends contrasted with IL-1β, which showed identical levels regardless of the disease stage (Figure 1B). A weak but significant correlation between hyperuricemia and neutrophil superoxide production induced by 1 mg/ml MSU crystals was also observed (Figure 1C). Consistent with this correlation, neutrophils from subjects with moderate to high serum uric acid produced significantly higher levels of superoxide compared to healthy controls over a range of MSU crystal concentrations (Figure 1D).

PBMC-derived soluble mediators and neutrophil superoxide production. Elevated levels of serum cytokines in patients with gout led us to investigate what effect cytokine production by MSU crystal-stimulated monocytes has on neutrophil superoxide production in gout. Proinflammatory cytokine production by MSU crystal-activated recruited monocytes has been reported to play a role in inflammation in acute gout15,25, and may contribute to the elevated levels of proinflammatory cytokines observed in sera of patients with gout29. Therefore, supernatant from blood monocytes activated by MSU crystals in vitro represents a physiologically relevant composition of the proinflammatory cytokines likely to be encountered by neutrophils in vivo. PBMC activated with MSU crystals produced large quantities of IL-1β, IL-6, IL-8, and TNF-α following exposure to MSU crystals (Figure 2A).

Purified human neutrophils were then exposed to culture supernatants from either unstimulated PBMC (conditioned media) or MSU crystal-stimulated PBMC (MSU-condi-
tioned media). Neutrophils incubated in conditioned or MSU-conditioned media alone exhibited no significant increase in the production of superoxide above controls (Figure 2B, 0 mg/ml MSU). However, following MSU crystal stimulation, neutrophils activated in MSU-conditioned media produced superoxide at significantly lower concentrations of MSU crystals compared to neutrophils cultured in other media (Figure 2B). Neutrophils activated in MSU-conditioned media with the alternative stimulus formyl methionylleucyl phenylalanine (fMLP) also showed a significant increase in superoxide production (Figure 2C).

Soluble mediators delay neutrophil apoptosis. To determine whether cytokine production by MSU crystal-activated PBMC affected neutrophil viability, neutrophils were cultured in either conditioned or MSU-conditioned media for 24 hours and analyzed for apoptosis by the expression of annexin-V and PI. In the absence of any stimuli, 46% of neutrophils were undergoing apoptosis after 24 h in culture (Figure 3A) compared to 23% of MSU crystal-stimulated neutrophils and 14% of neutrophils in MSU-conditioned media (Figure 3A). Neutrophils activated by MSU crystals in the presence of conditioned or MSU-conditioned media did not increase neutrophil survival further (Figure 3A).

Soluble mediators stimulate neutrophil IL-8 production. Next, we investigated whether the MSU-conditioned media could stimulate neutrophils to produce the neutrophil chemokine IL-8. As shown in Figure 3B, 60% of neutrophils cultured in conditioned media were IL-8-positive compared to 14% in control cultures, indicating that the MSU-conditioned media alone induced IL-8 production by neutrophils. The addition of MSU crystals increased the percentage of IL-8-positive neutrophils cultured in conditioned and nonconditioned media, but not MSU-conditioned media. Analysis of the mean fluorescence intensity of IL-8 in neutrophils treated with MSU-conditioned media or MSU crystals alone showed that MSU-conditioned media induced
significantly higher neutrophil IL-8 production compared to MSU crystal stimulation alone (Figure 3C).

Inflammatory cytokines and MSU crystal-induced neutrophils. To identify which inflammatory cytokines were contributing to contact-independent neutrophil activation and survival, neutrophils were cultured in MSU-conditioned media in the presence of different combinations of antibodies for the inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α.

Neutrophil sensitization to MSU crystal-induced superoxide production was significantly abrogated by anti-IL-8 and to a lesser extent by anti-IL-6 and anti-TNF-α, and was blocked completely in the presence of all 3 antibodies (Figure 4A). Neutrophil viability was partially decreased following treatment with anti-IL-6 (Figure 4B), while neutrophil IL-8 production was abrogated by anti-TNF-α (Figure 4C). Anti-IL-1β had no effect on any of the readouts measured. Neutrophils cultured in normal media containing IL-8 or TNF-α also exhibited elevated superoxide production and IL-8 production, respectively (Figure 5).

DISCUSSION
We report that neutrophils isolated from patients with intercritical-phase gout and from patients with chronic gout produced higher levels of MSU crystal-induced superoxide compared to neutrophils isolated from healthy volunteers. These results are similar to the pattern of neutrophil “priming” observed in both patients with RA and patients with PsA in response to fMLP restimulation, including individuals with treated RA in therapeutic remission.

Neutrophils isolated from participants with asymptomatic hyperuricemia also produced higher levels of MSU crystal-induced superoxide compared to healthy controls, showing that a prior attack of gout is not a prerequisite for priming of neutrophils for superoxide production. More importantly, protection from developing gout does not appear to rely on suppressed neutrophil responses to MSU crystals, as neutrophils from patients with gout and from asymptomatic patients with hyperuricemia exhibited a neutrophil “priming” phenotype.

The neutrophil priming observed in subjects with hyperuricemia and gout is not explained by the comorbid conditions that prime neutrophils for superoxide production, as the proportion of subjects with diabetes mellitus or cardiovascular disease was similar across all study groups. The asymptomatic hyperuricemia and acute and chronic gout groups did include a higher proportion of subjects with treated hypertension than the healthy control group. However, because hypertension was adequately treated, it is
Figure 3. Soluble mediators generated by peripheral blood mononuclear cells extend neutrophil viability and stimulate IL-8 production. A. Neutrophils (5 × 10^5 cells, 0.5 ml) were cultured in different conditioned media in the presence or absence of monosodium urate (MSU) crystals (200 µg/ml). After 24 h, neutrophils were harvested and analyzed for viability. B. Neutrophils (5 × 10^5 cells, 0.5 ml) were cultured in different conditioned media with 20 µl phosphate buffered saline (PBS) or in the presence of 200 µg/ml MSU crystals. After 4 h, neutrophils were analyzed by flow cytometry for intracellular IL-8 production. C. IL-8 mean fluorescent intensity (MFI) in neutrophils cultured (as in B) in either nonconditioned media containing 200 µg/ml MSU crystals (N. Media + MSU), or in MSU-conditioned media (M. Media). Values represent mean ± SEM. Statistical analyses by Student’s T test. ***p < 0.001.

Figure 4. The effect of cytokine-blocking antibodies on monosodium urate (MSU) crystal-conditioned media activation of neutrophils. A. Neutrophils (1.5 × 10^5 cells, 0.15 ml) were cultured in MSU crystal-conditioned media in the presence of neutralizing antibodies (2 h). Neutrophils were stimulated with MSU crystals (200 µg/ml, 1 h) and superoxide production was measured by reduction with the colorimetric dye WST-1 as measured at absorbance 450 nm. B. Neutrophils (5 × 10^5 cells, 0.5 ml) were cultured in different conditioned media (N. Media: nonconditioned media; M. Media: MSU crystal-conditioned media) in the presence of neutralizing antibodies. After 24 h, neutrophils were harvested and analyzed for viability by flow cytometry using annexin-V and propidium iodide staining. C. Neutrophils (5 × 10^5 cells, 0.5 ml) were cultured in different conditioned media in the presence of neutralizing antibodies. After 4 h, neutrophils were harvested and analyzed for intracellular IL-8 production by flow cytometry. Values represent mean ± SEM. Statistical analyses were performed by Tukey multiple comparisons test. ***p < 0.001, **p < 0.01, *p < 0.05.
unlikely to be responsible for priming of neutrophils for superoxide production. Instead, there is strong evidence that hyperuricemia plays a part in neutrophil priming.

We observed a weak trend toward higher serum levels of IL-8, IL-6, and TNF-α in some patient groups, including participants with asymptomatic hyperuricemia, compared to healthy controls. This trend indicated that local proinflammatory cytokine levels could also be contributing to the priming of neutrophil superoxide production. Neutrophils from healthy volunteers pretreated with TNF-α, GM-CSF, or IL-8 have been shown to exhibit enhanced superoxide production after stimulation with fMLP, with maximal priming seen after stimulation with a combination of cytokines.

In our study, exposure of neutrophils to supernatant from MSU crystal-stimulated monocytes was found to enhance neutrophil production of superoxide after both MSU crystal and fMLP stimulation, indicating that priming is not tuned for specific stimuli. Consistent with previous work, our results also showed that elevated neutrophil sensitivity to MSU crystal-induced superoxide production was driven primarily by the presence of IL-8 in the monocyte culture supernatant, with a smaller contribution from TNF-α and IL-6. It is important to note that, even in the presence of these “priming” cytokines, neutrophils required contact with MSU crystals to elicit superoxide generation. This requirement for MSU crystal contact would therefore restrict the production of superoxide to the tissue compartment or synovial space in which MSU crystals are present.

In the absence of direct MSU crystal stimulation, exposure to a proinflammatory cytokine environment increased neutrophil survival that was partially dependent on IL-6. IL-6 has been shown to play an important role in neutrophil survival in other inflammatory conditions. Elevated levels of IL-6 have been reported in sera and synovial fluids from patients with MSU crystal-inflamed joints and have been linked to MSU crystal-induced production of IL-6 by monocytes and synoviocytes. These data indicate a key role for local production of IL-6 in driving inflammation in gout by improving neutrophil survival and therefore neutrophil accumulation.

The TNF-α-dependent production of IL-8 by neutrophils cultured in MSU crystal-induced inflammatory mediators further emphasizes the influence of the inflammatory environment on the neutrophil. Based on these findings, local production of TNF-α has the potential to induce infiltrating neutrophils to produce IL-8, thereby augmenting neutrophil recruitment and priming for superoxide production. Indeed, it is highly likely that increased neutrophil survival and IL-8 production are 2 of the key factors responsible for driving the accumulation of MSU crystal-recruited neutrophils in vivo.

Neutrophils from both patients with gout and from individuals with asymptomatic hyperuricemia are primed for enhanced superoxide production after MSU crystal stimulation. Consistent with other inflammatory conditions, this priming phenomenon appears to be linked to the levels of IL-8 in the surrounding inflammatory environment, but may also be driven by exposure to elevated serum uric acid. Based on our findings, upon recruitment into the joint, primed neutrophils may be activated by MSU crystals directly to produce superoxide and IL-8 and indirectly by local production of TNF-α to augment neutrophil IL-8 production and recruitment. These data also highlight an important role for the local inflammatory cytokine environment in neutrophil function and survival in gout, independent of direct MSU crystal activation.

It does not appear that neutrophil sensitization to MSU
crystals is directly responsible for susceptibility to developing gout. Hyperuricemia alone is sufficient to prime neutrophils, yet the majority of these individuals remain asymptomatic. However, other immune cells associated with gout inflammation need to be profiled before we can make any definitive conclusions about whether susceptibility to, or protection from, developing gout results from differential immune cell function.

REFERENCES