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**Precipitation of Soluble Uric Acid is Necessary for *in vitro* Activation of the NLRP3 Inflammasome
in Primary Human Monocytes**

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Short running head: Hyperuricemia and NLRP3 Activation

Abstract:

Objective. To investigate the effects of soluble uric acid (UA) on expression and activation of the NOD-like receptor (NLR) pyrin domain containing protein 3 (NLRP3) inflammasome in human monocytes to elucidate the role of hyperuricemia in the pathogenesis of gout.

Methods. Primary human monocytes and the THP-1 human monocyte cell line were used to determine the effects of short- and long-term exposure to UA on activation of the NLRP3 inflammasome and subsequent interleukin-1 β (IL-1 β) secretion by enzyme linked immunosorbent assay (ELISA) and cell-based assays. Expression of key NLRP3 components in monocytes from patients with a history of gout were analysed by quantitative PCR.

Results. Precipitation of UA was required for the activation of the NLRP3 inflammasome and subsequent release of IL-1 β in human monocytes. Neither monosodium urate (MSU) crystals nor soluble UA had any effect on activation of the transcription factor, NF- κ B. Prolonged exposure of monocytes to soluble UA did not alter these responses. However, both MSU crystals and soluble UA did result in a 2-fold increase in reactive oxygen species (ROS). Gout patients (n=15) had significantly elevated serum UA concentrations compared to healthy individuals (n=16), yet secretion of IL-1 β and expression of NLRP3 inflammasome components in monocytes isolated from these patients were not different from healthy controls.

Conclusion. Despite recent reports indicating that soluble UA can prime and activate the NLRP3 inflammasome in human peripheral blood mononuclear cells (PBMCs), precipitation of soluble UA into MSU crystals is essential for *in vitro* NLRP3 signalling in primary human monocytes.

Introduction

Uric acid (UA) is a major antioxidant in the plasma that can help protect against free-radical induced oxidative stress. Indeed, this function of UA is thought to have led to the evolutionary loss of the uricase gene in humans resulting in much higher circulating concentrations of UA in humans than in other mammals (1, 2). Clinically relevant hyperuricemia is defined as a plasma UA concentration in excess of 6.8 mg/dL (3) which is at increased risk of precipitation to form potentially pro-inflammatory monosodium urate (MSU) crystals (4).

Hyperuricemia is associated with a number of pathological conditions including metabolic syndrome (5), hypertension (6), chronic kidney disease (7) and cardiovascular disease (8). It is also the biggest single risk factor for gout, the pathophysiology of which is primarily driven by the production of the pro-inflammatory cytokine IL-1 β released as a result of recognition of MSU crystals by mononuclear cells (9). Intriguingly, only about 10% of hyperuricaemic individuals ever develop gout (10), nor does the presence of MSU crystals within joints necessarily precipitate a gout attack (11).

In vivo processing and subsequent secretion of IL-1 β occurs via activation of the NOD-like receptor (NLR) pyrin domain containing protein 3 (NLRP3) inflammasome. This is a tripartite cytosolic complex formed of three proteins NLRP3, Apoptosis-associated Speck-like protein containing a CARD domain (ASC) and pro-caspase-1. Upon activation, NLRP3 oligomerises with the adaptor protein ASC which in turn mediates the recruitment of the inactive zymogen pro-caspase-1 (12). Oligomerisation of inflammasome components ultimately results in the autoproteolytic cleavage of pro-caspase-1 into its active form. Caspase-1 then cleaves pro-IL-1 β and pro-IL-18 into their active forms resulting in their secretion from cells (13).

It is generally accepted that activation of the NLRP3 inflammasome requires two distinct signals, although an alternative pathway has recently been identified in human monocytes in response to LPS (14). These two signals consist of a priming signal to induce transcription of both NLRP3 and pro-IL-1 β , and a second signal that induces oligomerisation of the inflammasome. Various ligands can induce

NLRP3 priming, including the toll-like receptor (TLR) 2 ligand Pam3CSK4 (Pam3) and the TLR4 ligand lipopolysaccharide (LPS) via activation of NF- κ B (15). A number of structurally diverse stimuli can function as the second signal but none have been demonstrated to interact directly with inflammasome proteins (16, 17). Possible mechanisms by which second signals activate the inflammasome include lysosomal degradation(18), cationic flux(19) and generation of reactive oxygen species (ROS) (20).

Despite hyperuricemia being the single greatest risk factor for the development of gout, the precise role of UA in its pathophysiology remains to be fully elucidated. Recent studies have shown that soluble UA can influence both the priming and activation of the inflammasome even in the absence of MSU crystal formation (21-23). However, many of these studies were completed in human PBMCs or mouse bone marrow derived macrophages which may not accurately reflect the responses of primary human monocytes.

It is important to fully define the role of UA in activating the NLRP3 inflammasome in these cells, as monocytes rather than PBMCs are the major producers of IL-1 β during an acute attack of gout (24). Thus, the aim of this study was to determine the effect of soluble UA on activation of NLRP3 in human monocytes to investigate how hyperuricemia can increase susceptibility to gout at the cellular level. We used a combination of human monocytic THP-1 cells and primary human monocytes from healthy controls and gout patients to investigate both the acute and longer-term effects of UA on expression, priming and activation of the NLRP3 inflammasome.

Materials and Methods

Reagents

Cell culture media, fetal bovine serum (FBS), and uric acid were purchased from Sigma-Aldrich (St Louis, MO). Opti-MEM media and penicillin/streptomycin were purchased from Thermo Fisher Scientific (East Grinstead, UK). MSU crystals, MCC950(25) and QUANTI-Blue reagent were purchased from Invivogen (San Diego, CA). Qiazol, QuantiTect Reverse Transcription kit, QuantiFast-SYBR green PCR kit were from Qiagen (Hilden, Germany).

Ethical approval

National Research Ethics Service approval (NRES reference: 15/NS/0083) was obtained for collection of human blood samples after receiving informed written consent. No patient identifiable information was ever accessed by researchers. For purchased blood products, local ethical approval was given by the Brighton and Sussex Medical School Research Governance and Ethics Committee (R&D REF number: 15/130/MUL).

Isolation and stimulation of primary human monocytes

Single donor plateletpheresis residues were obtained from the North London Blood Transfusion Centre (United Kingdom). PBMCs were isolated by density gradient separation using lympholyte-H cell separation media (VH Bio, UK) followed by isolation of monocytes by Percoll (Sigma-Aldrich, MO) density gradient centrifugation (26). Cells were stored in liquid nitrogen for future use.

The appropriate concentration of MSU crystals for stimulating cells was determined by titration to achieve activation of NLRP3 with no effects on viability (data not shown). Soluble UA concentrations were chosen to allow direct comparison with MSU crystal concentrations and were subsequently increased to represent a wider range of hyperuricemia. UA was dissolved in pre-warmed RPMI at a final concentration of 0.6-1.0 mg/ml and filter sterilized using 0.20 µm filters. Crystals were not detectable in preparations of uric acid, nor were crystals ever observed under any of the experimental

conditions used (Supplementary Fig. 1). For experiments, monocytes were resuspended in RPMI cell culture media containing 5% FCS and seeded at 3×10^5 cells/well in 96-well plates. For experiments involving pre-incubation of cells with soluble UA, cells were allowed to adhere for 2 hours and then incubated in RPMI (5% FCS) +/- 30mg/dL UA for 10-16 hours. Cells were then washed with RPMI and stimulants added for the specified time.

For some experiments, monocytes were isolated from 30 mL of blood collected from gout patients (n=15) or healthy volunteers (n=16). Gout patients were defined as those who had suffered an inflammatory arthritic attack that was clinically diagnosed as gout. The majority of these patients (10/16) were prescribed anti-inflammatory medication, with 50% prescribed allopurinol. PBMCs were isolated by density gradient separation using lympholyte-H followed by purification of monocytes using CD14⁺ magnetic bead (Miltenyi Biotec, Germany) positive isolation and stored in liquid nitrogen until use. Matched serum samples were collected from each donor and stored at -80 °C. For experiments, cells were seeded at 4×10^4 cells/well in 384-tissue culture plates and allowed to adhere for 2 hours, then stimulated for 18 hours in culture media containing Pam3 +/- MSU.

Culture and stimulation of THP-1 cells

The human THP-1 monocytic cell line was cultured in RPMI-1640 media supplemented with 10% (v/v) FCS and streptomycin/penicillin (100 µg/mL and 100 U/mL, respectively). THP-1 cells were seeded at 1.6×10^5 cells/well in 96-well plates and stimulated for 18 hours with Pam3, UA or MSU before collecting cell conditioned media for analyses. The THP1-Blue cell line (Invivogen, Toulouse, France) is derived from human THP-1 monocytes with stable integration of an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct and cultured as described for THP-1 cells with the addition of 100 µg/mL of Zeocin to maintain the reporter plasmid. For experiments requiring preincubation with UA, cells were cultured in the presence of 30 mg/dL of UA in complete RPMI for 24-48 hours and then seeded and stimulated for the specified times.

Measurement of NF-κB activation in THP-1 Blue cells

THP1-Blue cells were stimulated for 20 hours with 100 ng/mL Pam3, 10-40 mg/dL UA and/or 10-20 mg/dL of MSU crystals. SEAP levels were determined using Quanti-Blue assay (Invivogen) according to the manufacturer's instructions.

Quantification of IL-1 β

IL-1 β in cell supernatants was measured by ELISA using matched anti-human IL-1 β antibodies (R&D systems).

ROS measurements

ROS generation was detected using ROS-Glo assay (Promega) according to the manufacturer's instructions (27). Briefly, cells were stimulated for 2 hours with 100 ng/mL Pam3, 10 mg/dL of MSU or 30 mg/dL of UA in the presence of 25 μ M ROS-Glo H₂O₂ substrate and ROS levels determined by the addition of ROS-Glo detection reagent. Luminescence was measured by spectrophotometry.

Measurement of serum uric acid

Serum UA concentrations were measured using an Amplex Red-based uric acid/uricase assay kit (ThermoFisher) according to the manufacturer's instructions. Fluorescence of Amplex red reagent was measured at ex530/em590nm.

RNA extraction, reverse transcription and absolute RT-qPCR

Monocytes were washed twice in PBS, then RNA extracted using RNeasy kits (Qiagen) and reverse transcribed to cDNA using QuantiTect reverse transcription kits (Qiagen, UK) according to the manufacturer's instructions. The qPCR assays for absolute quantification of gene expression were purchased from qStandard (London, UK). Copy numbers for *NLRP3*, *CASP1*, *IL1 β* and *PYCARD* (gene name for ASC) were determined by Quantitative real-time RT-PCR (qPCR) using the QuantiFast SYBR Green PCR kit (Qiagen) on a Stratagene Mx3000 thermocycler (Agilent Technologies, UK) or Rotor-Gene thermocycler (Qiagen, Germany) under the following thermocycling program; 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds with an initial cycle of 95°C for 15 minutes. The following

primers were used; human Beta-2 Microglobulin (*B2M*), forward 5'-ctctctctttctggcctggag-3' and reverse 5'-accagacacatagcaattcag-3'; human ribosomal protein L32 (*RPL32*), forward 5'-catctccttctcggcatcat-3' and reverse 5'-accctgttgtaatgcctct-3'; human *CASP1*, forward 5'-atgcctgttctctgatgtgg-3' and reverse 5'-ctcttcaactctgccacaga-3'; human *IL1B*, forward 5'-gtaatgacaaaatacctgtggccttg-3' and reverse 5'-ttgggatctacactctccagc-3'; human *NLRP3*, forward 5'-gagatgagccgaagtggggttc-3' and reverse 5'-gcttctcacgtactttctgtacttct-3'; human *PYCARD*, forward 5'-gctaactgtctgcgcgacat-3' and reverse 5'-ccactcaacgtttgtgaccct-3'. Copy number for each gene of interest (GOI) and reference gene was calculated by interpolation from a standard curve ranging from 10^1 - 10^7 copies run on the same plate. *B2M* and *RPL32* were used as reference genes.

Statistical analysis

Differences between groups were assessed using unpaired t-tests, Mann-Whitney U-tests or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test after testing for differences in variation using the Brown-Forsythe test. Correlation analysis was performed using Pearson's correlation coefficient. The level of significance was set at $P < 0.05$. All analyses were conducted using GraphPad Prism version 7 (Graphpad software, San Diego, CA).

Results

Effect of MSU and soluble UA on activation of the NLRP3 inflammasome in human monocytes

THP-1 monocytes or primary human monocytes were used to investigate activation of the NLRP3 inflammasome in response to MSU crystals or soluble UA. The TLR2 ligand, Pam3, was used as a priming signal and secretion of IL-1 β used as a measure of NLRP3 activation. To confirm the activation of NLRP3, cells were stimulated in the presence of MCC950, a chemical inhibitor of NLRP3. In both THP-1 monocytes (Supplementary Fig. 2A) and primary human monocytes (Supplementary Fig. 2B) MCC950 inhibited IL-1 β secretion in response to MSU crystals by 90%, confirming NLRP3-dependant secretion. Small increases in secretion of IL-1 β were observed in response to Pam3 alone, with

significantly greater release of IL-1 β in the presence of MSU in both THP-1 monocytes (Fig. 1A) and primary human monocytes (Fig. 1C). In contrast, addition of soluble UA did not increase Pam3-induced IL-1 β secretion in either THP-1 cells (Fig. 1B) or primary human monocytes (Fig. 1D). THP-1 Blue cells were used to investigate whether MSU or soluble UA had any effect on priming of the NLRP3 pathway. Neither MSU (Fig. 1E) nor soluble UA (Fig. 1F) treatment alone had any effect on activation of NF- κ B in these cells, although the higher concentration of MSU did increase Pam3-induced NF- κ B.

In hyperuricemic individuals, circulating monocytes are exposed to elevated concentrations of soluble UA for prolonged periods. We therefore hypothesised that monocytes exposed to soluble UA prior to stimulation could have altered priming and activation of the NLRP3 inflammasome. To test this, primary human and THP-1 monocytes were exposed to soluble UA (30 mg/dL) for up to 48 hours. This concentration of soluble UA is well in excess of the accepted range for hyperuricaemia (>6.8 mg/dL). Longer incubation times were tested in THP-1 cells compared with primary human monocytes due to decreases in viability in primary cells after 24 hours culture. Pre-incubation of primary human monocytes with soluble UA for 10 hours (Fig. 2A), or THP-1 cells for up to 24-48 hours (Fig. 2B&C) had no effect on the release of IL-1 β from these cells. Furthermore, activation of NF- κ B in response to Pam3 +/- MSU was also unaffected by pre-incubation with soluble UA for 24 hours (Supplementary Fig. 3A&B) or 48 hours (Fig. 3D&E).

Induction of ROS by soluble uric acid and MSU crystals in primary human monocytes

To assess whether MSU crystals or soluble UA can induce ROS generation, we measured ROS levels in human monocytes. Initial experiments were done using catalase to decrease ROS or MSU to increase ROS to confirm that the ROS-Glo assay could indeed measure changes in ROS (Fig. 3A) and to determine the optimal timing for measurement of ROS in these cells (Fig. 3B). Short-term (2h) treatment with both MSU and soluble UA increased ROS generation in primary human monocytes (Fig. 3C), an effect which was not seen when cells were pre-incubated with 30 mg/dL of soluble UA for 16 hours prior to stimulation with Pam3 +/- MSU or UA (Fig. 3D).

Activation of the NLRP3 inflammasome in monocytes isolated from gout patients

Susceptibility to gout attacks could be related to increased cellular sensitivity to MSU crystals. To test this hypothesis, monocytes were isolated from the blood of individuals with a previous history of gout attacks and stimulated for 18 hours with Pam3 +/- MSU crystals. Serum UA concentration was significantly higher in patients with gout ($n=15$) compared to healthy controls ($n=16$) (Fig. 4A), although only 27% of the subjects with gout were clinically hyperuricemic at the time of sampling.

There were no significant differences in IL-1 β secretion between monocytes from gout patients and healthy controls (Fig. 4B and C), although there was a trend towards increased responsiveness to MSU in monocytes from gout patients when the IL-1 β secretion data was normalised to secretion induced by Pam3 alone for each donor (Fig. 4D). However, IL-1 β secretion from these cells did not correlate with serum uric acid concentration (Supplementary Fig. 4). To determine whether this trend could be explained by increased expression of genes coding for components of the NLRP3 inflammasome, qPCR was used to determine constitutive expression levels of caspase-1 (*CASP1*), pro-IL-1 β (*IL1B*), ASC (*PYCARD*) and *NLRP3* in the absence of a stimulus. There were no differences in the expression of any of the genes tested between healthy controls and gout patients (Fig. 5A-D). Caspase-1 and pro-IL-1 β transcripts were expressed at 10-fold greater levels than the *NLRP3* transcripts. Finally, there was no correlation between serum UA concentration and expression of the inflammasome genes measured (Fig. 5D-F) in either healthy controls or gout patients.

Discussion

There are a limited number of studies assessing the effects of uric acid in primary human cells but the available data suggests that any effect of UA is cell-type specific and much more subtle than the well-documented effects of crystallized UA. Thus, UA had no effect on release of IL-1 β or TNF from human monocyte-derived macrophages but at concentrations of 50 mg/dL did induce secretion of IL-1 β from PBMCs (22). However, these results were not recapitulated in monocytes where incubation with UA

had no effect on IL-1 β similar to the results shown here, but did increase IL-1 β production when cells were subjected to stimulation with LPS +/- MSU after the initial incubation with UA (23).

Clearly, the responses of isolated monocytes are different to those of PBMCs, which is a mixed population of monocytes and lymphocytes and contain on average approximately 8-10% of monocytes. The differences in responses to uric acid between human monocytes and PBMCs cannot be explained simply by differences in expression of NLRP3 as this is substantially lower in lymphocytes than in monocytes (28). However, it has previously been shown that UA can activate T cells in an antigen-independent manner (29). Perhaps there are interactions between activated T cells and monocytes that cause the monocytes in the PBMC mixture to respond to UA by secreting IL-1 β , an effect not seen in pure monocyte cultures. This possibility deserves further investigation as it may provide important insights into the activation of monocytes during hyperuricemia.

The monocytes from gout patients studied here showed a tendency to produce more IL-1 β as a result of treatment with MSU. Serum UA concentration was higher in gout patients than in healthy donors and could indicate that constant exposure to UA may predispose monocytes to produce more IL-1 β upon stimulation, a theory supported by previous studies conducted with PBMCs (22, 30). However, despite the trend for increased responsiveness to Pam3 + MSU in the monocytes from gout patients observed here, this was not mirrored by increases in the levels of gene expression of components of the NLRP3 inflammasome nor did these levels correlate with serum uric acid concentration. These data suggest that there are sufficient levels of NLRP3 inflammasome components at the protein level to allow secretion of IL-1 β in the absence of upregulated gene expression. Importantly, it also suggests that exposure of monocytes to uric acid does not result in increased gene expression, perhaps explaining why hyperuricemia alone is insufficient to cause IL-1 β -mediated inflammation.

Increased expression of *NLRP3*, *PYCARD*, *CASPASE-1* and *IL-1 β* does occur in conditions such as atherosclerosis (31), Sjögren's syndrome (32) and preeclampsia (33), so it is intriguing that we found no increases in basal expression of these genes in gout patients, given the importance of IL-1 β in the

disease process in gout. Of note, expression of *NLRP3* was relatively low compared to both *IL-1B* and *CASP1* consistent with the requirement for TLR-induced transcriptional priming and translation of *NLRP3* prior to oligomerization and activation of the *NLRP3* inflammasome (15).

The increased ROS in response to UA in the monocytes studied here was intriguing, given the well-known antioxidant effects of UA (34). However, UA is also known to have pro-oxidant effects depending on context and location, functioning as a pro-oxidant in intracellular spaces and as an antioxidant in the extracellular environments (35). This raises the question of whether UA enters the monocytes over the course of the incubations or whether UA is exerting these effects on ROS by influencing the extracellular redox environment. Longer incubations with UA did not result in increased ROS in the cells and this could have been due to increases in cellular anti-oxidant activity as a compensatory measure. It is currently unclear whether or not UA can enter monocytes. UA is taken up by adipocytes (36) and VSMCs (37) via specific urate transporters and UA entry into these cell types results in increased ROS. Leukocytes do express the Glut9a urate transporter (38) that is associated with regulation of plasma urate levels, but there are no available data on whether Glut9a actually transports UA in these cells.

The fact that we see increased ROS in monocytes upon incubation with UA, together with the epigenetic changes that occur in monocytes incubated with UA (22) could point to intracellular effects of UA in these cells. However, both of these observed effects could also be mediated by UA-induced changes in the extracellular environment that causes changes in the redox state of membrane receptors/proteins that then exert intracellular effects.

The increases in ROS observed after incubation with UA did not influence IL-1 β secretion, suggesting that ROS are dispensable for activation of the *NLRP3* inflammasome. The role of ROS in IL-1 β production is controversial, with some studies showing that activation of the *NLRP3* inflammasome is reliant on ROS (39, 40), but others showing that ROS are dispensable in this process (19, 41, 42). Thus,

our findings are in agreement with the view that ROS are not involved in IL-1 β production in monocytes.

Our results are consistent with previously published work showing that incubation of primary human monocytes with UA did not result in IL-1 β secretion (23). We did not see any effect of UA on subsequent stimulation of cells with Pam3 +/- MSU as shown by others (23) because we only performed these experiments in THP-1 cells. The mechanism by which pre-incubation with UA increases IL-1 β secretion upon subsequent stimulation with Pam3 is via downregulation of IL-1Ra which allows increased binding of the IL-1 β produced to the IL-1R (22). Crucially, THP-1 cells do not express the IL-1R (43) and so cannot respond to IL-1 β in this manner. Thus, any downregulation of IL-1Ra in response to UA has no effect in our assays.

What then is the relationship between hyperuricaemia and susceptibility to gout? There was no correlation between serum UA and production of IL-1 β in monocytes from gout patients and our results also appear to demonstrate that crystallisation of UA into MSU is necessary for activating the NLRP3 inflammasome in these cells. One possibility is that hyperuricaemia results in higher levels of oxidative stress where there are greater levels of ROS than can be dealt with via antioxidant mechanisms. The relationship between oxidative stress and inflammation is complex, but these two states tend to potentiate each other (44, 45). It may be that there are less efficient antioxidant mechanisms operating in gout patients than in asymptomatic hyperuricaemic individuals resulting in higher levels of oxidative stress that could contribute to greater sensitivity to the existence of MSU crystals. As the presence of MSU crystals within joints does not always result in a gout attack it may be the case that a second trigger, perhaps increased oxidative stress, is required to activate the NLRP3 inflammatory cascade. Indeed, this may also explain the association between gout and metabolic syndrome which is also characterised by increased oxidative stress and increased inflammation (46).

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Figure legends:

Figure 1. Priming and activation of the NLRP3 inflammasome in human monocytes. THP-1 (A&B) or primary human monocytes (C&D) were stimulated for 18 h with Pam3 ± MSU crystals or soluble UA. IL-1 β secretion was determined by ELISA. THP1-blue monocytes (E&F) were stimulated for 20 h with Pam3, soluble UA or MSU crystals. NF- κ B activation was determined via QUANTI-Blue assay. Data represents means from 3-4 separate experiments (THP-1 and THP1-Blue cells) or 3-5 individual donors (primary monocytes) ± S.E.M. Significance determined by one-way ANOVA or unpaired t-test (A&B) (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Figure 2. Effect of pre-incubation with soluble UA on priming and activation of NLRP3 in human monocytes. (A) Primary human monocytes were incubated with RPMI or soluble UA (30mg/dL) for 10 h prior to treatments indicated for 12 h. THP-1 monocytes were incubated with soluble UA for 24 h (B) or 48 h (C) prior to indicated treatments for 18 h. IL-1 β secretion was determined by ELISA. THP1-Blue monocytes were pre-exposed to RPMI or soluble UA for 48 hours (D&E), followed by 20 h stimulation. NF- κ B activation was determined via QUANTI-Blue assay. Data represent means ± S.E.M for 4 separate donors or 3-4 separate experiments. Significance determined by unpaired t-test.

Figure 3. Production of ROS in primary human monocytes. Primary human monocytes were treated with catalase ± MSU crystals for 2 hours (A) or MSU (10 mg/dL) (B) for 6 hours and ROS levels measured by ROS-Glo assay. Primary human monocytes were exposed to Pam3 ± MSU or soluble UA for 2 h (C) or for 2 h following a 16 h pre-incubation (D) in RPMI or soluble UA. ROS generation was measured using the ROS-Glo luminescent based assay from Promega™. Data represent mean of triplicate measure from a single donor (A&B) or represent mean values ± S.E.M from 4-6 separate donors (C&D). Significance was determined by one-way ANOVA or unpaired t-test.

Figure 4. Serum UA concentration and stimulation of monocytes from gout patients or healthy controls. (A) Uric acid concentration in serum from donors was measured by Amplex red uric acid

assay. Monocytes were isolated from whole blood and exposed to Pam3 (B) or Pam3 + MSU crystals (C). IL-1 β secretion was determined by ELISA. IL-1 β secretion in response to MSU was analysed by normalisation to secretion induced by Pam3 alone (D). Data represent means from 3-6 repeats for each separate donor. Error bars represent average of all donors \pm S.E.M. Significance was analysed using a two-tail unpaired Mann-whitney test (*P<0.05).

Figure 5. Expression of NLRP3 inflammasome components in monocytes from gout patients or healthy controls. cDNA was prepared from human monocytes, which were isolated from whole blood. Expression of *CASP1* (A), *IL1B* (B), *NRLP3* (C) and *PYCARD* (D) was analysed by qPCR. Absolute copy numbers were calculated using standard curves for each gene of interest and normalised to expression of B2M and RPL32. Each point represents an average of 2-3 replicates. Error bars are derived from mean of all samples \pm S.E.M. Significance was analysed using a two-tail unpaired Mann-Whitney test or by Spearman's correlation coefficient analysis.