Fingolimod Phosphate (FTY720-P) Activates Protein Phosphatase 2A in Human Monocytes and Inhibits Monosodium Urate Crystal-Induced IL-1β Production

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Running Title:

PP2A is a novel therapeutic target in gout.

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Text pages: 36
Tables: 0
Figures: 5
References: 47
Abstract: 249 words
Introduction: 701 words
Discussion: 1,494 words
Abbreviations:

BMDM: Bone marrow derived macrophages; CD44: Cluster determinant 44; C\textsubscript{T}: Cycle threshold; ELISA: Enzyme linked immunosorbent assay; FBS: Fetal bovine serum; LDH: Lactate dehydrogenase; LPS: Lipopolysaccharide; I\kappa B\alpha: Inhibitor kappa B subunit alpha; IKK\beta: Inhibitor of nuclear factor kappa-B kinase subunit beta; IL-1\beta: Interleukin-1 beta; IL-8: Interleukin-8; MCP-1: Monocyte chemoattractant protein-1; MSU: Monosodium urate monohydrate; NLRP3: NOD-, LRR- and pyrin domain containing protein 3; NF-\kappa B: Nuclear factor kappa B; NSAIDs: Non-steroidal anti-inflammatory drugs; PBMCs: Peripheral blood mononuclear cells; PBS: Phosphate buffered saline; PP2A: Protein phosphatase 2A; PPP2CA: Protein phosphatase 2 catalytic subunit alpha; pro-IL-1\beta: pro-interleukin-1 beta; ROS: Reactive oxygen species; RT-qPCR: Real time quantitative polymerase chain reaction; sUA: Soluble uric acid; S1P: Sphingosine-1-phosphate; S1P\textsubscript{1}: Sphingosine-1-phosphate receptor 1; TLR: Toll-like receptor; TNF-\alpha: tumor necrosis factor alpha.

**Recommended section assignment:** Inflammation, Immunopharmacology and Asthma
Abstract

Gout is a chronic inflammatory arthritis caused by monosodium urate monohydrate (MSU) crystal deposits in joints of lower limbs. Phagocytic uptake of MSU crystals by joint-resident macrophages and recruited circulating monocytes results in IL-1β expression and production. Current acute gout treatments have serious toxicities and suffer suboptimal clinical outcomes. Protein phosphatase 2A (PP2A) plays an important role in regulating signaling pathways relevant to inflammation. We hypothesized that innate immune danger signals e.g. lipopolysaccharide (LPS) and soluble uric acid (sUA) prime human monocytes towards MSU crystal phagocytosis, and increased IL-1β production mediated by a reduction in PP2A activity and restoring PP2A activity exerts an anti-inflammatory effect in this setting. Priming monocytes with LPS + sUA increased cytosolic pro-IL-1β and mature IL-1β and enhanced MSU crystal phagocytosis and its downstream IL-1β expression (p<0.001). A combination of LPS + sUA priming and MSU crystals reduced PP2A activity in monocytes by 60% (p=0.013). PP2A catalytic subunit gene knockdown reduced PP2A activity and exacerbated MSU crystal induced IL-1β expression and secretion (p<0.0001). Fingolimod (FTY720) and its active metabolite, fingolimod phosphate (FTY720-P) were evaluated for their ability to activate PP2A in human monocytes over 24 h. FTY720 and FTY720-P activated PP2A to a similar extent, and maximal enzyme activity occurred at 24 h for FTY720 and at 6 h for FTY720-P. FTY720-P (2.5 μM) reduced pro-IL-1β production and IL-1β secretion in primed and MSU crystal stimulated monocytes (p<0.0001) without changing the magnitude of crystal phagocytosis. We conclude that PP2A is a promising new target in acute gout.
Significance Statement:

The activity of protein phosphatase 2A (PP2A) is implicated in the enhanced expression and production of IL-1β by human monocytes in response to priming with soluble uric acid, lipopolysaccharide and phagocytosis of monosodium urate monohydrate (MSU) crystals. Fingolimod phosphate activates PP2A in human monocytes and reduces cytosolic pro-IL-1β content and its conversion to biologically active IL-1β in human monocytes exposed to MSU crystals.
Introduction

Gout is a common and often debilitating inflammatory arthritis which is characterized by self-limiting episodes of rapid-onset acute flares of intense pain and inflammation, in the midst of asymptomatic periods which may range in duration between few days and several years (Grassi and DeAngelis, 2012; Pascart and Liote, 2019). The pathogenesis of gout is attributed to the deposition of monosodium urate monohydrate (MSU) crystals in joints of lower limbs (Busso and So, 2010; Pascual et al., 2015; Stewart et al., 2016). Uric acid is a byproduct of purine base metabolism and is predominantly excreted by the kidneys (Stewart et al., 2016). Owing to its low solubility, uric acid tends to precipitate in the form of needle-shaped crystals when serum uric acid levels surpass its saturation concentration (Martillo et al., 2014). Soluble uric acid may also have a direct proinflammatory effect on different cell types including peripheral blood mononuclear cells (PBMCs) and macrophages, which contributes to the clinical picture of gout (Crisan et al., 2016; Braga TT et al., 2017). Drugs indicated in acute gout include colchicine, NSAIDs and corticosteroids (Narang and Dalbeth, 2018). During the asymptomatic phase of gout, serum urate lowering therapies are prescribed to reduce the likelihood of an acute gout flare (Chaichian et al., 2014). The use of colchicine, NSAIDs and corticosteroids in acute gout is associated with considerable side effects and toxicities and a significant population of patients have relative contraindications for these drugs (Janssens et al., 2008; Van Echteld et al., 2014; Van Durme CM et al., 2015). Not surprisingly, inadequate control of acute gout is prevalent and suboptimal clinical outcomes with existing therapies highlight the need for new therapeutics with novel mechanisms of action (Hutton et al., 2009; Dalbeth et al., 2017).

Joint-resident macrophages initiate inflammation in acute gout following their phagocytosis of MSU crystals (Martin et al., 2009; Busso and So, 2010). Toll-like receptors 2 and 4 (TLR2 and
TLR4) and CD44 receptor mediate MSU crystal phagocytosis by macrophages (Liu-Bryan et al., 2005; Bousoik et al., 2020). In macrophages, MSU crystals activate the NOD-, LRR- and pyrin domain containing protein 3 (NLRP3) inflammasome, a multi-protein complex of the NLRP3 protein, ASC adaptor protein and recruited procaspase-1 enzyme (Amaral et al., 2012; Swanson et al., 2019). Procaspase-1 is converted to active caspase-1, which in turn catalyzes the conversion of pro-interleukin-1 beta (pro-IL-1β) to mature IL-1β (Swanson et al., 2019), which is the effector cytokine in acute gout (So et al., 2018). Chemokines e.g. monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) are also secreted in the joint in response to MSU crystals, resulting in the recruitment of peripherally circulating monocytes to the inflamed joint (Martin et al., 2009; Busso and So, 2010). Activated monocytes contribute to inflammation by virtue of MSU crystal phagocytosis and their differentiation into proinflammatory macrophages (Martin et al., 2011). Monocyte priming, activation and migration to inflamed joints in acute gout might be facilitated by TLR4 stimulation and hyperuricemia (Martin et al., 2011; Grainger et al., 2013).

Our lab has recently demonstrated that an antibody-mediated cleavage of CD44 extracellular domain inhibited MSU crystal phagocytosis by macrophages and independently attenuated IL-1β gene expression in a mechanism that involved the activation of protein phosphatase 2A (PP2A) (Bousoik et al., 2020). In that study, inhibition of PP2A in macrophages abolished the anti-inflammatory activity of the CD44 antibody (Bousoik et al., 2020). Thus, we aimed to comprehensively investigate the role of PP2A, a cytosolic serine/threonine phosphatase that is expressed in multiple organs and the immune system (Reynhaut and Janssens, 2019), in the activation of peripheral blood monocytes by MSU crystals. We hypothesized that soluble uric
acid (sUA) and lipopolysaccharide (LPS) facilitate peripheral blood monocyte activation by MSU crystals where PP2A is a critical regulator of this process. We evaluated whether priming of monocytes by sUA and LPS enhanced MSU crystal phagocytosis by THP-1 monocytes and downstream NLRP3 inflammasome activation, IL-1β expression and production. We also assessed the regulatory role of PP2A in monocyte activation using a combination of protein phosphatase catalytic subunit gene silencing and pharmacologic activation by fingolimod phosphate (FTY720-P) (Rahman et al., 2016), and we studied the impact of FTY720-P treatment on the production of pro-IL-1β and its subsequent conversion to mature IL-1β in MSU-challenged human monocytes.

**Materials and Methods**

Human THP-1 monocytes (ATCC, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) to a density of 1.5 x 10^6 cells/mL in 37°C and 5% CO₂. The human THP-1 leukemic cell line retains its monocytic properties including its phagocytic activity against sheep red blood cells (Tsuchiya et al., 1980). Throughout our experiments, THP-1 monocytes were used at 1.0 x 10^6 cells/well density in serum-free medium, unless otherwise indicated. We have utilized THP-1 monocytes between the 3rd and 9th passages. All our experiments were designed to address the aims of the study using a pre-determined sample size of 3-4 independent experiments with duplicate wells per experimental group. The pre-determined sample sizes were based on our previous experience evaluating the anti-inflammatory efficacy of anti-CD44 antibody in an MSU-stimulated bone marrow derived macrophage (BMDM) model, where a statistically and a biologically significant effect was obtained with 3-4 independent experiments (Bousoik et al., 2020). However, when assaying
intracellular mature IL-1β levels in human monocytes, we used a sample size of 5 independent experiments to account for the lower intracellular mature IL-1β levels vis-à-vis secreted IL-1β levels.

Assessment of phagocytic activity of THP-1 monocytes against FITC-labeled rabbit IgG-coated latex beads and monosodium urate monohydrate (MSU) crystals following priming with lipopolysaccharide (LPS) and soluble uric acid (sUA).

THP-1 monocytes were primed with LPS (10 ng/mL) (Invivogen, USA), soluble uric acid (sUA) (50 mg/dL; Sigma-Aldrich, USA) or a combination of LPS and sUA for 24 h. Uric acid was solubilized in warm serum-free RPMI 1640 medium. Latex beads-rabbit IgG-FITC complex (7.5μL/well) (Phagocytosis Assay Kit, Cayman Chemicals, USA) were added to THP-1 monocytes and incubated for 4 h at 37°C. Subsequently, cells were centrifuged for 5 min and washed three times with PBS and re-suspended in the assay buffer. Mean cell associated fluorescence, measured using a BD FACSVerse flow cytometer, was used to quantitatively determine the extent of latex beads phagocytosis by monocytes. Assessment of MSU crystal (pyrogen-free crystals, Invivogen, USA) phagocytosis by THP-1 monocytes ± LPS and/or sUA pre-treatments was performed as we have previously described (Qadri et al., 2018; Bousoik et al., 2020). We have utilized an indirect method of determining MSU phagocytosis by analyzing the change in monocyte side-scatter distribution due to crystal phagocytosis using a flow cytometer. Two regions of interest were identified; P1 representing the monocyte population in the absence of MSU exposure and P2 representing the monocyte population with increased side scatter attributed to MSU phagocytosis (Qadri et al., 2018; Bousoik et al., 2020). MSU crystal-
positive cells were calculated as the ratio of cells in the P2 region to the sum of cells in the P1 and P2 regions. We have previously validated this indirect method of MSU crystal phagocytosis assessment against the direct method of visualizing MSU crystal uptake by THP-1 macrophages under a microscope (Bousoik et al., 2020). Throughout our experiments, we utilized MSU crystals at 100 μg/mL, a concentration that induced significant IL-1β expression and production while causing less than 10% cytotoxicity (Bousoik et al., 2020). MSU crystals were incubated for 4 h with THP-1 monocytes that were previously primed with LPS and/or sUA followed by cell collection and centrifugation. THP-1 monocytes were washed with PBS and MSU phagocytosis was determined as described above and expressed as MSU crystal-positive monocytes (%).

Interleukin-1 beta (IL-1β) expression, secretion, protein phosphatase 2A (PP2A) expression and activity in THP-1 monocytes that were primed with lipopolysaccharide (LPS) and/or soluble uric acid (sUA) and subsequently stimulated with monosodium urate monohydrate (MSU) crystals.

THP-1 monocytes were primed with LPS and/or sUA as described above. Subsequently, cells were stimulated with MSU crystals for 6 h followed by RNA isolation, cDNA synthesis and RT-qPCR as previously described (Qadri et al., 2018). Genes of interest included IL-1β (Hs01555410_m1), protein phosphatase 2 catalytic subunit alpha (PPP2CA) (Hs00427260_m1) and GAPDH (Hs02786624_g1) was used as a reference gene. All primers and probes are commercially available (ThermoFisher Scientific, USA). The cycle threshold (Ct) values of genes of interest were normalized to the Ct value of GAPDH in the same sample, and the relative expression in the different experimental groups compared to untreated controls.
was computed using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Secreted IL-1β levels were determined using an ELISA (R&D Systems, USA). In a separate set of experiments, priming with LPS and sUA was performed as described above followed by a 6 h MSU crystal incubation. THP-1 monocytes were subsequently lysed using RIPA buffer supplemented with a protease inhibitor (ThermoFisher Scientific). Protein levels were quantified using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). A total of 5 μg protein was utilized to determine PP2A activity using the PP2A immunoprecipitation kit (Sigma Aldrich, USA). Protein samples were incubated with anti-PPP2CA antibody (4μL) and protein A agarose slurry (30μL) in a total volume of 120μL assay buffer for 3 h at 4°C. Subsequently, agarose beads were washed three times with tris buffered saline and then beads were incubated with a phosphopeptide substrate for 10 min at 37°C. The supernatant was then incubated with a malachite green phosphate detection solution and absorbance was measured at 650 nm.

Protein phosphatase 2 catalytic subunit alpha (PPP2CA) silencing in THP-1 monocytes and its impact on interleukin-1 beta (IL-1β) expression and secretion following stimulation with monosodium urate monohydrate (MSU) crystals.

THP-1 monocytes were cultured in Opti-MEM reduced serum medium (ThermoFisher Scientific) and treated with PPP2CA siRNA or scramble siRNA (negative control) (ThermoFisher Scientific) to a final concentration of 3μM in Lipofectamine 3000 transfection reagent (9μL per well; ThermoFisher Scientific) for 24 h at 37°C. PPP2CA silencing was confirmed using a combination of RT-qPCR and PP2A activity as described above. Following
PPP2CA silencing, THP-1 monocytes were treated with MSU crystals for 6 h and IL-1β expression and production were determined as described above.

Protein phosphatase 2A (PP2A) activity in THP-1 monocytes following treatment with fingolimod hydrochloride prodrug (FTY720) or its active metabolite fingolimod phosphate (FTY720-P).

Fingolimod hydrochloride (FTY720) and fingolimod phosphate (FTY720-P) (Cayman Chemicals, USA) were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 500μM and used to treat THP-1 monocytes at a final concentration of 2.5μM. FTY720-P at 2.5μM was previously shown to produce maximal PP2A activation in A549 cells over a 6 h treatment period (Rahman et al., 2016). THP-1 monocytes were treated for 1, 2, 6 and 24 h and PP2A activity was measured as described above. Total PP2A activity over 24 h following incubation with FTY720 or FTY720-P was determined by computing the area under the curve (AUC) of liberated phosphate and the time of maximal PP2A activity (T_{max}) was also determined using Prism 8 software (GraphPad, USA).

Impact of fingolimod phosphate (FTY720-P) treatment on monosodium urate monohydrate (MSU) crystal phagocytosis and downstream activation of NLRP3 inflammasome, interleukin-1 beta (IL-1β) expression, pro-IL-1β production, generation and secretion of mature IL-1β in THP-1 monocytes.
THP-1 monocytes were primed with LPS + sUA as described above. THP-1 monocytes were subsequently pre-treated with FTY720-P (2.5μM) for 3 h prior to the addition of MSU crystals for another 3 h to determine cellular NLRP3 protein, caspase-1 activity, pro-IL-1β and mature IL-1β levels. Alternatively, MSU crystals were incubated with THP-1 monocytes for 6 h followed by assaying cell viability, MSU crystal phagocytosis, PPP2CA and IL-1β expression, PP2A activity and secreted IL-1β levels. THP-1 cell viability (25,000 cells per well) was determined using the lactate dehydrogenase (LDH) release assay (LDH Assay Kit; Abcam; USA) and percent cytotoxicity was calculated. PPP2CA and IL-1β expression levels, PP2A activity and secreted IL-1β concentrations were determined as described above. Commercially available ELISA kits were used to determine cellular NLRP3 (MyBioSource, USA), pro-IL-1β (Abcam), and mature IL-1β (R&D Systems) contents and analyte protein levels were normalized to total protein in each treatment group. Caspase-1 activity was determined using a caspase-1 specific fluorometric assay (Caspase-1 Assay, Abcam), corrected for total protein and normalized to caspase-1 activity in untreated control monocytes. In these assays, we utilized 10μg total protein per experimental group.

Statistical analyses

Statistical analyses of gene expression data were performed using ΔCt values (Ct target gene-Ct GAPDH) for genes of interest in each experimental group and data were graphically presented as fold change compared to untreated controls. AUC values are reported as means along with their 95% confidence intervals (CI). Continuous variables were initially evaluated to determine if they satisfy the requirements of parametric statistical tests. Statistical comparisons of multiple
groups were performed using analysis of variance (ANOVA) for parametric data and ANOVA on the ranks for non-parametric data followed by post-hoc Tukey’s test. A p value of < 0.05 was considered statistically significant. Data are graphically represented as scatter plot bar graphs with mean ± standard deviation indicated.

**Results**

*Priming THP-1 monocytes with lipopolysaccharide (LPS) and soluble uric acid (sUA) increased their phagocytic activity against latex beads and monosodium urate monohydrate (MSU) crystals and resulted in enhanced interleukin-1 beta (IL-1β) expression and production, and a parallel reduction in protein phosphatase 2A (PP2A) activity.*

A representative flow cytometry histogram qualitatively depicting a rightward shift in LPS + sUA primed THP-1 cell associated fluorescence following incubation with FITC-conjugated latex beads is indicative of enhanced bead phagocytosis (figure 1A). LPS + sUA primed THP-1 monocytes had a mean cell associated fluorescence that was higher than the corresponding mean of control THP-1 monocytes (the effect size was a 26% increase in cell associated fluorescence; \( p=0.016 \)) (figure 1B). In contrast, priming with LPS or sUA alone did not significantly alter cell associated fluorescence compared to untreated control THP-1 monocytes (\( p>0.05 \) for both comparisons). LPS + sUA priming also increased MSU crystal phagocytosis by THP-1 monocytes compared to control cells (\( p<0.0001 \)) (representative flow cytometry plot in figure 1C; quantitative data in figure 1D). Priming with LPS alone did not increase MSU phagocytosis by THP-1 monocytes (\( p>0.05 \)), while priming with sUA increased MSU phagocytosis by THP-1 monocytes (\( p<0.0001 \)) (representative flow cytometry plots in figure 1C; quantitative data in
The downstream effect of MSU phagocytosis by LPS + sUA primed THP-1 monocytes was a significant induction of IL-1β gene expression ($p<0.0001$ vs. control THP-1 monocytes; figure 1E) and production ($p<0.0001$ vs. control THP-1 monocytes; figure 1F). Similarly, the downstream effect of MSU crystal phagocytosis in sUA primed THP-1 monocytes was a significant elevation in IL-1β gene expression and production ($p<0.01$ vs. control THP-1 monocytes for both comparisons). MSU crystals did not change $PPP2CA$ gene expression among the different experimental groups ($p>0.05$ for all comparisons; figure 1G). However, MSU crystals reduced PP2A activity in sUA-primed THP-1 monocytes ($p=0.003$; figure 1H) and LPS + sUA primed THP-1 monocytes ($p=0.013$; figure 1H) compared to control THP-1 monocytes. The magnitude of reduction in PP2A activity in LPS + sUA primed monocytes following incubation with MSU crystals was approximately 60%.

$PPP2CA$ gene silencing enhanced interleukin-1 beta (IL-1β) gene expression and production in monosodium urate monohydrate (MSU) crystals stimulated THP-1 monocytes.

$PPP2CA$ siRNA treatment resulted in a significant reduction in $PPP2CA$ expression, approximating 62%, in THP-1 monocytes ($p<0.0001$; figure 2A). In contrast, scramble siRNA treatment did not alter $PPP2CA$ expression in THP-1 monocytes ($p>0.05$). $PPP2CA$ siRNA treatment reduced PP2A activity in THP-1 monocytes ($p=0.005$; figure 2B), with a mean 53% reduction in enzyme activity. MSU crystals did not induce IL-1β gene expression or production in control THP-1 monocytes ($p>0.05$ vs. untreated control monocytes for both comparisons; figure 2C & 2D). However, $PPP2CA$ gene silencing increased IL-1β expression and production.
(p<0.001 vs. MSU-treated control monocytes; p<0.05 vs. MSU-treated scramble siRNA monocytes; figure 2C & 2D).

Fingolimod hydrochloride (FTY720) and its active metabolite, fingolimod phosphate (FTY720-P) increased protein phosphatase 2A (PP2A) activity in THP-1 monocytes.

FTY720 and FTY720-P increased PP2A activity as early as 1 h following incubation with THP-1 monocytes and this effect continued up to 24 h (figure 3A & 3B). The calculated AUC of FTY720 was 11,597 pmol*hr (95% CI: 7,681 to 15,513), whereas the calculated AUC of FTY720-P was 10,030 pmol*hr (95% CI: 8,768 to 11,292). There was no difference in the total amount of liberated phosphate over 24 h between FTY720 treated and FTY720-P treated THP-1 monocytes (p>0.05). Peak PP2A activity in FTY720-treated monocytes occurred at 24 h while peak PP2A activity in FTY720-P-treated monocytes occurred at 6 h. We utilized FTY720-P to activate PP2A in follow-up experiments and pre-treated THP-1 monocytes with FTY720-P for 3 h prior to adding MSU crystals to allow for maximal PP2A activation.

Fingolimod phosphate (FTY720-P) treatment restored protein phosphatase 2A (PP2A) activity in monosodium urate monohydrate (MSU) crystals stimulated THP-1 monocytes and reduced interleukin-1 beta (IL-1β) gene expression and production without altering MSU crystal phagocytosis by monocytes.

To rule out the possibility that the effect of FTY720-P on THP-1 monocytes is due to a cytotoxic effect, we studied cell cytotoxicity in response to MSU crystal challenge ± FTY720-P (figure...
4A). In LPS + sUA primed THP-1 monocytes, MSU crystals increased cell cytotoxicity compared to unprimed THP-1 monocytes \((p=0.024)\), while FTY720-P treatment did not result in a cytotoxic effect in THP-1 monocytes \((p>0.05\) between MSU and MSU + FTY720-P groups; \(p>0.05\) between MSU (LPS+sUA) and MSU (LPS+sUA) + FTY720-P groups). Representative flow cytometry plots depicting the impact of FTY720-P treatment on MSU crystal phagocytosis are presented in figure 4B. FTY720-P treatment did not change the extent of MSU crystal phagocytosis by THP-1 monocytes \((p>0.05\) between MSU and MSU + FTY720-P groups; \(p>0.05\) between MSU (LPS+sUA) and MSU (LPS+sUA) + FTY720-P groups; figure 4C). Furthermore, FTY720-P treatment did not alter \(PPP2CA\) gene expression in THP-1 monocytes \((p>0.05\) between MSU and MSU + FTY720-P groups; \(p>0.05\) between MSU (LPS+sUA) and MSU (LPS+sUA) + FTY720-P groups; figure 4D). FTY720-P partially restored PP2A activity in LPS + sUA primed THP-1 monocytes following MSU crystal incubation \((p=0.04\) between MSU (LPS+sUA) + FTY720-P and MSU (LPS+sUA) groups; figure 4E). However, PP2A activity in MSU (LPS+sUA) + FTY720-P group remained lower than the corresponding activity in control THP-1 monocytes \((p=0.008\); figure 4E). FTY720-P treatment reduced IL-1\(\beta\) gene expression in MSU-treated THP-1 monocytes \((p<0.001\) between MSU (LPS+sUA) + FTY720-P and MSU (LPS+sUA) groups; figure 4F). Secreted IL-1\(\beta\) levels with FTY720-P treatment were reduced by 71%, which was both biologically and statistically significant \((p<0.0001\) between MSU (LPS+sUA) + FTY720-P and MSU (LPS+sUA) groups; figure 4G).

Monosodium urate monohydrate (MSU) crystals increased cytosolic pro-interleukin-1 beta (Pro-IL-1\(\beta\)) levels without inducing NLRP3 or caspase-1 activation in THP-1 monocytes and this effect was reversed with fingolimod phosphate (FTY720-P) treatment.
Cytosolic NLRP3 protein levels in THP-1 monocytes did not change as a result of LPS + sUA priming and subsequent addition of MSU crystals ($p>0.05$ among control (untreated), control (LPS+sUA) and MSU (LPS+sUA) groups; figure 5A). Similarly, caspase-1 activity did not change in primed THP-1 monocytes ± MSU crystals ($p>0.05$ among control (untreated), control (LPS+sUA) and MSU (LPS+sUA) groups; figure 5B). FTY720-P treatment did not change cytosolic NLRP3 level or capase-1 activity ($p>0.05$ between MSU (LPS+sUA) and MSU (LPS+sUA) + FTY720-P groups; figures 5A & 5B). LPS + sUA priming induced pro-IL-1β protein in THP-1 monocytes ($p<0.0001$ between control (untreated) and control (LPS+sUA) groups; figure 5C). The induction of pro-IL-1β by LPS + sUA resulted in an increase in intracellular mature IL-1β content in THP-1 monocytes ($p<0.0001$ between control (untreated) and control (LPS+sUA) groups; figure 5D). MSU crystals increased pro-IL-1β content, with an effect size approximating 84% ($p=0.009$ between MSU (LPS+sUA) and control (LPS+sUA) groups; figure 5C), whereas FTY720-P treatment reduced it by 69% ($p<0.001$ between MSU (LPS+sUA) and MSU (LPS+sUA) + FTY720-P groups; figure 5C). Similarly, MSU crystals increased mature IL-1β content, with an effect size approximating 79% ($p<0.0001$ between MSU (LPS+sUA) and control (LPS+sUA) groups; figure 5D), whereas FTY720-P treatment reduced it by 60% ($p<0.001$ between MSU (LPS+sUA) and MSU (LPS+sUA) + FTY720-P groups; figure 5D). In addition, FTY720-P also reduced pro-IL-1β and mature IL-1β generated by LPS + sUA priming of THP-1 monocytes ($p<0.0001$ between control (LPS+sUA) and FTY720-P (LPS+sUA) groups; figure 5C and 5D).

**Discussion**
In this study, we investigated the priming effect of sUA and LPS alone and in combination on the activation of human monocytes by MSU crystals and found that a combination of sUA and LPS enhanced MSU crystal uptake by monocytes. In the absence of a priming signal, monocytes were limited in their capacity to internalize MSU crystals and consequently release IL-1β. The enhanced uptake of urate crystals by monocytes secondary to LPS and sUA priming translated to a dramatic increase in IL-1β expression and production, and that was associated with a significant reduction in PP2A activity without a change in its catalytic subunit expression level. Our findings provide a proof-of-concept that PP2A activity in monocytes is impaired in the setting of acute inflammation. While little is known about the exact mechanism of PP2A activity impairment under inflammatory conditions, one potential mechanism is the accumulation of reactive oxygen species (ROS) leading to the oxidation of critical thiol groups in PP2A’s active site (Elgenaidi and Spiers, 2019). While we have not specifically studied the role of ROS in regulating PP2A activity in human monocytes, this mechanism may be biologically relevant in acute gout. Phagocytosis of MSU crystals by monocytes and macrophages results in the generation of ROS, which contributes to the activation of the NLRP3 inflammasome and IL-1β secretion (Pascual et al., 2015). In our study, the extent of reduction in PP2A activity was dependent on the magnitude of MSU crystal phagocytosis by THP-1 monocytes and thus it is reasonable to postulate a role for crystal-induced ROS generation in reducing PP2A activity in our cell system.

PP2A has a complex structure where approximately 30% of its content in mammalian cells exists as a dimer of a catalytic C subunit (further subdivided into α and β isoforms) and a structural A subunit (Kremmer et al., 1997; Reynhaut and Janssens, 2019). The core dimer of the enzyme
can further associate with a number of regulatory B subunits (Kremmer et al., 1997; Reynhaut and Janssens, 2019). PP2A was shown to regulate the innate immune response by macrophages where a conditional knockout of PP2A catalytic subunit enhanced TNF-α expression in LPS-stimulated murine BMDMs (Sun et al., 2017). We observed a similar role for PP2A in monocytes where IL-1β induction was evident following PP2A catalytic subunit gene knockdown. Furthermore, partial restoration of PP2A activity in MSU crystal stimulated monocytes was associated with diminution of IL-1β secretion. The mechanism by which PP2A regulates IL-1β expression is likely related to its role in regulating the nuclear factor kappa B (NF-κB) signaling axis (Bousoik et al., 2020). PP2A regulates NF-κB pathway activation via dephosphorylation of IKKβ, IκBα, and NF-κB p65 subunit (Tsuchiya et al., 2017). We have also shown that in macrophages, a PP2A inhibitor increased NF-κB nuclear translocation following MSU crystal stimulation (Bousoik et al., 2020). Therefore, it appears that PP2A is critical to the activation of cells from the monocyte/macrophage lineage by damage associated molecular patterns (DAMPs) whereby a reduction in PP2A activity results in immune cell activation by low-level exposure to innate immune signals which otherwise may not be sufficient to trigger inflammation.

Hyperuricemia is causally linked to the pathogenesis of a number of chronic diseases that have low-grade inflammation as a common feature e.g. chronic kidney disease, type 2 diabetes, coronary heart disease and hypertension (Heinig and Johnson, 2006; Johnson et al., 2013; Athyros and Mikhailidis, 2014; Zuo et al., 2016). The mechanism by which sUA causes inflammation is not entirely understood, but NLRP3 inflammasome activation may be contributory (Braga et al., 2017). The classical inflammasome activation pathway is often
described as a two-signal process with signal 1 mediated by IL-1β or TLR2/4 receptor agonists (Yang et al., 2019). At this step, transcriptional priming occurs resulting in increased cellular pro-IL-1β and the inflammasome components, while signal 2 is triggered by DAMPs e.g. MSU crystals which results in K⁺ efflux, activation of the NLRP3 inflammasome and generation of mature IL-1β (Yang et al., 2019). To evaluate whether NLRP3 inflammasome activation contributed to IL-1β release by human monocytes, we studied NLRP3 protein, pro-IL-1β, mature IL-1β contents and caspase-1 activity following MSU challenge. We failed to detect NLRP3 protein induction or caspase-1 activation upon priming of monocytes but observed increased pro-IL-1β production. NLRP3 content and capsase-1 activity remained unchanged with MSU crystal exposure, and the increase in cellular and secreted IL-1β levels caused by MSU crystals was due to a greater production of pro-IL-1β. The lack of NLRP3 induction in our MSU stimulated monocytes is in agreement with earlier observations, which suggested that NLRP3 inflammasome activation in monocytes is non-classical in nature with a gradual onset and no pyroptosome formation or pyroptotic cell death (Crisan et al., 2016; Gaidt et al., 2016; Braga et al., 2017). Pharmacologic inhibition of the NLRP3 inflammasome is a promising strategy to treat a variety of inflammatory diseases (Mangan MS et al., 2018). However, such therapeutic approach may not be useful in gout as the expression levels of NLRP3 inflammasome components in circulating monocytes from gout patients were shown not to be different from those of normal subjects (Albers BM et al., 2019). Rather, an anti-inflammatory effect in urate crystal stimulated monocytes can be achieved independent of the NLRP3 inflammasome as demonstrated with FTY720-P where it reduced IL-1β secretion by monocytes without altering caspase-1 activity or NLRP3 protein level.
FTY720 is an orally available structural analog of sphingosine that undergoes phosphorylation in vivo by sphingosine kinase 2, an enzyme that is widely expressed in cells of the immune system, to generate the active metabolite FTY720-P (Billich et al., 2003; Nofer et al., 2007; Chun and Hartung, 2010). FTY720-P modulates the egress of lymphocytes from lymphoid tissues due to its interaction with the sphingosine-1-phosphate (S1P) receptors, and this effect is clinically beneficial in patients with multiple sclerosis (Chun and Hartung, 2010). Independent of its interaction with S1P receptors, FTY720-P activates PP2A due to its ability to bind SET protein, an endogenous PP2A inhibitor that binds to its catalytic subunit, and hence FTY720-P acts to prevent SET and PP2A interaction (Sangodkar et al., 2016). FTY720 activated PP2A in THP-1 cells as sphingosine kinase 2 is highly expressed in monocytes, and thus FTY720 would have been readily converted to FTY720-P (Sangodkar et al., 2016). Both FTY720 and FTY720-P activated PP2A to a similar extent, as the total amounts of phosphate liberated from a phosphopeptide substrate were similar over a 24 h period. However, the effect of FTY720-P on PP2A activation appeared to be more immediate and peak PP2A activity was achieved at an earlier time point. The efficacy of FTY720-P was biologically significant with approximately 70% reduction in IL-1β secretion. FTY720-P neither modified the extent of MSU crystal phagocytosis nor did it exhibit a cytotoxic effect in THP-1 monocytes and therefore, it is unlikely that the observed effect of the drug was an artifact of changes in crystal phagocytosis or cell viability. FTY720-P is also known to cause sustained S1P receptor 1 (S1P₁) subtype cellular internalization and degradation in a mechanism that involves β-arrestin recruitment (Sykes et al., 2014). Our knowledge of S1P’s function in circulating monocytes under normal and inflammatory conditions is extremely limited. In contrast, our understanding of the role of S1P in tissue macrophage homeostasis has materialized over the past few years (Weigert et al., 2019).
Tissue macrophages express the five S1P receptors and S1P has a complex role in regulating macrophage phagocytosis and phenotype polarization (Weigert et al., 2019). In this study, we did not investigate whether S1P$_1$ signaling was implicated in the anti-inflammatory effect of FTY720-P, since it would not have been technically feasible given the lack of knowledge of the role of S1P$_1$ in monocytes, and the complex pharmacology of S1P$_1$ modulators (Weigert et al., 2019).

One limitation of our study is that we utilized a monocytic cell line to conduct our experiments in lieu of primary monocytes derived from patients with acute or chronic gout. The level of PP2A activity in monocytes from gout patients relative to normal volunteers remains unknown and deciphering how monocytic PP2A activity changes over time in patients with gout will be important to further appreciate the role of PP2A in regulating circulating monocyte activation and trafficking to inflamed joints. We have limited our priming protocol to 24 h to simulate an acute gout flare. Since endotoxin tolerance is a well described phenomenon in monocytes, prolonging the duration of priming beyond 24 h might have caused a suppressed IL-1β release upon subsequent MSU crystal exposure and thus potentially diminish the effect size of FTY720-P treatment (Shi et al., 2015). In conclusion, sUA and LPS combined to prime human monocytes and increase IL-1β expression and secretion, while independently enhancing MSU crystal phagocytosis and downstream IL-1β expression. The activation of monocytes by MSU crystals was associated with a reduction in PP2A activity, and PP2A’s catalytic subunit gene knockdown activated monocytes towards MSU crystals. Restoration of PP2A activity by FTY720-P was associated with an anti-inflammatory effect in MSU challenged monocytes. We conclude that PP2A is a novel therapeutic target in acute gout treatment.
Authorship Contributions

Participated in research design: Elsaid, ElSayed, Qadri

Conducted experiments: ElSayed, Qadri

Performed data analysis: Elsaid, ElSayed, Qadri

Wrote or contributed to the writing of the manuscript: Elsaid, ElSayed, Qadri
References


Footnotes

This work was supported by the National Institutes of Health National Institute of Arthritis and Musculoskeletal and Skin Diseases [Grant R01AR067748].

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No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Figure 1 Impact of lipopolysaccharide (LPS) and soluble uric acid (sUA) pre-treatments on the phagocytic activity of human THP-1 monocytes against latex beads and monosodium urate monohydrate (MSU) crystals and corresponding downstream interleukin-1 beta (IL-1β) gene expression and production, protein phosphatase 2 catalytic subunit alpha (PPP2CA) gene expression and protein phosphatase 2A (PP2A) activity. LPS (10 ng/mL) and sUA (50 mg/mL) pre-treatments were performed for 24 h. Phagocytic analysis of MSU crystals (100 μg/mL) by THP-1 monocytes was performed following a 4 h incubation and the percentage of MSU crystals-positive monocytes was determined using flow cytometry. Two regions of interest were identified: P1 representing monocytes in the absence of MSU crystals and P2 representing monocytes with increased side scatter due to MSU crystal phagocytosis. The percentage of MSU crystals-positive monocytes was determined as P1/(P1+P2). IL-1β and PPP2CA gene expressions, IL-1β protein levels and PP2A activity were determined following a 6 h incubation with MSU crystals. Data represent three to four independent experiments with duplicate wells per group. Statistical comparisons were performed using ANOVA followed by post-hoc Tukey’s test. p<0.05 was considered significant. ns: non-significant; * p<0.05; ** p<0.01; **** p<0.0001.

A. A representative flow cytometry plot showing enhanced cell-associated fluorescence in LPS + sUA pre-treated THP-1 monocytes compared to untreated control THP-1 monocytes following incubation with IgG-FITC labeled beads.

B. LPS + sUA pre-treatments increased latex beads phagocytosis by THP-1 monocytes.
C. Representative flow cytometry plots depicting monocyte populations with increased side scatter attributed to MSU crystal phagocytosis. LPS + sUA pre-treatments increased the monocyte population in the P2 region compared to no pre-treatments.

D. sUA alone and LPS + sUA pre-treatments increased MSU crystal phagocytosis by THP-1 monocytes.

E. sUA alone and LPS + sUA pre-treatments increased IL-1β gene expression in MSU-stimulated THP-1 monocytes.

F. sUA alone and LPS + sUA pre-treatments increased IL-1β secretion in MSU-stimulated THP-1 monocytes.

G. MSU crystals did not alter PPP2CA expression in THP-1 monocytes that were previously treated with LPS + sUA.

H. sUA alone and LPS + sUA pre-treatments reduced PP2A activity in MSU-stimulated THP-1 monocytes. sUA and LPS + sUA treatments did not alter PP2A activity in THP-1 monocytes not challenged with MSU crystals.

**Figure 2** Impact of protein phosphatase 2A (PP2A) catalytic subunit gene silencing on monosodium urate monohydrate (MSU) crystal-induced interleukin-1 beta (IL-1β) expression and production by human THP-1 monocytes. Gene silencing was performed using protein phosphatase 2 catalytic subunit alpha (PPP2CA) siRNA. PPP2CA or scramble siRNA treatments were conducted for 24 h and gene knockdown was confirmed by PPP2CA expression and corresponding PP2A activity. IL-1β expression and production were determined following incubation of scramble siRNA or PPP2CA siRNA-treated THP-1 monocytes with MSU crystals (100µg/mL) for 6 h. Data represent three to four independent experiments with duplicate wells
per group. Statistical comparisons were performed using ANOVA followed by post-hoc Tukey’s test. \( p<0.05 \) was considered significant. ns: non-significant; * \( p<0.05 \); ** \( p<0.01 \); *** \( p<0.001 \).

A. Mean \( PPP2CA \) expression was approximately 60% lower in \( PPP2CA \)-siRNA treated THP-1 monocytes compared to control THP-1 monocytes. \( Scramble \) siRNA treatment did not alter \( PPP2CA \) expression.

B. Mean PP2A activity was 53% lower in \( PPP2CA \)-siRNA treated THP-1 monocytes compared to control THP-1 monocytes. \( Scramble \) siRNA treatment did not alter PP2A activity.

C. \( PPP2CA \)-siRNA treatment increased IL-1\( \beta \) expression in THP-1 monocytes following MSU crystal stimulation.

D. \( PPP2CA \)-siRNA treatment increased IL-1\( \beta \) production in THP-1 monocytes following MSU crystal stimulation.

**Figure 3** Protein phosphatase 2A (PP2A) activity in THP-1 monocytes following treatment with fingolimod (FTY720; 2.5\( \mu \)M) (A) or its active metabolite fingolimod phosphate (FTY720-P; 2.5\( \mu \)M) (B). PP2A activity was determined following immunoprecipitation of PP2A from cell lysate and its activity was expressed as picomoles (pmol) of liberated phosphate. The area under the curve (AUC), calculated as mean with 95% confidence interval (CI) of liberated phosphate with FTY720 treatment was 11,597 pmol*hr. (95% CI: 7,681 to 15,513), which was not different from an AUC of 10,030 pmol*hr. (95% CI: 8,768 to 11,292) with FTY720-P treatment. Maximal PP2A activity in FTY720 and FTY720-P-treated monocytes was observed at 24 h (A).
and at 6 h (B), respectively. Data represent three independent experiments with duplicate wells per time point. Statistical comparisons were performed using ANOVA followed by post-hoc Tukey’s test. \( p<0.05 \) was considered significant. * \( p<0.05 \); ** \( p<0.01 \); *** \( p<0.001 \); **** \( p<0.0001 \).

**Figure 4** Impact of fingolimod phosphate (FTY720-P; 2.5\( \mu \)M) treatment on interleukin-1 beta (IL-1\( \beta \)) expression and production in monosodium urate monohydrate (MSU) crystal challenged THP-1 monocytes. THP-1 monocytes were pre-treated with lipopolysaccharide (LPS; 10 ng/mL) and soluble uric acid (sUA; 50 mg/mL) for 24 h followed by MSU crystals (100\( \mu \)g/mL). The viability of THP-1 monocytes in response to MSU ± FTY720-P treatment was determined using the lactate dehydrogenase (LDH) release assay. Phagocytosis of MSU crystals was determined using flow cytometry. Two regions of interest were identified: P1 representing monocytes in the absence of MSU crystals and P2 representing monocytes with increased side scatter due to MSU crystal phagocytosis. The percentage of MSU crystals-positive monocytes was determined as P1/(P1+P2). Protein phosphatase 2A (PP2A) regulation by FTY720-P was evaluated by a combination of protein phosphatase 2 catalytic subunit alpha (PPP2CA) gene expression and PP2A activity. Data represent three to four independent experiments with duplicate wells per treatment. Statistical comparisons were performed using ANOVA followed by post-hoc Tukey’s test. \( p<0.05 \) was considered significant. ns: non-significant; * \( p<0.05 \); ** \( p<0.01 \); *** \( p<0.001 \); **** \( p<0.0001 \).

A. FTY720-P treatment did not alter THP-1 monocyte viability.
B. Representative flow cytometry plots depicting monocyte populations with increased side scatter attributed to MSU crystal phagocytosis. FTY720-P treatment did not appear to change the localization of MSU crystals-positive monocytes in the P2 region.

C. FTY720-P treatment did not alter MSU crystal phagocytosis by THP-1 monocytes.

D. FTY720-P treatment did not alter PPP2CA expression in THP-1 monocytes.

E. FTY720-P treatment increased PP2A activity in MSU crystal challenged THP-1 monocytes.

F. FTY720-P treatment reduced IL-1β expression.

G. FTY720-P treatment reduced IL-1β production.

Figure 5 Regulation of the NLRP3 (Nod-, LRR-and pyrin domain-containing protein 3) protein, caspase-1 activity, pro-interleukin-1 beta (Pro-IL-1β) and mature IL-1β cytosolic levels in THP-1 monocytes that were pre-treated with lipopolysaccharide (LPS; 10 ng/mL) and soluble uric acid (sUA; 50 mg/mL) for 24 h followed by monosodium urate monohydrate (MSU) crystals (100 μg/mL) ± fingolimod phosphate (FTY720-P; 2.5μM) treatments. THP-1 monocytes were pre-treated with FTY720-P for 3 h and analyses were conducted at 3 h following MSU challenge. NLRP3, Pro-IL-1β and IL-1β protein levels were determined by ELISA. Data represent three to five independent experiments with duplicate wells per treatment. Statistical comparisons were performed using ANOVA followed by post-hoc Tukey’s test. p<0.05 was considered significant. ns: non-significant; ** p<0.01; *** p<0.001; **** p<0.0001.

A. NLRP3 protein content did not change with MSU crystal exposure.

B. Caspase-1 activity did not change with MSU crystal exposure.
C. FTY720-P treatment reduced cytosolic pro-IL-1β levels.

D. FTY720-P treatment reduced cytosolic mature IL-1β levels.
Fig. 3

A

FTY720

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*

B

FTY720-P

***

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PP2A Activity (pmol of liberated phosphate)

Control 1 h 3 h 6 h 24 h

Control 1 h 3 h 6 h 24 h