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

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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 toward MSU crystal phagocytosis and that in-creased 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 hours. FTY720 and FTY720–P activated PP2A to a similar extent, and maximal enzyme activity occurred at 24 hours for FTY720 and at 6 hours 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 and 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 that is characterized by self-limiting episodes of rapid-onset acute flares of intense pain and inflammation in the midst of asymptomatic periods that may range in duration from a few days to several years (Grassi and DeAngelis, 2012; Pascart and Liòtè, 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 and macrophages, which contributes to the clinical picture of gout (Crișan et al., 2016; Braga et al., 2017). Drugs indicated in acute gout include colchicine, nonsteroidal anti-inflammatory drugs, 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 flare.
gout flare (Chaichian et al., 2014). The use of colchicine, nonsteroidal anti-inflammatory drugs, 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). 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 after their phagocytosis of MSU crystals (Martin et al., 2009; Busso and So, 2010). Toll-like receptor (TLR) 2, 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 NLRP3 inflammasome, a multiprotein complex of the NLRP3 protein, apoptosis-associated speck-like protein containing a C-terminus caspase recruitment domain (ASC), 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 prointerleukin-1β (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 and interleukin-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 laboratory 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 (Reynhout 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 and that 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 (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% FBS to a density of 1.5 × 10^6 cells per milliliter at 37°C and 5% CO₂. The human THP-1 leukemia cell line retains its monocytic properties, including its phagocytic activity against sheep red blood cells (Teu- chiya et al., 1980). Throughout our experiments, THP-1 monocytes were used at a density of 1.0 × 10^6 cells per well in serum-free medium unless otherwise indicated. We have used THP-1 monocytes between the third and ninth passages. All our experiments were designed to address the aims of the study using a predetermined sample size of three to four independent experiments with duplicate wells per experimental group. The predetermined 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, in which a statistically and a biologically significant effect was obtained with three to four independent experiments (Bousoik et al., 2020). However, when assaying intracellular mature IL-1β levels in human monocytes, we used a sample size of five independent experiments to account for the lower intracellular mature IL-1β levels vis-à-vis secreted IL-1β levels.

#### Activity of PP2A in THP-1 Monocytes against FITC-Labeled Rabbit IgG-Coated Latex Beads and MSU Crystals after Priming with LPS and sUA

THP-1 monocytes were primed with LPS (10 ng/ml) (Invivogen), sUA (50 mg/dl); Sigma-Aldrich), or a combination of LPS and sUA for 24 hours. Uric acid was solubilized in warm serum-free RPMI 1640 medium. Latex beads-rabbit IgG-FITC complex (7.5 μl/well) (Phagocytosis Assay Kit; Cayman Chemicals) were added to THP-1 monocytes and incubated for 4 hours at 37°C. Subsequently, cells were centrifuged for 5 minutes and washed three times with PBS and resuspended in the assay buffer. Mean cell-associated fluorescence, measured using a BD FACVerse flow cytometer, was used to quantitatively determine the extent of latex bead phagocytosis by monocytes. Assessment of MSU crystal (pyrogen-free crystals; Invivogen) phagocytosis by THP-1 monocytes ± LPS and/or sUA pretreatments was performed as we have previously described (Qadri et al., 2018; Bousoik et al., 2020). We have used 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 used 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 hours 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 (%).

#### IL-1β Expression and Secretion and PP2A Expression and Activity in THP-1 Monocytes that were Primed with LPS and/or sUA and Subsequently Stimulated with MSU Crystals

THP-1 monocytes were primed with LPS and/or sUA as described above. Subsequently, cells were stimulated with MSU crystals for 4 hours, followed by RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction as previously described (Qadri et al., 2018). Genes of interest included IL-1β (Hs01555410_m1) and protein phosphatase 2 catalytic subunit α (PPP2CA) (Hs00427260_m1), and GAPDH (Hs00265956_g1) was used as a reference gene. All primers...
and probes are commercially available (ThermoFisher Scientific). The cycle threshold ($C_t$) values of genes of interest were normalized to the $C_t$ value of GAPDH in the same sample, and the relative expression in the different experimental groups compared with untreated controls was computed using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001). Secreted IL-1β levels were determined using an ELISA (R&D Systems). In a separate set of experiments, priming with LPS and sUA was performed as described above, followed by a 6-hour 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 used to determine PP2A activity using the PP2A immunoprecipitation kit (Sigma-Aldrich). Protein samples were incubated with anti-PPP2CA antibody (4 μl) and protein A agarose slurry (30 μl) in a total volume of 120 μl assay buffer for 3 hours 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 minutes at 37°C. The supernatant was then incubated with a malachite green phosphatase detection solution, and absorbance was measured at 650 nm.

PPP2CA Silencing in THP-1 Monocytes and Its Impact on IL-1β Expression and Secretion after Stimulation with 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 3 μM in LipofectAMINE 3000 transfection reagent (9 μl/well; ThermoFisher Scientific) for 24 hours at 37°C. PPP2CA silencing was confirmed using a combination of real-time quantitative polymerase chain reaction and PP2A activity as described above. After PPP2CA silencing, THP-1 monocytes were treated with MSU crystals for 6 hours, and IL-1β expression and production were determined as described above.

PP2A Activity in THP-1 Monocytes after Treatment with Fingolimod Hydrochloride Prodrug (FTY720) or Its Active Metabolite Fingolimod Phosphate (FTY720-P). Fingolimod hydrochloride (FTY720) and fingolimod phosphate (FTY720-P) (Cayman Chemicals) were dissolved in DMSO to a concentration of 500 μM and used to treat THP-1 monocytes at a final concentration of 2.5 μM. FTY720-P at 2.5 μM was previously shown to produce maximal PP2A activation in A549 cells over a 6-hour treatment period (Rahman et al., 2016). THP-1 monocytes were treated for 1, 2, 6, and 24 hours, and PP2A activity was measured as described above. Total PP2A activity over 24 hours after incubation with FTY720 or FTY720-P was determined by computing the area under the curve (AUC) of liberated phosphate, and the time of peak PP2A activity was also determined using Prism 8 software (GraphPad).

Impact of Fingolimod Phosphate (FTY720-P) Treatment on MSU Crystal Phagocytosis and Downstream Activation of NLRP3 Inflammasome, IL-1β Expression, Pro-IL-1β Production, and 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 pretreated with FTY720-P (2.5 μM) for 3 hours prior to the addition of MSU crystals for another 3 hours to determine cellular NLRP3 protein, caspase activity, pro-IL-1β, and mature IL-1β levels. Alternatively, MSU crystals were incubated with THP-1 monocytes for 6 hours, 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/well) was determined using the lactate dehydrogenase (LDH) release assay (LDH Assay Kit; Abcam), 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), 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 used 10 μg total protein per experimental group.

Statistical Analyses. Statistical analyses of gene expression data were performed using ΔCt values ($C_t$ target gene − $C_t$ GAPDH) for genes of interest in each experimental group, and data were graphically presented as fold change compared with untreated controls. AUC values are reported as means along with their 95% confidence intervals (CI). Continuous variables were initially evaluated to determine whether they satisfy the requirements of parametric statistical tests. Statistical comparisons of multiple groups were performed using ANOVA for parametric data and ANOVA on the ranks for nonparametric 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 ± S.D. indicated.

Results

Priming THP-1 Monocytes with LPS and sUA Increased Their Phagocytic Activity against Latex Beads and MSU Crystals and Resulted in Enhanced IL-1β Expression and Production and a Parallel Reduction in PPP2A Activity. A representative flow cytometry histogram qualitatively depicting a rightward shift in LPS + sUA–primed THP-1 cell-associated fluorescence after incubation with FITC-conjugated latex beads is indicative of enhanced bead phagocytosis (Fig. 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; $P = 0.016$) (Fig. 1B). In contrast, priming with LPS or sUA alone did not significantly alter cell-associated fluorescence compared with untreated control THP-1 monocytes ($P > 0.05$ for both comparisons). LPS + sUA priming also increased MSU crystal phagocytosis by THP-1 monocytes compared with control cells ($P < 0.0001$) (representative flow cytometry plots in Fig. 1C; quantitative data in Fig. 1D). Priming with LPS alone did not increase MSU phagocytosis by THP-1 monocytes ($P > 0.05$), whereas priming with sUA increased MSU phagocytosis by THP-1 monocytes ($P < 0.0001$) (representative flow cytometry plots in Fig. 1C; quantitative data in Fig. 1D). 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; Fig. 1E) and production ($P < 0.0001$ vs. control THP-1 monocytes; Fig. 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; Fig. 1G). However, MSU crystals reduced PP2A activity in sUA-primed THP-1 monocytes ($P = 0.003$; Fig. 1H) and LPS + sUA–primed THP-1 monocytes ($P = 0.013$; Fig. 1H) compared with control THP-1 monocytes. The magnitude of reduction in PP2A activity in LPS + sUA–primed monocytes after incubation with MSU crystals was approximately 60%.

PPP2CA Gene Silencing Enhanced IL-1β Gene Expression and Production in MSU Crystal–Stimulated THP-1 Monocytes. PPP2CA siRNA treatment resulted in a significant reduction in PPP2CA expression, approximating 62%, in THP-1 monocytes ($P < 0.0001$; Fig. 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 \((P = 0.005; \text{Fig. 2B})\), with a mean 53\% reduction in enzyme activity. MSU crystals did not induce IL-1\(\beta\) gene expression or production in control THP-1 monocytes \((P > 0.05)\) vs. untreated control monocytes for both comparisons; \text{Fig. 2, C and D}). However, PPP2CA gene silencing increased IL-1\(\beta\) expression and production \((P < 0.001\) vs. MSU-treated control monocytes; \(P < 0.05\) vs. MSU-treated scramble siRNA monocytes; \text{Fig. 2, C and D}).

**Fingolimod Hydrochloride (FTY720) and Its Active Metabolite, Fingolimod Phosphate (FTY720-P), Increased PP2A Activity in THP-1 Monocytes.** FTY720 and FTY720-P increased PP2A activity as early as 1 hour after incubation with THP-1 monocytes, and this effect continued up to 24 hours (\text{Fig. 3, A and B}). The calculated AUC of FTY720 was 11,597 pmol*h (95\% CI: 7681–15,513), whereas the calculated AUC of FTY720-P was 10,030 pmol*h (95\% CI: 8768–11,292). There was no difference in the total amount of liberated phosphate over 24 hours between FTY720-treated and FTY720-P-treated THP-1 monocytes \((P > 0.05)\). Peak PP2A activity in FTY720-treated monocytes occurred at 24 hours, whereas peak PP2A activity in FTY720-P-treated monocytes occurred at 6 hours. We used FTY720-P to activate PP2A in follow-up experiments and pretreated THP-1 monocytes with FTY720-P for 3 hours prior to adding MSU crystals to allow for maximal PP2A activation.

**Fingolimod Phosphate (FTY720-P) Treatment Restored PP2A Activity in MSU Crystal-Stimulated THP-1 Monocytes and Reduced IL-1\(\beta\) 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 \((P < 0.05)\). In LPS + sUA–primed THP-1 monocytes, MSU crystals increased cell cytotoxicity compared with unprimed THP-1 monocytes \((P = 0.024)\), whereas...
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 Fig. 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; Fig. 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; Fig. 4D]. FTY720-P partially restored PP2A activity in LPS + sUA–primed THP-1 monocytes after MSU crystal incubation \(P = 0.04\) between MSU (LPS + sUA) + FTY720-P and MSU (LPS + sUA) groups; Fig. 4E]. However, PP2A activity in the MSU (LPS + sUA) + FTY720-P group remained lower than the corresponding activity in control THP-1 monocytes \(P < 0.008\); Fig. 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; Fig. 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; Fig. 4G].

**Fig. 2.** Impact of PP2A catalytic subunit gene silencing on MSU crystal–induced IL-1\(\beta\) expression and production by human THP-1 monocytes. Gene silencing was performed using PPP2CA siRNA. PPP2CA or scramble siRNA treatments were conducted for 24 hours, and gene knockdown was confirmed by PPP2CA expression and corresponding PP2A activity. IL-1\(\beta\) expression and production were determined after incubation of scramble siRNA or PPP2CA siRNA–treated THP-1 monocytes with MSU crystals (100 \(\mu\)g/ml) for 6 hours. 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. \(\ast P < 0.05\); \(\ast\ast P < 0.01\); \(\ast\ast\ast P < 0.001\); \(\ast\ast\ast\ast P < 0.0001\).

**Fig. 3.** PP2A activity in THP-1 monocytes after treatment with fingleidom (FTY720; 2.5 \(\mu\)M) (A) or its active metabolite, fingleidom 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 of liberated phosphate. The AUC, calculated as mean with 95\% CI of liberated phosphate with FTY720 treatment, was 11,597 pmol/h (95\% CI: 7681–15,513), which was not different from an AUC of 10,630 pmol/h (95\% CI: 5768–11,192) with FTY720-P treatment. Maximal PP2A activity in FTY720 and FTY720-P–treated monocytes was observed at 24 hours (A) and at 6 hours (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. \(\ast P < 0.05\); \(\ast\ast P < 0.01\); \(\ast\ast\ast P < 0.001\); \(\ast\ast\ast\ast P < 0.0001\).
Fig. 4. Impact of fingolimod phosphate (FTY720-P; 2.5 μM) treatment on IL-1β expression and production in MSU crystal–challenged THP-1 monocytes. THP-1 monocytes were pretreated with LPS (10 ng/ml) and sUA (50 mg/ml) for 24 hours, followed by MSU crystals (100 μg/ml). The viability of THP-1 monocytes in response to MSU ± FTY720-P treatment was determined using the LDH release assay. Phagocytosis of MSU crystals was determined by flow cytometry using forward scatter area (FSC-A) and side scatter area (SSC-A). 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 crystal–positive monocytes was determined as P1/(P1 + P2). PP2A regulation by FTY720-P was evaluated by a combination of 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. **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 crystal–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. ns, not significant.
induced pro-IL-1β protein in THP-1 monocytes \( P < 0.0001 \) between control (untreated) and control (LPS + sUA) groups; Fig. 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; Fig. 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; Fig. 5C), whereas FTY720-P treatment reduced it by 69% \( P < 0.001 \) between MSU (LPS + sUA) and MSU (LPS + sUA) + FTY720-P (LPS + sUA) groups; Fig. 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; Fig. 5C).

### 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. Although 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). Although 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; thus, it is reasonable to postulate a role for crystal-induced ROS generation in reducing PP2A activity in our cell system.

**Fig. 5.** Regulation of the NLRP3 protein, caspase-1 activity, pro-IL-1β, and mature IL-1β cytosolic levels in THP-1 monocytes that were pretreated with LPS (10 ng/ml) and sUA (50 mg/ml) for 24 hours followed by MSU crystals (100 μg/ml) + fingolimod phosphate (FTY720-P; 2.5 μM) treatments. THP-1 monocytes were pretreated with FTY720-P for 3 hours, and analyses were conducted at 3 hours after 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. **\( P < 0.01 \); ***\( P < 0.001 \); ****\( P < 0.0001 \). (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. ns, not significant.
PP2A has a complex structure in which 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; Reynhout and Janssens, 2019). The core dimer of the enzyme can further associate with a number of regulatory B subunits (Kremmer et al., 1997; Reynhout and Janssens, 2019). PP2A was shown to regulate the innate immune response by macrophages where a conditional knockout of PP2A catalytic subunit enhanced tumor necrosis factor-α expression in LPS-stimulated murine BMDMs (Sun et al., 2017). We observed a similar role for PP2A in monocytes in which IL-1β induction was evident after 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 κB (NF-κB) signaling axis (Bousoi et al., 2020). PP2A regulates NF-κB pathway activation via dephosphorylation of inhibitor of nuclear factor κB kinase subunit β, inhibitor κB subunit α, and NF-κB p65 subunit (Tsuchiya et al., 2017). We have also shown that, in macrophages, a PP2A inhibitor increased NF-κB nuclear translocation after MSU crystal stimulation (Bousoi 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 whereby a reduction in PP2A activity results in immune cell activation by low-level exposure to innate immune signals that 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 classic 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, and signal 2 is triggered by damage-associated molecular patterns, 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 after 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 caspase-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 nonclassic in nature with a gradual onset and no pyroptosome formation or pyroptotic cell death (Crané 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 et al., 2018). However, such a therapeutic approach may not be useful in gout, as the expression levels of NLRP3 inflammasome components in circulating monocytes from patients with gout were shown not to be different from those of normal subjects (Alberts 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, which 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 because of 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 because of 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; 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-hour 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 on PP2A activity appeared to be more immediate, and peak PP2A activity was achieved at an earlier time point. The efficacy of FTY720-P on PP2A activity appeared to be more immediate, and peak PP2A activity was achieved at an earlier time point. The efficacy of FTY720-P on PP2A activity appeared to be more immediate, and peak PP2A activity was achieved at an earlier time point. The efficacy of FTY720-P on PP2A activity appeared to be more immediate, and peak PP2A activity was achieved at an earlier time point.
regulating circulating monocyte activity and trafficking to inflamed joints. We have limited our priming protocol to 24 hours to simulate an acute gout flare. Since endotoxin tolerance is a well described phenomenon in monocytes, prolonging the duration of priming beyond 24 hours might have caused a suppressed IL-1β release upon subsequent MSU crystal exposure and thus potentially diminish the effect size of PFTY720-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 toward MSU crystals. Restoration of PP2A activity by PFTY720-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: Qadri, ElSayed, Elsaid.
Conducted experiments: Qadri, ElSayed.
Performed data analysis: Qadri, ElSayed, Elsaid.
Wrote or contributed to the writing of the manuscript: Qadri, ElSayed, Elsaid.

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Martin WJ, Shaw O, Liu X, Steiger S, and Harper JL (2011) Monosodium urate crystal-induced injury to monocytes is dependent on 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 toward MSU crystals. Restoration of PP2A activity by PFTY720-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.

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