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P2Y₆ Receptor Signaling Pathway Mediates Inflammatory Responses Induced by Monosodium Urate Crystals

Hideya Uratsuji,* Yayoi Tada,* Tomohiko Kawashima,* Masahiro Kamata,* Carren Sy Hau,* Yoshihide Asano,* Makoto Sugaya,* Takafumi Kadono,* Akihiko Asahina,† Shinichi Sato,* and Kunihiko Tamaki*

Gout occurs in individuals with hyperuricemia when monosodium urate (MSU) crystals precipitate in tissues and induce acute inflammation via phagocytic cells such as monocytes. MSU crystals have been demonstrated in skin diseases such as tophaceous gout or psoriasis; however, the importance of MSU crystals in the skin is totally unknown. In this study, we found that MSU crystals, through P2Y₆ receptors, stimulated normal human keratinocytes (NHK) to produce IL-1β, IL-8/CXCL8, and IL-6. P2Y₆ receptor expression increased in MSU-stimulated NHK. Both P2Y₆-specific antagonist and P2Y₆ antisense oligonucleotides significantly reduced neutrophil influx in both mouse air pouch and peritonitis models. Thus, these results indicate that the P2Y₆ receptor signaling pathway may be a potential therapeutic target for MSU-associated inflammatory diseases, such as tophaceous gout.


Uric acid is the natural end product of purine nucleotide catabolism and is normally present in plasma in soluble form. However, beyond maximal solubility, uric acid can crystallize. These monosodium urate (MSU) crystals are well known to be the cause of gout. Gout is generally associated with hyperuricemia and is characterized by deposition of MSU crystals within joints or skin, stimulating acute inflammation. In gout, MSU crystals stimulate the production of inflammatory cytokines such as IL-1, TNF-α, and IL-6 and chemotactic factors for neutrophils such as IL-8/CXCL8 and S100A8/A9 (1–6). Because the MSU crystal-mediated pathways in monocytes/macrophages are being intensely investigated, the mechanisms are becoming clear. Recent observations point out that ingestion of MSU crystals by phagocytes stimulates the production of inflammatory cytokines and chemokines through activation of the inflammasome (7, 8).

Uric acid is released from injured or infected cells and precipitates and forms MSU crystals. These MSU crystals are known to act as danger signals that activate the immune system (9). Because epidermis is located in the most external part of the body compared with other organs, it is most frequently exposed to various stimuli and Ags such as UV irradiation, physical obstruction, pathogens, allergens, and so on. In addition, apoptosis and necrosis of keratinocytes (KC) is observed in many inflammatory skin diseases including atopic eczema, contact dermatitis, lichen planus, and toxic epidermal necrosis. Therefore, cell injury or necrosis frequently occurs in KC, and, as a result, MSU would easily precipitate within epidermis. Because uric acid is the last metabolic product of nucleic acids, MSU could also appear in skin diseases showing epidermal hyperproliferation. In fact, MSU crystals have been found in the epidermis in psoriatic lesions, in which epidermal hyperproliferation is known (10, 11).

However, precisely how MSU crystals trigger inflammation in the epidermis remains unknown. Therefore, we were prompted to examine how the MSU crystals might stimulate KC to produce cytokines and chemokines. In this study, we showed that MSU crystals could induce the production of inflammatory cytokines and chemokines and that their production was mainly mediated by the P2Y₆ receptor pathway. Furthermore, we showed that P2Y₆ receptors were essential in MSU-induced inflammation not only in KC but also in THP-1 cells, a human monocytic cell line, and in vivo in an MSU-induced inflammation model.

Materials and Methods

Cells

Normal human keratinocytes (NHK) were purchased from Kurabo (Osaka, Japan). The cells were cultured in serum-free medium, Humedia-KG2 (Karabo), supplemented with insulin (10 μg/ml), human recombinant epidermal growth factor (0.1 ng/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), and bovine pituitary extract (0.4%, v/v). THP-1 cells were purchased from American Type Culture Collection (Manassas, VA). THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin–streptomycin. THP-1 cells were treated for 3 h with 0.5 μM PMA (Sigma, St. Louis, MO) the day before stimulation. After PMA treatment, THP-1 cells were washed with PBS. This treatment increases the phagocytic properties of the cells and induces a constitutive production of pro–IL-1β. The next day, THP-1 cells were washed with PBS, and OptiMEM (Invitrogen, Carlsbad, CA) was added for stimulation.

Reagents

MSU crystals were purchased from Alexis (Lausen, Switzerland). Caspase-1 inhibitor (z-YVAD-FMK), U-73122, and U-73433 were purchased from

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The online version of this article contains supplemental material.

Abbreviations used in this article: IL-1RA, IL-1 receptor antagonist; KC, keratinocytes; MSU, monosodium urate; NHK, normal human keratinocytes; P8, propidium iodide; PLC, phospholipase C; RB2, Reactive Blue 2; siRNA, small interfering RNA.
Cytokine detection by ELISA

NHK were plated at 2.0 × 10^4 cells/well in a 24-well plate. When they reached 80% confluence, the medium was removed, and fresh medium was applied along with MSU (50, 100, 200, 500 μg/ml). After 24 h, the supernatants were collected. NHK were pretreated with IL-1RA, anti-TNF receptor, anti–TNF-α, suramin, RB2, MRS2578, U-73122, and U-73343 for 1 h before adding MSU (200 or 500 μg/ml). The supernatants were collected after stimulation with MSU for 24 h. IL-1a, IL-1b, TNF-α, IL-8/CXCL8, and IL-6 concentrations in the supernatants were measured by ELISA (R&D Systems) according to the manufacturer’s protocol. IL-1b concentration was measured by ELISA purchased from MBL (Nagoya, Japan). The effect of reagents on NHK viability was examined by trypan blue staining or MTT assay using MTT cell count kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s protocol. THP-1 cells were plated at 4.0 × 10^5 cells/well in a 24-well plate after PMA stimulation. The cells were pretreated with z-VAD-fmk, MRS2578, and U-73122 for 1 h before applying MSU (100 μg/ml). The supernatants were collected after stimulation with MSU for 6 h. IL-1b concentration in the supernatants was measured by ELISA (R&D Systems) according to the manufacturer’s protocol.

mRNA expression analysis

Total RNA was extracted with RNeasy kit (Qiagen, Valencia, CA). DNAse I (Invitrogen) was used to avoid genomic DNA contamination. We synthesized cDNA from total RNA (1 μg) using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). We performed PCR analysis as templates of the synthesized cDNA. Nucleotide sequences for RT-PCR are shown in Table I. The mixture was pre-denatured for 5 min at 94°C and then subjected to 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, then 72°C for 10 min. Amplified DNA fragments were judged from 2% agarose gel electrophoresis. For real-time RT-PCR, we used the ABI Prism 7000 sequence detection system (Applied Biosystems, Carlsbad, CA). A 50-μl reaction mixture containing cDNA diluted in RNase-free water and 2.5 μl TaqMan Gene Expression Assays Inventoried was mixed with 25 μl TaqMan Universal PCR Master Mix (2×; Applied Biosystems). Gene Expression Assays Inventoried consists of a 20× mix of unlabelled PCR primers and TaqMan MGB probes (FAM dye labeled). Assay IDs Hs99999902_m1, Hs00602548_m1, and Hs99999905_m1 were used for IL-1a, P2Y6, and GAPDH, respectively. The reaction conditions were designed as followed: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension. The threshold cycle, the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was subsequently determined. Expression was normalized to mRNA for GAPDH.

Immunoblot analysis

Cells were disrupted in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerophosphate) with 1 mM PMSF (Nacalai Tesque), 1 mg/ml leupeptin (Peptide Institute, Institute, Louisville, KY), and 1 mM sodium orthovanadate (Sigma). The concentrations of the extracted proteins were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The samples were mixed with equal volume of 2× sample buffer (100 mM Tris HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 1% 2-mercaptoethanol, v/v), boiled for 3 min, and separated by 12.5% SDS-PAGE (3 μg of protein per lane). After transfer to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), the membrane was incubated in blocking buffer (5% skim milk in 25 mM Tris/0.02% KCl/0.8% NaCl, pH 7.4; Tris-buffered saline) for 1 h at room temperature, followed by an appropriate primary Ab overnight at 4°C. The membrane was washed and incubated with a secondary Ab for 30 min, and the bands were visualized using a chemiluminescence method (LuminGLO Reagent and Peroxide; Cell Signaling). We used rabbit polyclonal anti-human P2Y6 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-human β-actin Ab (Cell Signaling, Danvers, MA) as primary Ab and anti-rabbit IgG HRP-linked Ab (Cell Signaling) as secondary Ab.

Treatment with antisense oligonucleotides

Antisense or sense oligonucleotides against P2Y6 protein were synthesized as phosphorothioate-modified oligonucleotides and were purified by high-pressure liquid chromatography. The oligonucleotides were P2Y6 antisense (5′-CATGTTGCCATCCATGCATGCG-3′), and P2Y6 sense (5′-GCTGATGGA- ATGGGACAAATG-3′). NHK were transfected with a final volume of 0.5 μM of the indicated oligonucleotides premixed with FuGENE 6 (Roche Diagnostics, Basel, Switzerland) in KG2 for 24 h. The medium was aspirated, and the cells were cultured with KG2 for 24 h and then treated with MSU (200 μg/ml) for 24 h. THP-1 cells were washed twice in PBS and cultured in serum-free RPMI (1640 medium). Cells were supplemented with 2.5% (w/v) oligofectamine (Invitrogen) and various concentrations of oligonucleotides and were incubated for 4 h. Then, 4 volumes of complete culture medium was added and cultured overnight. The next day, the cell medium was replaced with OptiMEM, and the cells were treated with MSU (100 μg/ml) for 6 h.

Transfection with small interfering RNA

All transfections were done using Lipofectamine 2000 following the manufacturer’s instructions (Invitrogen). P2Y6 small interfering RNA (siRNA; M-004579-02-0005; Thermo Scientific Dharmacon) or negative control siRNA (D-001210-05-05; Thermo Scientific Dharmacon) was transfected into 30–50% subconfluent NHK in 6-well plates with Lipofectamine 2000. Medium was changed after 6 h. After another 24 h, fresh medium with MSU 200 μg/ml was infused. After 24 h of incubation with MSU, P2Y6 mRNA and protein expressions were assayed by RT-PCR and immunoblot, respectively. Cell supernatants were collected, and IL-1α and IL-8/CXCL8 levels were assessed by ELISA.

In vivo MSU-induced mouse inflammation model

Air pouches were created on the backs of 8-wk-old female BALB/c mice (Japan SLC, Hamamatsu, Japan). Three milliliters of filtered air was injected s.c., followed by a second injection of 3 ml of filtered air after 3 d. Seven days after the first injection, 2 mg MSU crystals in 1 ml PBS or 1 ml PBS alone was injected into the air pouches. MR2578 or PBS was injected into the air pouch 1 h before MSU injection. After 9 h, the pouch fluid was harvested by injecting 1 ml PBS. Peritonitis was induced by injection of 1 mg MSU in 1 ml PBS. Equal volumes of PBS were injected into mice and served as negative controls. Six hours after injection, peritoneal cavities were harvested with 8 ml PBS. To analyze the involvement of P2Y6 receptors, mice were injected with MR2578 (5 μg/body) in 1 ml PBS 1 h before MSU. The harvested cells were aspirated with anti-mouse CD16/CD32 mAb 2.4G2 (BD Biosciences, Franklin Lakes, NJ) to block FcγRIIa receptors and stained with FTTC-conjugated Ly-6G (BD Biosciences). After propidium iodide (PI) was added to exclude dead cells, the cells were analyzed using a FACScan flow cytometer equipped with CellQuest software (FACSCalibur; BD Biosciences). The number of neutrophils was determined by measuring the number of Ly-6G+cells (−) cells. All animal experiments were approved by the Animal Research Committee of the University of Tokyo.

Data analysis and statistics

Data represent mean ± SEM. Statistical differences between two groups were determined using two-tailed Student and Aspin–Welch t tests following F-test. Differences were considered to be significant at p < 0.05.

Results

IL-1α plays a central role in MSU-induced inflammation in human KC

In monocytes, MSU is reported to induce the production of various inflammatory cytokines and chemokines including IL-1β and IL-18 (2–4). In NHK, MSU also induced the production of small but significant amounts of TNF-α and IL-1β and large amounts of IL-8/CXCL8 together with IL-18 and IL-6 (Fig. 1A). These cytokine productions were induced in a time-dependent manner. When we used soluble uric acid or uricase-digested MSU for stimulation, IL-8/CXCL8 production from NHK was impaired (Supplemental Fig. 1). These findings indicated that MSU crystals can induce the production of inflammatory cytokines and chemokines from KC, as from monocytes.

Because the activation of IL-1R is known to be essential for propagation of the inflammatory cascade triggered by MSU crystals, we next studied the importance of IL-1R in the MSU-induced production of cytokines by NHK. IL-1RA significantly inhibited the production of both IL-8/CXCL8 and IL-6 from
MSU-stimulated NHK (Fig. 1B). TNF-α is also known to be an inflammation-inducing cytokine; however, neither anti–TNF-α Ab nor anti-TNF receptor Ab inhibited the production of these cytokines, although these Abs inhibited TNF-α–induced IL-8/CXCL8 production (data not shown). These results showed that IL-1 plays a central role in inducing IL-8/CXCL8 and IL-6 production from
MSU-stimulated NHK. Although IL-1β appears to be the dominant form of IL-1 produced by monocytes, macrophages, and dendritic cells, IL-1α predominates in the epithelial cells, including KC. Thus, we investigated the production of IL-1α by MSU-stimulated NHK. We observed large amounts of IL-1α secreted from the MSU-stimulated NHK compared with the amounts of

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**FIGURE 2.** MSU increases P2Y6 receptor expression in NHK. A. The mRNA expression of P2Y receptors was compared between NHK stimulated with MSU for 24 h and no stimulation. Human reference cDNA was used as positive control for expression of P2Y receptors. RT-PCR analysis was performed using primer sets in Table I. B. Quantification of P2Y6 receptor mRNA expression in MSU-stimulated NHK normalized by GAPDH using real-time RT-PCR. Data show mean ± SEM of three independent experiments. Student t test was used to calculate p values. C. Comparison of P2Y6 receptor protein expression in NHK between no stimulation and stimulated by MSU (200 μg/ml) using immunoblot analysis.
IL-1β (Fig. 1A, 1C). MSU not only increased the IL-1α secretion but also increased IL-1α mRNA expression in a dose- and time-dependent manner (data not shown). Furthermore, anti–IL-1α neutralizing Ab, but not anti–IL-1β Ab, significantly suppressed the production of IL-8/CXCL8 and IL-6 (Fig. 1D). Additionally, when we treated NHK with caspase-1 inhibitor z-YVAD, the production of IL-1β was inhibited, however, IL-1α, IL-8/CXCL8, and IL-6 production was not (Supplemental Fig. 2). Thus, these results indicate that IL-1α-mediated signaling through IL-1R is pivotal for subsequent cytokine and chemokine secretion, such as IL-8/CXCL8 and IL-6, from NHK after MSU stimulation.

**MSU induces inflammation via P2Y receptors in human KC**

Our next interest was how MSU crystals stimulate KC. Although KC are capable of phagocytosing melanosomes (12), the result using electron microscopy did not suggest any phagocytosis of

![FIGURE 3. MSU crystals induce inflammation via P2Y6 receptors in NHK. A, IL-1α, IL-8/CXCL8, and IL-6 concentration in supernatants of NHK stimulated by MSU (200 μg/ml) for 24 h in the presence of MRS2578. B, P2Y6 receptor protein expression in NHK treated with the indicated oligonucleotides. C, IL-1α, IL-8/CXCL8, and IL-6 concentration in supernatants of NHK stimulated by MSU (200 μg/ml) for 24 h after treatment with the indicated oligonucleotides. D, IL-1α, IL-8/CXCL8, and IL-6 concentration in supernatants of NHK stimulated by MSU (200 μg/ml) for 24 h in presence of U-73122 or U-73343. Data show mean ± SEM of three or four independent experiments. A and D, *p < 0.05, **p < 0.01 (significant differences from the response to MSU alone). C, Student or Aspin–Welch t test was used to calculate p values.](http://www.jimmunol.org/)

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MSU crystals by NHK (Supplemental Fig. 3), and cytochalasin D, a phagocytosis inhibitor (8), did not suppress the production of IL-1α, IL-8/CXCL8, and IL-6 (data not shown). We speculated that NHK might be stimulated by MSU in a different manner from phagocytes; therefore, we attempted to identify the cell surface receptors for MSU on KC. IL-1RA, which inhibited IL-8/CXCL8 and IL-6 production from NHK, did not inhibit IL-1α production by MSU-stimulated NHK (data not shown), indicating that IL-1R is not the cell surface receptor for MSU on NHK. We next examined the role of P2 receptors in the MSU-induced cytokine production by NHK, as uric acid is a breakdown product of purines (ATP, GTP, and nucleic acids), and P2 receptors sense extracellular nucleotides such as ATP, ADP, UTP, and UDP (13). We first focused on the P2X7 receptors, as P2X7 receptor antagonists but not P2Y6 antagonists (13). The P2Y6 receptors mediate MSU-induced inflammation in vivo.

**P2Y6 receptors mediate MSU-induced inflammation in human KC**

To identify which of the P2Y receptors might be responsible for the stimulation of NHK by MSU, we first attempted to identify P2Y receptors expressed in NHK (Table I). As shown in Fig. 2A, the results of RT-PCR analysis revealed that NHK expressed the mRNA for P2Y1,2,6,11. MSU stimulation of NHK enhanced or induced the expression of P2Y6,12,14 but not other P2Y receptor subtypes. Among these P2Y receptors, we focused on the P2Y6 receptors, as their mRNA expression was enhanced after MSU stimulation, and both suramin and RB2 are known to be P2Y6 antagonists but not P2Y14 antagonists (13). The P2Y6 receptors were expressed constitutively in NHK, and we confirmed that MSU stimulation enhanced their expression at both mRNA and protein levels by real-time RT-PCR and immunoblot, respectively (Fig. 2B, 2C). To examine the role of P2Y6 receptors in MSU-induced production of cytokines by NHK, we used MRS2578, a specific P2Y6 antagonist (16), and P2Y6 antisense oligonucleotides. MRS2578 significantly inhibited the MSU-induced production of IL-1α, IL-8/CXCL8, and IL-6 by NHK (Fig. 3A). The P2Y6 antisense oligonucleotide decreased the P2Y6 protein expression (Fig. 3B). Furthermore, treatment of NHK with P2Y6 antisense significantly inhibited the MSU-induced cytokine production (Fig. 3C). To complement the experiments with antisense oligonucleotides, we also examined the effect of P2Y6 siRNA. The P2Y6 siRNA decreased both the P2Y6 mRNA and protein expression (Fig. 4A, 4B). As shown in Fig. 4C, P2Y6 siRNA inhibited IL-1α and IL-8/CXCL8 production. The P2Y6 receptors are known to be coupled to the activation of phospholipase C (PLC) (13). Consistent with this, U-73122, a PLC inhibitor, significantly suppressed the MSU-induced production of IL-1α, IL-8/CXCL8, and IL-6 by NHK, whereas U-73343, the inactive analogue of U-73122, did not (Fig. 3D). These results indicated that a functional P2Y6–PLC pathway plays a pivotal role in the MSU-induced inflammatory cytokine and chemokine production by KC.

**P2Y6 receptors mediate MSU-induced inflammation in THP-1 cells**

In acute gouty inflammation, it is reported that MSU crystals internalized by monocytes activate the caspase-1–activating NALP3 inflammasome, subsequently resulting in the production of IL-1β. In fact, MSU induced a large amount of IL-1β production from THP-1 cells (Fig. 5A) compared with that induced from NHK (Fig. 1A). We investigated whether P2Y6 receptors are also important in this MSU-induced IL-1β release by monocytes. P2Y6 protein expression in THP-1 cells was increased by MSU stimulation (Fig. 5A). A caspase-1–specific inhibitor (z-YVAD) completely suppressed MSU-induced IL-1β production in THP-1 cells (Fig. 5B), indicating that MSU-induced IL-1β production from THP-1 cells is dependent on caspase-1 activation, which is consistent with previous reports (17). The specific P2Y6 antagonist, MRS2578, almost completely inhibited the MSU-induced production of IL-1β by THP-1 cells (Fig. 5B). Furthermore, the treatment of THP-1 cells with P2Y6 antisense oligonucleotides significantly inhibited MSU-induced IL-1β production (Fig. 5C). Moreover, U-73122 significantly suppressed the MSU-induced IL-1β production by THP-1 cells (Fig. 5D). Thus, these results showed that the P2Y6–PLC signaling pathway mediates MSU-induced inflammatory responses not only in KC but also in monocytes.

**P2Y6 receptors mediate MSU-induced inflammation in vivo**

Finally, we examined whether the P2Y6 receptors may also be responsible for MSU-induced inflammation in vivo. In the mouse air pouch model, MSU induced neutrophil infiltration into the air pouch (17). The number of neutrophils infiltrating into the air pouch was assayed by staining the cells with neutrophils marker Ly-6G. As shown in Fig. 6A, MSU enhanced the infiltration of neutrophils into the air pouch, which was significantly inhibited by MRS2578 (Fig. 6B). We also investigated the effect of MRS2578 using MSU-induced peritonitis model, as there were some previous reports showing the different effect of reagents.
between these two models (1, 17). MSU induced neutrophil infiltration into the peritoneum, which was significantly suppressed by MRS2578 as expected (Fig. 6C). Thus, these findings led to the conclusion that P2Y6 receptors are pivotal for MSU-induced inflammation in vivo.

Discussion
One of the central observations in this report is that MSU-induced inflammatory cytokine and chemokine production is predominantly regulated by the G protein-coupled P2Y6 receptors–PLC signaling pathway in both KC and monocytes. Some cell surface receptors have been proposed to be involved in MSU-induced inflammation. They are TLR2/4 and CD14 on macrophages (17, 18), CD16 and complement receptors on neutrophils (19), and membrane integrins (GPIIb/IIIa) on platelets (20). In contrast, Ng et al. (21) recently reported that in the case of dendritic cells, direct binding of MSU to the cell membrane, not onto the dendritic cell receptor, is important. In the current study, we investigated the role of P2 receptors in MSU-induced inflammation. P2 receptors are classified into P2X and P2Y receptors based on their molecular structure and signal transduction pathways. P2X receptors are ligand-gated ion channels, and seven receptor subtypes exist (P2X1–7). P2Y receptors, which are G protein-coupled metabotropic receptors, have eight receptor subtypes (P2Y1,2,4,6,11–14) in humans. P2Y1,2,4,6,11 are coupled to PLC via Gq/11 protein, whereas P2Y12,13,14 are coupled to adenylate cyclase via Gi protein (13). The activation of P2Y receptors, which are distributed widely among various tissues, leads to cell proliferation, differentiation, and inflammation in many kinds of cells. Among the P2Y receptors on KC, the P2Y2 receptors are reported to be involved in KC proliferation (22) and inhibition of KC migration in wounds (23). In addition, ATP and UTP induce the production of IL-6 from KC through P2Y receptors (24), and ATP enhances the production of IL-8/CXCL8 from IFN-γ–stimulated KC (25). Although expression of P2Y6 receptors on KC has been controversial (26), we demonstrated that the expression of P2Y6 receptors was increased at both mRNA and protein levels in MSU-stimulated KC. Furthermore, MSU-induced IL-8/CXCL8 and IL-6 production was inhibited by P2Y6 antagonist and P2Y6 antisense oligonucleotides. These results strongly suggest that KC express functional P2Y6 receptors under MSU stimulation. Consistent with this, epithelial cells in lung and intestine are demonstrated to produce IL-8/CXCL8 via P2Y6 receptors (27, 28).

Regarding monocytes, THP-1 cells also express functional P2Y6 receptors. THP-1 cell activation through the endogenous ligand, UDP, leads to IL-8/CXCL8 production (29). Furthermore, UDP upregulates the mRNA expression of TNF-α, IL-8/CXCL8, and IL-1β in another monocytenotic cell line, U937, when U937 is stably transfected with P2Y6 receptors (30). UDP is shown to facilitate the phagocytosis in a P2Y6 receptor-dependent manner in microglia (31); therefore, phagocytosis of MSU by monocytes could be facilitated via P2Y6 receptor signaling. It is known that IL-1β released from MSU-stimulated monocytes triggers the induction of acute gouty inflammation. Our current study showed that P2Y6...
receptors play a pivotal role in IL-1β release from MSU-stimulated monocytes in vitro.

Moreover, we showed that P2Y$_6$ antagonist significantly inhibited the infiltration of neutrophils in both air pouch and peritoneal cavities. Our results clearly demonstrate that P2Y$_6$ are major receptors mediating the neutrophilic gouty inflammation in vivo. P2Y$_6$ receptors are reported to be involved in inflammatory bowel diseases, as their expression is upregulated in human T cells infiltrating the bowel of affected patients. There have been no reports suggesting the role of P2Y$_6$ receptors in gout; however, recent reports have suggested that the activation of protein kinase C is important for neutrophil activation by MSU crystals in gout. P2Y$_6$ receptors are coupled to the G$_{q/11}$ protein, which activates PLC and results in the activation of protein kinase C. Therefore, this report may implicate the involvement of P2Y$_6$ receptors in gout. We investigated several possibilities to explain the mechanism. First, P2Y$_6$ receptors may act as a receptor for MSU. To examine whether MSU crystals can directly bind to P2Y$_6$ receptors, we assessed the calcium mobilization by MSU stimulation using 1321N1 astrocytoma transfected with P2Y$_6$ receptors; however, we could not detect the calcium flux immediately after MSU stimulation (data not shown), suggesting that MSU crystals do not bind directly to P2Y$_6$ receptors. We next investigated the possibility that UDP produced by MSU stimulation contributes to cytokine production via P2Y$_6$ receptors, as UDP stimulation has been reported to induce the production of IL-8/CXCL8 from lung epithelial cells. However, UDP did not induce IL-1α, IL-8/CXCL8, or IL-6 production from NHK (data not shown), which is consistent with a previous report. Furthermore, UDP (1, 10, 100, 1000 μM) did not induce IL-1β production from THP-1 cells (data not shown). We also considered the possibility that MSU induces ATP release from NHK, which subsequently activates KC. However, this was unlikely, because ATP did not induce IL-1α or IL-8/CXCL8 production from NHK (data not shown). These results indicate that MSU crystals act on cells via the P2Y$_6$ receptor-signaling pathway through unknown mechanisms. Further investigation is needed to clarify the exact mechanism by which MSU signals through P2Y$_6$ receptors.

Another major finding in this study is that MSU crystals can induce various inflammatory cytokines and chemokines from human KC, in which the IL-1α and IL-1R pathway plays a pivotal role. MSU crystals have recently been reported to induce IL-1β by activating caspase-1 through the formation of NALP3 inflammasome in monocytes and to induce other inflammatory cytokines including TNF-α, IL-6, and IL-8/CXCL8 through the IL-1R–MyD88–dependent pathway. Watanabe et al. have shown that KC also have all the necessary components to form NALP3 inflammasome and that it can be formed by skin irritants and UV B, which results in IL-1β release. In this study, MSU induced the production of IL-1β from KC; however, the amount of IL-1β produced by KC was much smaller than that by monocytes. Furthermore, when we treated NHK with caspase-1 inhibitor z-YVAD, the production of IL-1β was inhibited; however, IL-1α, IL-8/CXCL8, and IL-6 production was not. These data demonstrated that there might be pathways that could be activated by P2Y$_6$ receptors to promote inflammatory signals, without involving inflammasomes in KC. In contrast, a large amount of IL-8/CXCL8 and IL-6 was produced from MSU-stimulated KC in an IL-1R–dependent manner. Therefore, we focused on IL-1α, which shares the receptor with IL-1β.

IL-1α, a major subtype of IL-1 secreted by KC, can be quickly released in case of epidermal infection or injury. IL-1α produced by KC can induce other inflammatory cytokines and chemokines through the autocrine pathway. Overexpression of IL-1α in murine epidermis produces spontaneous inflammatory lesions, suggesting the potency of IL-1α to drive skin inflammation. MSU crystals induced a large amount of IL-1α from KC. Furthermore, IL-1RA and anti–IL-1α neutralizing Ab significantly inhibited the production of IL-6 and IL-8/CXCL8 by

FIGURE 6. The effect of P2Y$_6$ antagonist (MRS2578) in MSU-induced neutrophil infiltration in vivo. A, Representative dot plots of Ly-6G expression on the harvested cells in the air pouch model. The Ly-6G(+)PI(−) gate represents neutrophils. B and C, The number of neutrophils in air pouch (B) or peritoneal cavities (C). Data show mean ± SEM: n = 5 mice per group (B); n = 3 mice per group (C). Student t test was used to calculate p values.

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KC. Thus, these results indicate that IL-1α, but not IL-1β, is essential for MSU-induced inflammation in KC, which is different from monocytes. To our knowledge, this is the first report to reveal the relationship between MSU crystals and IL-1α. A previous report has shown that the ingestion of MSU crystals by phagocytosis is central to MSU-induced gouty inflammation (8); therefore, it is interesting that MSU crystals can induce inflammation in epithelial cells such as KC through the IL-1α/IL-1R pathway without phagocytosis.

In conclusion, we showed that P2Y6 receptors mediate MSU-induced inflammation in both KC and monocytes and that P2Y6 antagonist suppressed neutrophil infiltration in both mouse air pouch and peritonitis models. We also demonstrated in this study that MSU-induced inflammatory cytokine and chemokine production by KC was regulated by the IL-1α/IL-1R pathway. Thus, our current findings could provide the possibility that P2Y6 receptor-signaling pathway is a potential therapeutic target for MSU-associated inflammatory diseases. Regarding skin diseases other than tophaceous gout, skin disorders showing epidermal proliferation or apoptosis and necrosis of KC, such as psoriasis or atopic eczema, could also be the target.

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Disclosures
The authors have no financial conflicts of interest.

References

P2Y6 RECEPTOR MEDIATES RESPONSES BY MSU

Supplement Figure 1

IL-8/CXCL8 production from keratinocytes was impaired when stimulated with soluble uric acid or uricase digested MSU. NHK were co-cultured with MSU crystals (200 µg/ml), soluble uric acid (200 µg/ml) or uricase treated MSU (240 µg MSU treated with 20 U uricase for 2 h in 25 °C) for 24 h. IL-8/CXCL8 concentration in the supernatant was measured by ELISA. Data show mean ± SEM of four independent experiments.
Supplement Figure 2
Caspase-1 inhibitor inhibited IL-1β but not IL-1α, IL-8/CXCL8 and IL-6 production from MSU stimulated NHK
NHK were pretreated with caspase-1 inhibitor Z-YVAD (10, 100 μM) for 1 h, and stimulated with MSU crystals (200 μg/ml) for 24 h. The cytokine concentration in the supernatant was measured by ELISA. Data show mean ± SEM of four independent experiments. Significant differences from the response to MSU alone: asterisk, P<0.01.
Supplement Figure 3

MSU crystals are ingested by THP-1 cells but not by keratinocytes. THP-1 cells or NHK were co-cultured with MSU crystals (100 or 400 μg/ml) for 2 or 24 h, respectively. Cells were collected, fixed in 3% glutaraldehyde, 0.1 M cacodylate buffer, postfixed in osmium tetroxide, dehydrated, and embedded in Spurr Resin. The cells was observed by a JEM 100SX (JEOL, Tokyo) electron microscope.