



## Research Paper

# Ugonin U stimulates NLRP3 inflammasome activation and enhances inflammasome-mediated pathogen clearance



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## ABSTRACT

The NOD-like receptor pyrin domain 3 (NLRP3) inflammasome contains Nod-like receptors, a subclass of pattern recognition receptors, suggesting that this complex has a prominent role in host defenses. Various structurally diverse stimulators activate the NLRP3 inflammasome through different signaling pathways. We previously reported that ugonin U (UgU), a natural flavonoid isolated from *Helminthostachys zeylanica* (L) Hook, directly stimulates phospholipase C (PLC) and triggers superoxide release in human neutrophils. In the present study, we showed that UgU induced NLRP3 inflammasome assembly and subsequent caspase-1 and interleukin (IL)-1 $\beta$  processing in lipopolysaccharide-primed human monocytes. Moreover, UgU elicited mitochondrial superoxide generation in a dose-dependent manner, and a specific scavenger of mitochondrial reactive oxygen species (ROS) diminished UgU-induced IL-1 $\beta$  and caspase-1 activation. UgU induced Ca<sup>2+</sup> mobilization, which was inhibited by treatment with inhibitors of PLC or inositol triphosphate receptor (IP<sub>3</sub>R). Blocking Ca<sup>2+</sup> mobilization, PLC, or IP<sub>3</sub>R diminished UgU-induced IL-1 $\beta$  release, caspase-1 activation, and mitochondrial ROS generation. These data demonstrated that UgU activated the NLRP3 inflammasome activation through Ca<sup>2+</sup> mobilization and the production of mitochondrial ROS. We also demonstrated that UgU-dependent NLRP3 inflammasome activation enhanced the bactericidal function of human monocytes. The ability of UgU to stimulate human neutrophils and monocytes, both of which are professional phagocytes, and its capacity to activate the NLRP3 inflammasome, which is a promising molecular target for developing anti-infective medicine, indicate that UgU treatment should be considered as a possible novel therapy for treating infectious diseases.

## 1. Introduction

Recognition of pathogenic microbes is the first and critical step of the host defense system, initiating a series of responses that are required for clearance of pathogens. The innate immune system utilizes various pattern recognition receptors (PRR), including Toll-like receptors, nucleotide-binding oligomerization domain-like receptors (NOD-

like receptors, NLRs), and RIG-like receptors, to identify pathogens or other danger signals [1]. The inflammasome contains NLRs, which are key cytosolic sensors, suggesting that the inflammasome plays a prominent role in intracellular sensing of various pathogen-associated molecular patterns (PAMPs) or host-derived signals of cell stress (danger-associated molecular patterns, DAMPs) [2,3]. Indeed, in response to PAMPs or DAMPs, the inflammasome is activated and

**Abbreviations:** ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium concentration; DAMPs, danger-associated molecular patterns; IP<sub>3</sub>, inositol triphosphate; IP<sub>3</sub>R, inositol triphosphate receptor; LPS, lipopolysaccharide; NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate; NLRs, nucleotide-binding oligomerization domain-like receptors; PBMcs, peripheral blood mononuclear cells; PLC, phospholipase C; PBS, phosphate buffered saline; PRP, pattern recognition receptors; PAMP, pathogen-associated molecular patterns; ROS, reactive oxygen species; PMA, phorbol-12-myristate-13-acetate; UgU, ugonin U, 4''a,5'',6'',7'',8''a-hexahydro-5,3',4'-trihydroxy-5'',5'',8''a-trimethyl-4H-chromeno [2'',3'':7,6]flavone; UP-LPS, ultrapure lipopolysaccharide

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induces immune responses that restrict pathogen replication [2,4,5].

As a cytoplasmic multi-molecular complex, the inflammasome serves as a platform for activation of caspase-1, which subsequently converts cytokine precursors into their mature forms [6]. The most characterized inflammasome is the NLRP3 inflammasome, which is composed of a sensing apparatus (NOD-like receptor pyrin domain 3, NLRP3), an adaptor (apoptosis-associated speck-like protein containing a caspase recruitment domain, ASC) and the pro-form of cytokine converting enzyme (pro-caspase-1) [7]. Numerous structurally diverse stimulators activate the NLRP3 inflammasome through different signaling pathways, including  $K^+$  efflux [7–11], reactive oxygen species (ROS) production [12–15],  $Ca^{2+}$  mobilization [11,16–19], mitochondrial destabilization [17,20–23], and lysosome rupture [24,25]. Therefore, it is critical that the molecular mechanisms by which novel stimulators activate the NLRP3 inflammasome are delineated in a context-specific manner.

The NLRP3 inflammasome plays a key role in host defenses against pathogenic bacteria and viruses [4,26]. This has stimulated research aimed at identifying chemical agents or natural products that can activate the complex and therefore modulate innate immunity and host defense [27–30]. We previously reported that ugonin U (UgU, 4''a,5'',6'',7'',8'',8''a-hexahydro-5,3',4'-trihydroxy-5'',5'',8''a-trimethyl-4H-chromeno [2'',3'':7,6]flavone, Fig. 1A) is the first identified natural flavonoid that directly stimulates phospholipase C (PLC) and induces a respiratory burst in human neutrophils [31]. UgU is isolated from *Helminthostachys zeylanica* (L) Hook, a pteridophyte with several medicinal properties including pain relief, detoxification, germ killing, and wound healing [32,33]. Moreover, UgU-induced PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate ( $IP_3$ ), which then promotes  $Ca^{2+}$  release from the endoplasmic reticulum. UgU can evoke  $Ca^{2+}$  mobilization and ROS production, both of which are signaling mediators involved in NLRP3 inflammasome activation. In this article, we evaluate the immuno-modulatory effects of UgU in human monocytes, which constitute another phagocyte subtype. We found that UgU induces  $Ca^{2+}$  mobilization, further promoting a series of signaling cascades that eventually activate the NLRP3 inflammasome in human monocytes. Furthermore, we show that UgU facilitates the bactericidal function of human monocytes to kill intracellular bacteria via activation of the NLRP3 inflammasome.

## 2. Materials and methods

### 2.1. Extraction of UgU

UgU (Fig. 1A) was extracted from rhizomes of *H. zeylanica* (L) Hook as previously described in detail [31]. In brief, the dried rhizomes of *H. zeylanica* (L) Hook were extracted with methanol (MeOH). The concentrated MeOH extract was consecutively partitioned with n-hexane,  $CHCl_3$ , and ethanol acetate (EtOAc). The EtOAc-soluble fraction was subjected to Sephadex LH-20 column chromatography. After loading the sample, it was eluted with MeOH to yield three fractions (Et1–Et3). Fraction Et2 was repeatedly separated by LH-20 column chromatography and reverse-phase high-performance liquid chromatography (MeOH: H<sub>2</sub>O (0.05% TFA): MeCN, 70:20:10; flow rate, 2 ml/min; UV detector, 300 nm) to yield UgU (4.5 mg; retention time, 44.42 min). The molecular formula was confirmed to be  $C_{25}H_{26}O_6$  by high-resolution fast-atom bombardment mass spectrometry.

### 2.2. Reagents

UgU was provided by Dr. Chih-Chuang Liaw, Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Taiwan. Ficoll-Paque was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Anti-NLRP3, anti-ASC, anti-pro-IL-

$1\beta$ , and anti- $\alpha$ -tubulin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Hank's balanced salt solution (HBSS) was obtained from Gibco (Grand Island, NY, USA). Fura-2-acetoxymethyl ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR, USA). Nitrocellulose membranes were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Immobilon Western chemiluminescence HRP substrate was purchased from Millipore Corporation (Billerica, MA, USA). BAPTA-AM, Bay 11-7082, gentamicin, RO 31-8220, and U73122 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xestospongin C (XeC) and Z-YVAD-FMK were purchased from Abcam (Cambridge, MA, USA). MitoSOX Red was purchased from Invitrogen (Waltham, MA, USA).

### 2.3. Cell preparation

This study was approved by the Institutional Review Board at Chang Gung Memorial Hospital, and written informed consent was acquired from every volunteer. Human monocytes were purified from peripheral blood mononuclear cells (PBMCs) using a modified cell culture flask adherence method [34,35]. Briefly, human whole blood was drawn from healthy donors (aged 20–30 years) who did not have any infection and did not take medicine within the week before sample collection. We then isolated PMBCs from whole blood using Ficoll-Paque density gradient centrifugation. PMBCs ( $2 \times 10^7$  cells/ml) were suspended in serum-free DMEM (10 ml) and seeded into 10-cm<sup>2</sup> culture disks for 3 h (37 °C, 5% CO<sub>2</sub>). The non-adherent cells were discarded by washing three times with warm phosphate buffered saline (PBS). The remaining adherent cells were incubated in cold 0.02% EDTA/PBS solution for 10 min and then detached by repeated aspiration and gentle expulsion of cold PBS. Isolated monocytes ( $1 \times 10^6$  cells/ml) were then suspended in DMEM. Cellular viability was determined by trypan blue exclusion.

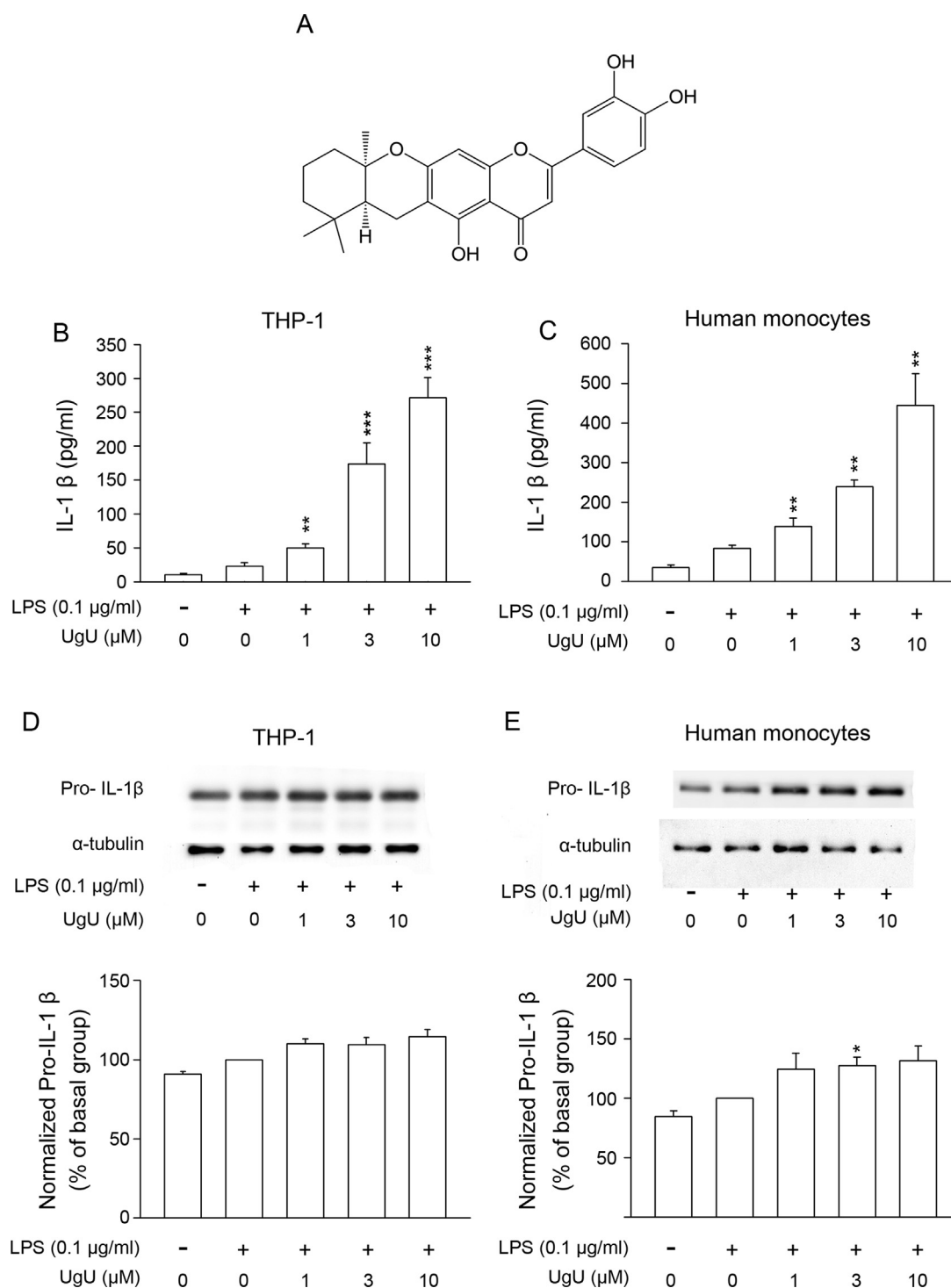
The human monocytic cell line THP-1 (ATCC) and THP-1 with reduced NLRP3 activity (THP-1-NLRP3def; InvivoGen; San Diego, CA, USA) were maintained in RPMI-1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/l sodium bicarbonate, complemented with 10% fetal calf serum, 50 mM 2-mercaptoethanol, and 1% penicillin-streptomycin at 37 °C and 5% CO<sub>2</sub>. For experiments, THP-1 and THP-1-NLRP3def were differentiated with phorbol-12-myristate-13-acetate (PMA, 100 nM) for 3 h and then rest for 21 h before cell stimulation [6].

### 2.4. Cell stimulation

Activation of the NLRP3 inflammasome occurs via a two-step process. To upregulate NLRP3 inflammasome activity and enhance transcription of the pro-IL-1 $\beta$  gene (signal 1), differentiated THP-1 cells (dTHP-1), differentiated THP-1-NLRP3def (dTHP-1-NLRP3def), and human monocytes were primed with ultrapure lipopolysaccharide (UP-LPS, 0.1  $\mu$ g/ml) for 3 h. In some experiments, different treatments using Z-YVAD-FMK (10  $\mu$ M), U73122 (1  $\mu$ M), XeC (5  $\mu$ M), BAPTA-AM (10  $\mu$ M), diphenyleneiodonium (DPI, 10  $\mu$ M), MitoTEMPO (10  $\mu$ M), Bay 11-7082 (10  $\mu$ M), or RO 31-8220 (1  $\mu$ M) were added for various times (XeC, 30 min; other inhibitors, 15 min). UgU (1–10  $\mu$ M) or nigericin (2.5  $\mu$ M), which offer signal 2 (secretion of IL-1 $\beta$ ) in our experiments, were added and incubated for 60 min (UgU) or 15 min (nigericin) for ELISA and western blot analyses, and for 30 min (UgU) or 15 min (nigericin) in other experiments.

### 2.5. ELISA

Cells were cultured and treated as described above. Supernatants were harvested and frozen for human IL-1 $\beta$  quantification using an ELISA assay kit (BD Biosciences) according to the manufacturer's instructions [36].



**Fig. 1. Ugonin U (UgU) induces IL-1 $\beta$  secretion in THP-1 and human monocytes.** (A) Chemical structure of UgU. (B, C) Concentration-dependent effects of UgU on IL-1 $\beta$  secretion. THP-1 ( $5 \times 10^5$  cells/ml) were differentiated with phorbol-12-myristate-13-acetate (PMA, 100 nM) for 3 h and then rested for 21 h. Differentiated THP-1 cells (dTHP-1,  $5 \times 10^5$  cells/ml) (B) and human monocytes ( $5 \times 10^5$  cells/ml) (C) were primed with ultrapure LPS (UP-LPS, 0.1  $\mu$ g/ml) for 3 h. Various concentrations of UgU (1, 3, and 10  $\mu$ M) were added and incubated for 60 min. Supernatants were collected for human IL-1 $\beta$  quantification using an ELISA assay. All data are expressed as the means  $\pm$  SEM ( $n=4$ ). \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to the basal value. (D, E) UgU induced a slight increase in pro-IL-1 $\beta$ . LPS-primed dTHP-1 (D) and human monocytes (E) were treated with various concentrations of UgU for 60 min. Cells were lysed and pro-IL-1 $\beta$  was assayed by western blotting. Blots are representative of four independent experiments. All data are expressed as the means  $\pm$  SEM ( $n=4$ ). \* $p < 0.05$ , compared to the basal value (UgU=0  $\mu$ M).

## 2.6. Western blot and immunoprecipitation

The treated cells were lysed with a buffer containing 50 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM EDTA, 2 mM  $\text{Na}_3\text{VO}_4$ , 10 mM p-nitrophenyl phosphate, 5%  $\beta$ -mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 1% protease inhibitor cocktail (Sigma-Aldrich, St.

Louis, MO, USA), and 1% Triton X-100. After sonication, cell lysates were centrifuged at 14,000 rpm at 4  $^\circ\text{C}$  for 20 min. The supernatants were boiled for 5 min and then electrophoresed on SDS-acrylamide gels. Proteins were transferred to nitrocellulose membranes. After being blocked with 5% nonfat milk in a mixture of tris-buffered saline and Tween 20 (TBS-T), the membranes were incubated with diluted

primary antibodies, including anti-pro-IL-1 $\beta$  and anti- $\alpha$ -tubulin, at 4 °C overnight. The membranes were washed with 0.05% TBS-T and incubated with diluted horseradish peroxidase-conjugated secondary antibodies for 1 h. After being washed, the labeled protein was measured using an enhanced chemiluminescence system (Amersham Biosciences; Buckinghamshire, U.K.). Protein bands were analyzed using a BioSpectrum Imaging System (UVP; Upland, CA, USA) [31].

The lysates from LPS-primed dTHP-1 cells treated with UgU (10  $\mu$ M) were incubated with diluted anti-ASC antibodies (sc-22514, Santa Cruz Biotechnology) for 8 h at 4 °C under gentle rotation. Protein A/G PLUS-agarose beads (sc-2003, Santa Cruz Biotechnology) were added, and the immune complexes were pulled down over 1 h at 4 °C. Unbound proteins were removed by washing the beads with SDS-buffer. The bead-bound proteins were then boiled for 5 min, fractionated by SDS-PAGE, and analyzed by western blot using NLRP3 or ASC antibodies [37]. The procedures for analysis of protein bands were the same as those for western blots.

### 2.7. Determination of active caspase-1

An active caspase-1-specific fluorescent detection reagent, FLICA (FAM-YVAD-FMK, Immunochemistry Technologies, Bloomington, MN, USA), was used to analyze caspase-1 processing [38]. FLICA reagent specifically binds to the active form of caspase-1 and inhibits further activation once added. LPS-primed dTHP-1 cells and human monocytes were pretreated with or without inhibitors including U73122 (1  $\mu$ M), BAPTA-AM (10  $\mu$ M), or RO 31-8220 (1  $\mu$ M) for 15 min; or XcC (5  $\mu$ M) for 30 min before UgU stimulation. Cells were then washed and media were replaced with medium containing FLICA reagent and then incubated for 1 h. Next, the cells were washed three times to rinse away unbound FLICA. The green fluorescent signal, which is a marker of caspase-1 activity, was analyzed by flow cytometry (BD Accuri C6; BD Biosciences, San Jose, CA, USA).

### 2.8. Measurement of cytosolic Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>])

LPS-primed human monocytes (5 $\times$ 10<sup>5</sup> cells/ml) were incubated with the Ca<sup>2+</sup>-sensitive dye Fura-2/AM (2  $\mu$ M) at 37 °C for 30 min, followed by centrifugation and resuspension in HBSS solution without CaCl<sub>2</sub>. The Fura-2/AM-labeled cells were treated with U73122 (1  $\mu$ M, 15 min), XcC (5  $\mu$ M, 30 min), or DMSO, followed by addition of UgU (10  $\mu$ M). We then used a fluorospectrophotometer (F-4500; Hitachi, Tokyo, Japan) with a dual-wavelength program to measure [Ca<sup>2+</sup>] (excitation wavelength, 340/380 nm; emission wavelength, 505 nm). At the end of each assay, we sequentially added Triton X-100 (0.05%) and ethylene glycol tetraacetic acid (20 mM) to get maximum and minimum values of fluorescence [39].

### 2.9. Assessment of mitochondrial superoxide

LPS-primed dTHP-1 cells (5 $\times$ 10<sup>5</sup> cells/ml) and human monocytes (5 $\times$ 10<sup>5</sup> cells/ml) were incubated in phenol red-free RPMI-1640 medium containing a mitochondrial superoxide specific indicator (MitoSOX Red reagent, 2.5  $\mu$ M) at 37 °C for 30 min [40]. Cells were washed and suspended in warm HBSS. The labeled cells were then treated as described above. MitoSOX fluorescence intensity was then measured by flow cytometry (BD Accuri™ C6) [41].

### 2.10. Determination of cAMP concentrations

The concentration of cAMP was measured using an enzyme immunoassay kit (GE Healthcare, Little Halfont, Buckinghamshire, UK). Cells were incubated with different concentrations of UgU, DMSO, or prostaglandin (3  $\mu$ M, as positive control) for 5 min. Dodecyltrimethylammonium bromide (0.5%) was used to hydrolyze cell membranes and release intra-cellular cAMP. Samples were cen-

trifuged at 3000 $\times$ g for 5 min at 4 °C to acquire cleared supernatants, which were then used to determine cAMP levels according to the manufacturer's instructions [42,43].

### 2.11. Gentamicin protection assay

A gentamicin protection assay was performed to assess intracellular bacteria survival in human monocytes [38]. One day before the experiment, isolated human monocytes were cultured in 24-well plates (5 $\times$ 10<sup>5</sup> cells/ml/well), and bacteria were prepared by growing *Staphylococcus aureus* in Luria-Bertani (LB) broth at 37 °C with shaking. On the day of the experiment, bacteria were centrifuged at 10,000 $\times$ g for 5 min and washed three times with PBS. Bacterial density was determined by measuring the optical density of a 3-ml aliquot of the suspensions in cuvettes at 670 nm. Human monocytes were primed with UP-LPS (0.1  $\mu$ g/ml) for 3 h and then infected with *S. aureus* at a multiplicity of infection (MOI) of 10. To increase the infection rate, plates were centrifuged for 15 min at 100 $\times$ g and incubated at 37 °C in 5% CO<sub>2</sub> for 20 min. Infected human monocytes were washed three times with warm HBSS to eliminate non-adherent bacteria. To kill extracellular bacteria, cells were then cultured in DMEM medium containing gentamicin (200  $\mu$ g/ml) for 30 min. After that, medium was replaced with fresh DMEM with or without UgU (1–10  $\mu$ M). The treated monocytes were incubated for 3 or 20 h, followed by washing once with HBSS and lysis with 1% Triton X-100. The survival of intracellular bacteria was quantified by spreading the serially-diluted lysate on LB agar plates and counting the colony forming units (CFUs) 24 h later.

### 2.12. Statistical analysis

To obtain results suitable for statistical analysis, 4–5 samples were used for each independent experiment. All results are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical comparisons were made between two groups using Student's *t*-test. Values of *p* less than 0.05 were considered statistically significant.

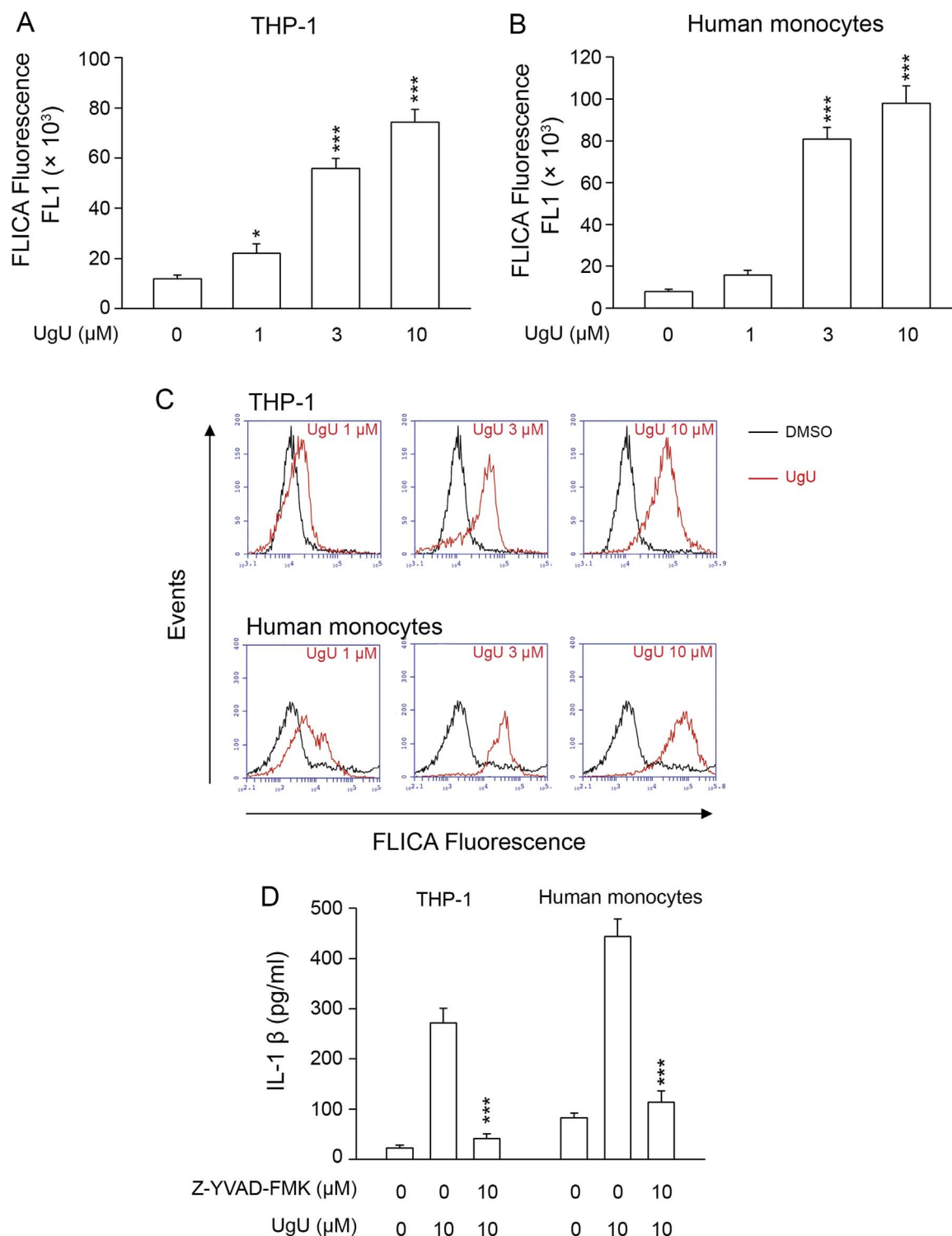
## 3. Results

### 3.1. UgU induces IL-1 $\beta$ secretion in LPS-primed human monocytes

To investigate the role of UgU in NLRP3 inflammasome activation, we first examined whether UgU induces IL-1 $\beta$  as a downstream target of the complex. Secreted IL-1 $\beta$  increased induced by UgU (1–10  $\mu$ M, 60 min) in a dose-dependent manner in LPS-primed dTHP-1 and human monocytes (Fig. 1B, C). Western blotting analysis revealed that UgU induced a slight increase in pro-IL-1 $\beta$  (Fig. 1D, E); however, the increase in pro-IL-1 $\beta$  was much lower than that in mature IL-1 $\beta$  (Fig. 1), suggesting that UgU promotes IL-1 $\beta$  processing.

### 3.2. UgU stimulates caspase-1 and NLRP3 inflammasome activation

Next, our experiments utilizing a specific active caspase-1 fluorescent indicator, YVAD-FLICA, revealed that UgU dose-dependently induces caspase-1 activation in LPS-primed dTHP-1 and human monocytes (Fig. 2A, B, and C). Moreover, the caspase-1 specific inhibitor, Z-YVAD-FMK, reduced IL-1 $\beta$  secretion in both cells types, confirming that active caspase-1 is required for IL-1 $\beta$  secretion following UgU stimulation (Fig. 2D). In addition, we used THP-1-NLRP3def to verify which inflammasome was induced in response to UgU. We found that UgU-dependent IL-1 $\beta$  secretion and caspase-1 activation were abolished in these cells (Fig. 3A, B and Supplementary Fig. 1). Consistent with this, IL-1 $\beta$  secretion and caspase-1 activation in THP-1 were also inhibited by Bay 11-7082, a compound that reportedly inhibits NLRP3 inflammasome activation (Fig. 3C, D and Supplementary Fig. 1) [44,45]. Moreover, an immunoprecipitation

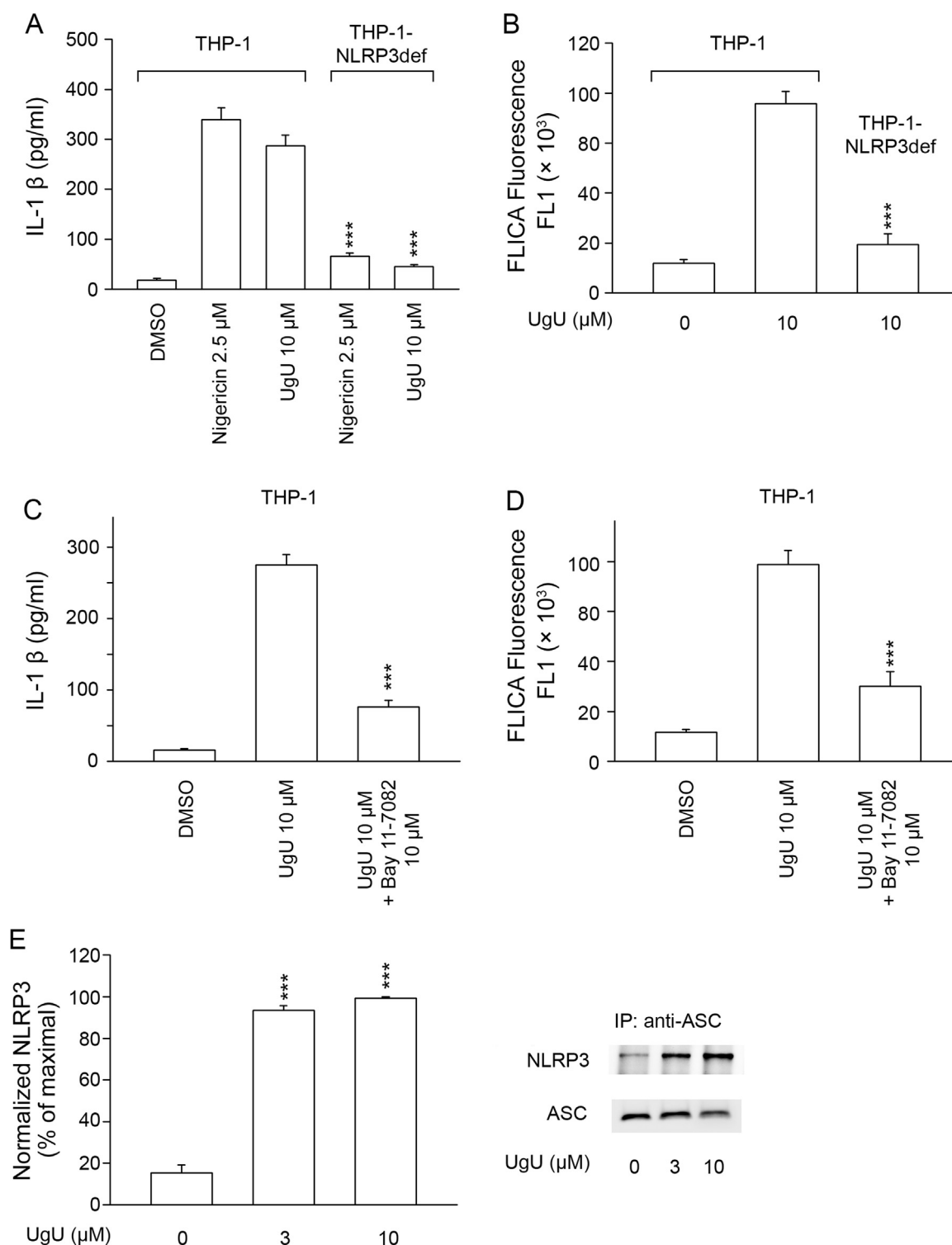


**Fig. 2.** UgU activates caspase-1 in THP-1 and human monocytes, which is essential for IL-1 $\beta$  secretion. (A) LPS-primed dTHP-1 ( $5 \times 10^5$  cells/ml) and (B) LPS-primed human monocytes ( $5 \times 10^5$  cells/ml) were incubated with the indicated concentrations of UgU for 30 min. Cells were stained with a fluorescent detection reagent specific for activated caspase-1 (FLICA reagent, FAM-YVAD-FMK). Fluorescence was monitored by flow cytometry. All data are expressed as the means  $\pm$  SEM ( $n=4$ ). \* $p < 0.05$ , \*\*\* $p < 0.001$  compared to the basal value (UgU=0  $\mu\text{M}$ ). (C) Representative images from one of four independent experiments are shown. (D) The caspase-1 specific inhibitor, Z-YVAD-FMK (10  $\mu\text{M}$ ), was added 15 min before LPS-primed dTHP-1 ( $5 \times 10^5$  cells/ml, left) and human monocytes ( $5 \times 10^5$  cells/ml, right) were stimulated with UgU (10  $\mu\text{M}$ ). IL-1 $\beta$  secretion decreased in the presence of a caspase-1-specific inhibitor in both cells. All data are expressed as the means  $\pm$  SEM ( $n=4$ ). \*\*\* $p < 0.001$  compared to the control (UgU=10  $\mu\text{M}$ ).

assay demonstrated that UgU enhanced the ASC-NLRP3 interaction in LPS-primed dTHP-1 cells (Fig. 3E). These assays prove that the NLRP3 inflammasome, but not other inflammasomes, is responsible for UgU-induced IL-1 $\beta$  secretion.

### 3.3. UgU elicits mitochondrial ROS production

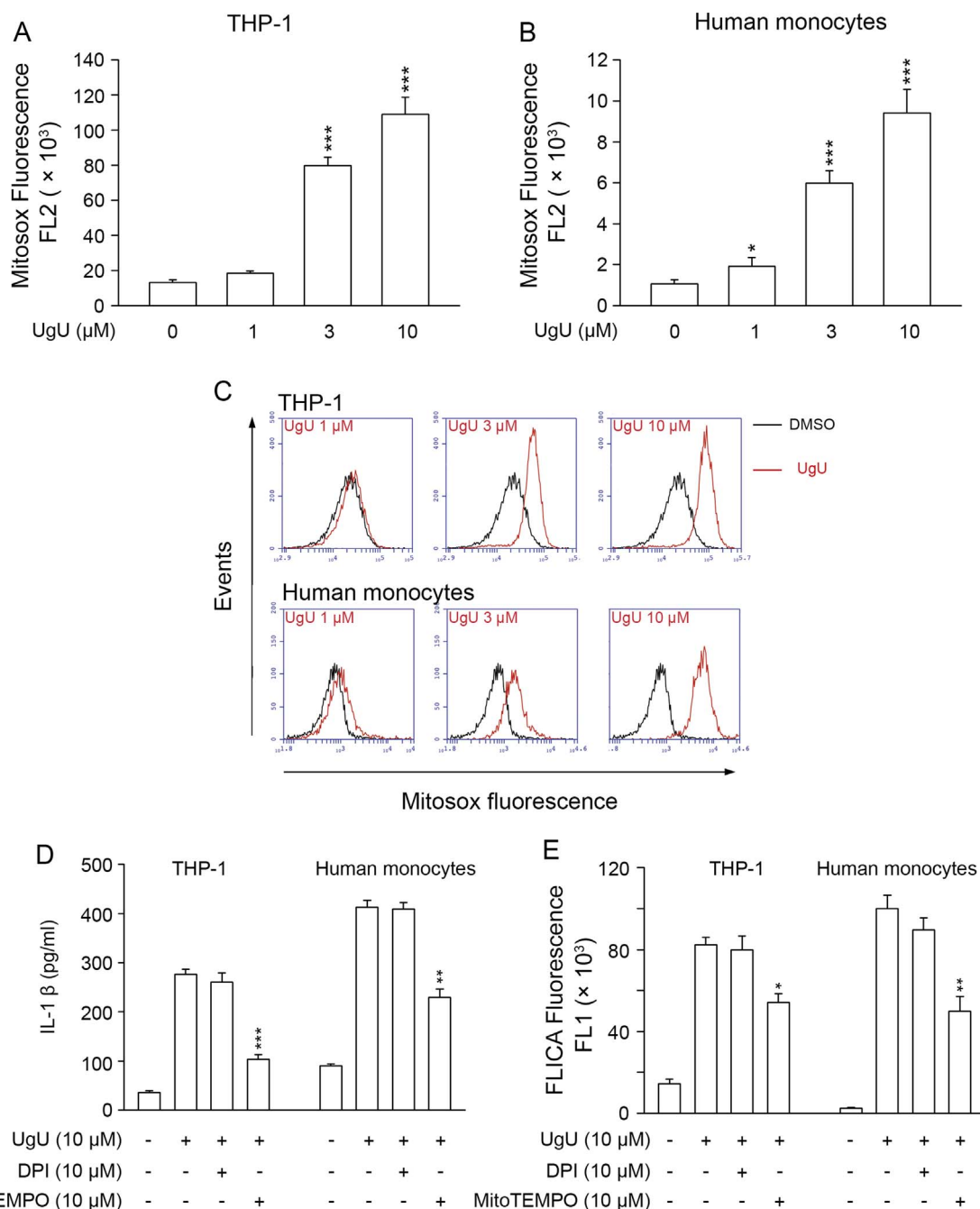
A mitochondrial superoxide-specific fluorescent probe (MitoSOX Red) was used to evaluate whether UgU induces mitochondrial super-



**Fig. 3. NLRP3 inflammasome is responsible for UgU-induced caspase-1 activation and IL-1 $\beta$  secretion.** (A, B) IL-1 $\beta$  secretion and caspase-1 activation were abolished in NLRP3-deficient THP-1 cells or (C, D) THP-1 treated with an NLRP3 inhibitor (Bay 11-7082). In IL-1 $\beta$  analyses, LPS-primed dTHP-1 and LPS-primed dTHP-1-NLRP3def were incubated with UgU (10  $\mu$ M, 60 min) or nigericin (2.5  $\mu$ M, 15 min), respectively. In activated caspase assays, LPS-primed dTHP-1 and LPS-primed dTHP-1-NLRP3def were incubated with UgU (10  $\mu$ M, 30 min) or nigericin (2.5  $\mu$ M, 15 min), respectively. Bay 11-7082 (10  $\mu$ M) were added 15 min before cell stimulation. Data are expressed as the mean  $\pm$  SEM (n=4). \*\*\* $p$  < 0.001 compared to the control. (E) An immunoprecipitation assay demonstrated the ASC-NLRP3 interaction. LPS-primed dTHP-1 ( $5 \times 10^5$  cells/ml) were stimulated with UgU (10  $\mu$ M). Cell lysates were immunoprecipitated with anti-ASC antibodies, followed by incubation with protein A/G agarose beads for 1 h at 4  $^{\circ}$ C. The bead-bound proteins were eluted and analyzed by western blot for NLRP3 or ASC. Data are expressed as the mean  $\pm$  SEM (n=4). \*\*\* $p$  < 0.001 compared to the basal value (UgU=0  $\mu$ M). Blots are representative of four independent experiments.

oxide. This experiment revealed that UgU elicited mitochondrial superoxide generation in a dose-dependent manner in LPS-primed dTHP-1 cells and human monocytes (Fig. 4A, B, and C). To further verify the origin of ROS during UgU-induced NLRP3 inflammasome

activation, we used a mitochondrial-target antioxidant (MitoTEMPO) and a NADPH oxidase (NOX) ROS inhibitor (DPI) in the following experiments. The results showed that MitoTEMPO, but not DPI, diminished IL-1 $\beta$  and caspase-1 activation (Fig. 4D, E, and



**Fig. 4.** UgU elicits mitochondrial superoxide generation in a dose-dependent manner in LPS-primed dTHP-1 cells and human monocytes. (A) LPS-primed dTHP-1 cells ( $5 \times 10^5$  cells/ml, upper pane) and (B) LPS-primed human monocytes ( $5 \times 10^5$  cells/ml, lower panel) were incubated in medium containing mitochondrial superoxide specific indicator (MitoSOX Red reagent, 2.5 μM) at 37 °C for 30 min. The labeled cells were then stimulated with UgU (1, 3, and 10 μM) for 30 min. MitoSOX fluorescent intensity was measured by flow cytometry. Data are expressed as the means  $\pm$  SEM (n=4). \* $p < 0.05$ , \*\*\* $p < 0.001$  compared to the basal value (UgU=0 μM). (C) Representative images from one of four independent experiments are shown. (D) IL-1β secretion and (E) caspase-1 activation was inhibited by a mitochondrial-specific antioxidant (MitoTEMPO), but not a NADPH oxidase (NOX) ROS inhibitor (DPI), in LPS-primed dTHP-1 cells (left) and human monocytes (right). Data are expressed as the means  $\pm$  SEM (n=4). \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to the control (UgU=10 μM).

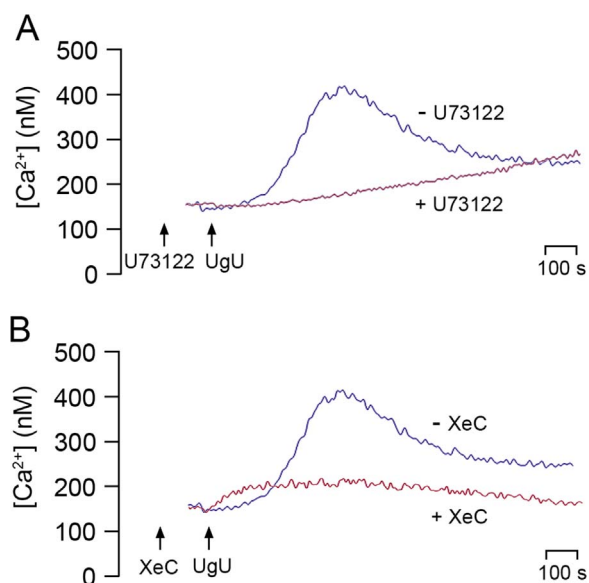
Supplementary Fig. 2).

### 3.4. UgU-induced NLRP3 inflammasome activation is dependent on $[Ca^{2+}]$ mobilization

$Ca^{2+}$  mobilization plays a critical role in NLRP3 inflammasome activation [17,18]. In this study, we demonstrated that UgU induced intracellular  $[Ca^{2+}]$ , (Black line, Fig. 5A, B). We previously reported that UgU stimulated respiratory burst in human neutrophils via the PLC/inositol triphosphate ( $IP_3$ )/ $Ca^{2+}$  pathway [31]. We observed that

in the human monocytes, UgU-induced  $Ca^{2+}$  mobilization was inhibited by treatment with inhibitors of PLC (U73122, 1 μM, 15 min) or the  $IP_3$  receptor ( $IP_3$ R, XeC, 5 μM, 30 min).

Furthermore, UgU-induced IL-1β release, caspase-1 activation, and mitochondrial ROS production were inhibited by the PLC inhibitor (U73122),  $IP_3$ R inhibitors (XeC), or the  $Ca^{2+}$  chelator (BAPTA-AM), but not by a PKC inhibitor (RO 31-8220) (Figs. 6, 7, and Supplementary Fig. 3). These data suggest that UgU-induced NLRP3 inflammasome activation is  $Ca^{2+}$ -dependent, and that the  $Ca^{2+}$  release induced by UgU is mediated through a PLC/ $IP_3$  pathway. In contrast,



**Fig. 5.** Ugu induces an increase in intracellular calcium ( $\text{Ca}^{2+}$ ). (A, B) Representative traces (black) of the effects of Ugu on intracellular  $\text{Ca}^{2+}$  mobilization in human monocytes are shown. LPS-primed human monocytes ( $5 \times 10^5$  cells/ml) were labeled with Fura-2/AM and then stimulated with Ugu ( $10 \mu\text{M}$ ) in  $\text{Ca}^{2+}$ -free HBSS. Fluorescence was monitored at  $37^\circ\text{C}$ . A phospholipase C (PLC) inhibitor (U73122,  $10 \mu\text{M}$ , 15 min) (A, red) or inositol triphosphate receptor ( $\text{IP}_3\text{R}$ ) inhibitor (Xestospingon C, XeC,  $5 \mu\text{M}$ , 30 min) (B, red) was added before human monocytes were stimulated with Ugu. Representative images from one of five independent experiments are shown.

PKC did not play a role in Ugu-associated inflammasome activation. Additionally, Ugu did not affect cAMP levels in LPS-primed dTHP-1 compared with the positive control  $\text{PGE}_1$  (data not shown).

### 3.5. Ugu augments intracellular bacteria clearance activity of human monocytes

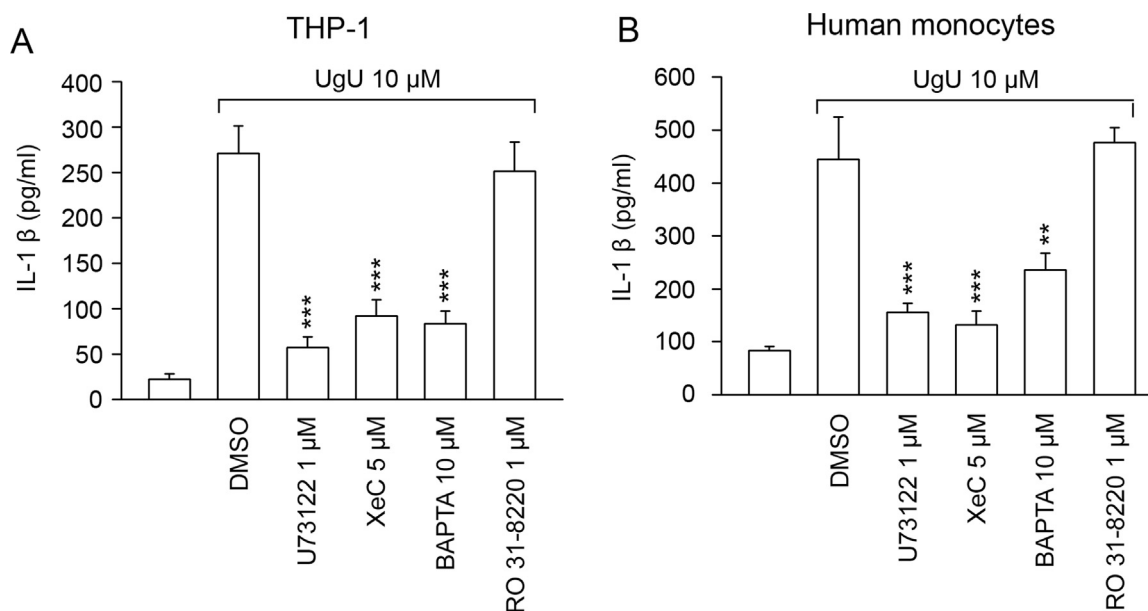
A gentamicin protection test was employed to evaluate whether Ugu affects the intracellular survival of *S. aureus* within human monocytes. *S. aureus* CFUs were higher at 20 h after infection than at 3 h after infection (data not shown), indicating that *S. aureus* had

replicated within the cell. The CFU number was lower in the group of LPS-primed human monocytes that were treated with Ugu. Moreover, the decrease in intracellular *S. aureus* caused by Ugu was partially reversed by an NLRP3 inflammasome inhibitor (Bay 11-7082) and caspase-1 inhibitor (Y-VZAD-FMK) (Fig. 8). These studies illustrate that Ugu-enhanced germicidal activity of human monocytes against intracellular bacteria is mediated by NLRP3 inflammasome activation. Fig. 9.

## 4. Discussion

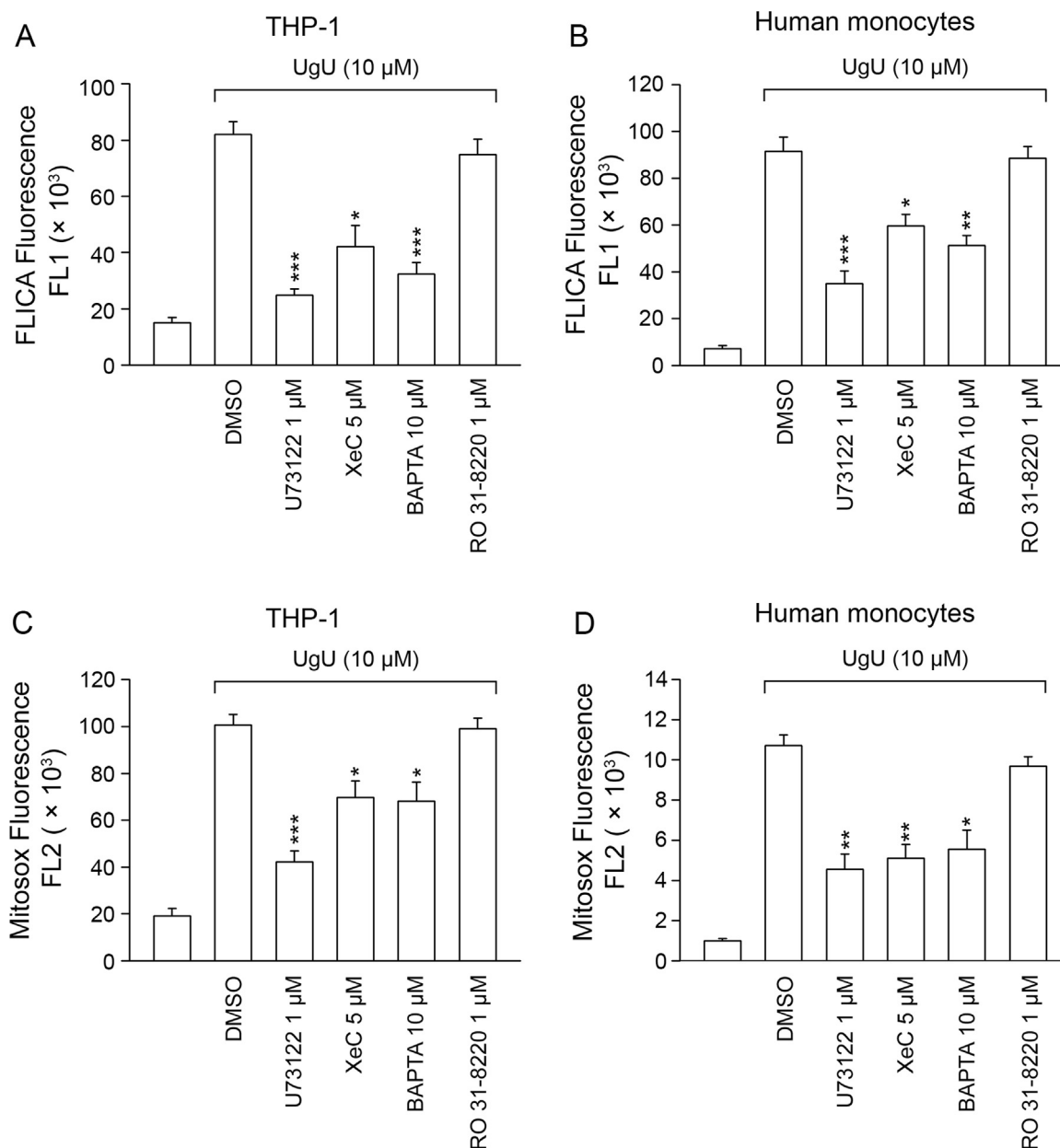
The NLRP3 inflammasome, which contains the evolutionarily ancient immunity receptor, NLR, is a key player in the host defense system [4]. Various structurally distinct stimulators activate the NLRP3 inflammasome, and employ diverse activation mechanisms to do so. In response to insults that cause cell stress, the inflammasome signaling cascade elicits the production of inflammatory cytokines and antimicrobial factors. There is a significant interest in chemical compounds or natural products that can activate the NLRP3 inflammasome, attributable to its significant role in protection against invading pathogens. In the current study, we demonstrate that Ugu, a flavonoid extracted from *H. zeylanica* (L) Hook, provokes IL-1 $\beta$  secretion by activating the NLRP3 inflammasome.

Many pathways that lead to the NLRP3 inflammasome converge on ROS production [12,46]. Mitochondria and NOX are two major sources of intracellular ROS [47,48], and mitochondrial-derived ROS contribute to NLRP3 inflammasome activation [17,20,49]. However, the role of NOX-derived ROS in the process is still controversial [46,50–52]. In our study, a mitochondrial-target antioxidant (MitoTEMPO) abolished IL-1 $\beta$  release and caspase-1 activation induced by Ugu, while a NOX inhibitor (DPI) had no effect. These results indicate that stimulation of the NLRP3 inflammasome by Ugu is dependent upon mitochondrial ROS, but independent of NOX-derived ROS. Moreover, our data showed that Ugu induced a slight increase in pro-IL-1 $\beta$ . Previous studies have shown that IL-1 $\beta$  induces pro-IL-1 $\beta$  production in an autocrine fashion [53]. ROS also play a role in NF- $\kappa\text{B}$  activation, which further increases pro-IL-1 $\beta$  production [54]. These data provide possible mechanisms by which Ugu promotes a small increase in pro-IL-1 $\beta$  production. Furthermore, we previously reported that Ugu is the first identified natural flavonoid as a PLC activator [31].



**Fig. 6.** Ugu-induced IL-1 $\beta$  secretion is  $\text{Ca}^{2+}$ -dependent, and the source of  $\text{Ca}^{2+}$  is through PLC/ $\text{IP}_3$  pathway. IL-1 $\beta$  release induced with Ugu in (A) LPS-primed dTHP-1 cells and (B) human monocytes was inhibited by a PLC inhibitor (U73122,  $1 \mu\text{M}$ ), an  $\text{IP}_3\text{R}$  inhibitor (XeC,  $5 \mu\text{M}$ ), or a calcium chelator (BAPTA-AM,  $10 \mu\text{M}$ ), but not a protein kinase C (PKC) inhibitor (RO 31-8220,  $1 \mu\text{M}$ ). All data are expressed as the means  $\pm$  SEM ( $n=4$ ). \*\*\* $p < 0.001$  compared to the control (Ugu= $10 \mu\text{M}$ ).



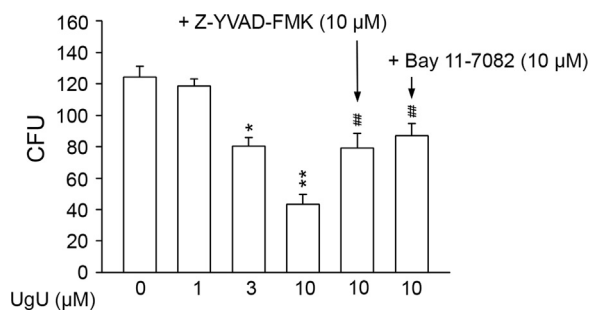


**Fig. 7. UgU-induced caspase-1 activation and mitochondria ROS production are inhibited when PLC, IP<sub>3</sub>R, or Ca<sup>2+</sup> mobilization is blocked.** (A, B) Caspase-1 activation and (C, D) mitochondrial ROS production induced with UgU in LPS-primed dTHP-1 cells (A, C) and LPS-primed human monocytes (B, D) were inhibited by a PLC inhibitor (U73122, 1 μM), an IP<sub>3</sub>R inhibitor (XeC, 5 μM), or a calcium chelator (BAPTA-AM, 10 μM), but not a PKC inhibitor (RO 31-8220, 1 μM). Data are expressed as the means ± SEM (n=5). \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 compared to the control (UgU = 10 μM).

Activation of PLC is an important pathway for intracellular Ca<sup>2+</sup> mobilization [55]. PLC metabolizes membrane phosphoinositides and produces IP<sub>3</sub>, which then binds with IP<sub>3</sub>R on the endoplasmic reticulum and releases Ca<sup>2+</sup> into the cytoplasm. Our study illustrates that UgU induces Ca<sup>2+</sup> mobilization in human monocytes. Blocking Ca<sup>2+</sup> mobilization inhibits UgU-induced mitochondrial ROS production. Consistent with other studies [17], our results provide evidence that Ca<sup>2+</sup> mobilization induces the production of mitochondrial ROS, which are important intermediaries of the NLRP3 inflammasome activation. In addition, our experiments showed that the Ca<sup>2+</sup> chelator, BAPTA-AM, did not completely inhibit caspase-1 activation and mitochondrial ROS production. Ca<sup>2+</sup> may have a direct activating effect on NLRP3 inflammasome [11,16–19]. Therefore, a parallel pathway of Ca<sup>2+</sup> activation of the NLRP3 inflammasome cannot be ruled out.

Antibiotics, one of the most important therapeutic agents in the

history of human medicine, have significantly reduced infection-associated morbidity and mortality. However, with the emergence of multiple antibiotic-resistant microbes, severe infectious diseases caused by these bacteria pose a serious problem to medical caregivers and academics in clinical research fields. As such, there is intense research into the molecular mechanisms by which bacteria develop antibiotic resistance and into the delineation of novel strategies to kill such pathogens. The inflammasome is an evolutionarily conserved cytosolic sensor that plays a critical role in host defenses against bacteria and viruses [5,26,30,56]. In response to pathogens, inflammasomes are activated and can induce immune responses that restrict pathogen replication. Therefore, the inflammasome is a potential molecular target for developing novel anti-bacteria agents [29,30]. In the current study, we show that UgU promotes the NLRP3 inflammasome-mediated innate immune response and enhances clearance of engulfed bacterial pathogens. Moreover, UgU-enhanced bactericidal



**Fig. 8. UgU augments the intracellular bacteria clearance activity of human monocytes.** UgU-induced dose-dependent reductions in colony forming units (CFUs) are shown. The decrease in CFUs caused by UgU was partially reversed by an NLRP3 inflammasome inhibitor (Bay 11-7082) and caspase-1 inhibitor (Y-VZAD-FMK). Cells were cultured in DMEM containing gentamicin (200 μg/ml) for 30 min. After that, medium was replaced with fresh DMEM with or without UgU (1, 3, and 10 μM). The treated monocytes were incubated for 20 h, followed by washing once with HBSS and then lysis with 1% Triton X-100. The survival of intracellular bacteria was determined by counting CFUs, which were formed 24 h after spreading the serially-diluted lysate on LB agar plates. Data are expressed as the means ± SEM (n = 3). \**p* < 0.05, \*\**p* < 0.01, compared to the basal value (UgU=0 μM). ##*p* < 0.01, compared to the UgU=10 μM group.

activity was reduced by NLRP3 inflammasome or caspase-1 inhibition, suggesting that UgU-induced NLRP3 inflammasome activation contributes to the antibacterial effects.

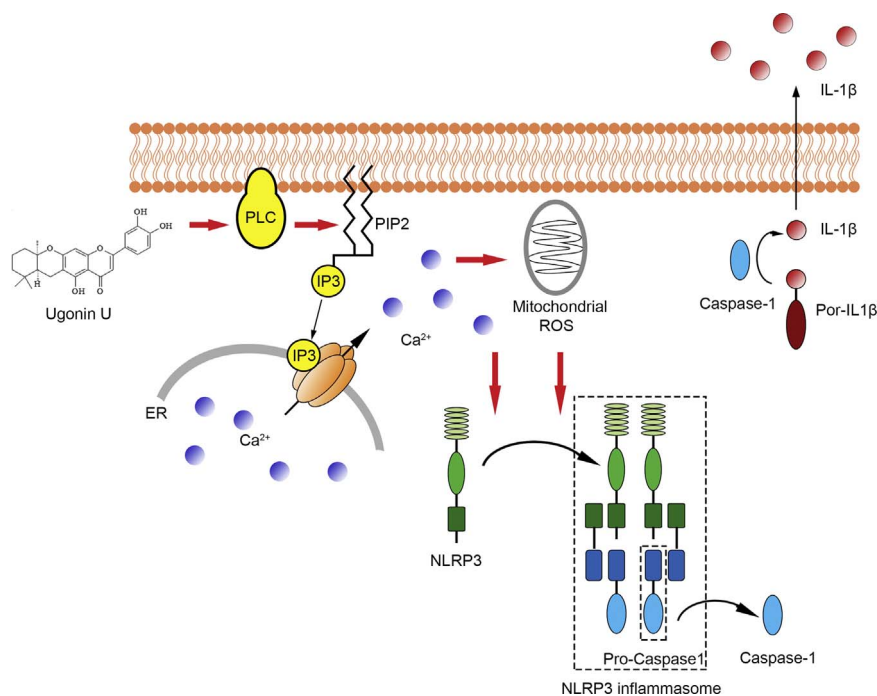
To date, however, specific molecular mechanisms linking inflammasome activation and intracellular bacteria clearance have remained unclear. A previous study reported that NLRP3 inflammasome and caspase-1 activation contribute to phagosome acidification and maturation, which are critically important for the clearance of engulfed bacteria [38]. Caspase-1 is activated and recruited to bacteria-containing phagosomes by the activated NLRP3 inflammasome [38]. Various studies show that microbes or their subcellular components negatively regulate the inflammasome in order to evade host defenses [5,38,57,58]. For instance, live bacteria can actively neutralize the acidity of phagosomes to escape inflammasome-associated bactericidal activities [38,59]. Accordingly, we infer that UgU augments the

bactericidal function of human monocytes by activating the NLRP3 inflammasome. In some respects, this is similar to the activity of nigericin, a potent NLRP3 inflammasome activator that attenuates viral infection in elderly mice with impaired NLRP3 inflammasome activity [30]. Moreover, we have reported that UgU activates human neutrophils and induces generation of ROS, which are toxic products that help eliminate intracellular pathogens. Taken together, these data suggest that UgU has the potential to be developed as a novel anti-infective drug, since it can activate the bactericidal activity of human neutrophils and monocytes. However, to clarify the effectiveness of UgU in treating microbial infections, more rigorous animal studies should be performed.

To conclude, we demonstrate that UgU activates the NLRP3 inflammasome and mature IL-1β secretion through a PLC/Ca<sup>2+</sup>/mitochondrial ROS cascade, whereas NADPH ROS were not involved. The activation facilitates the killing of engulfed bacteria by human monocytes. Thus, to the best of our knowledge, UgU is the first described natural flavonoid that activates human neutrophils and monocytes; both these cell types are expert microbial killers and are essential for the resolution of infections. Since the NLRP3 Inflammasome is a target for development of anti-infective medicines [29,30,60], we expect that further investigation of UgU will facilitate the development of novel anti-infective agents. Studies of UgU will also enhance our understanding of the pharmacology of flavonoids.

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**Fig. 9.** Schematic diagram illustrating the proposed signaling pathway involved in UgU-induced NLRP3 inflammasome assembly and the following molecular events in human monocytes. UgU induces IL-1β release via the PLC/IP<sub>3</sub>/Ca<sup>2+</sup>/mitochondrial ROS pathway in human monocytes. UgU-induced Ca<sup>2+</sup> may have a direct activating effect on NLRP3 inflammasome. PKC, cAMP, and NOX-derived ROS are not involved.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2016.12.018.

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