P2X7 receptor-stimulation causes fever via PGE2 and IL-1β release

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Nonstandard abbreviations:

BzATP: 3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate; ETYA: 5,8,11,14-Eicosatetraynoic acid; BMDM: Bone marrow delivered macrophages; Ac-YVAD-AOM: Caspase-1 inhibitor IV; COX: Cyclooxygenase; EGTA: Ethylene glycol tetra-acetic acid; HPBM: Human peripheral blood monocytes; LDH: Lactate dehydrogenase; LPS: Lipopolysaccharide; LT: Leukotriene; MAPK: Mitogen activated protein kinase; NLRP3: NLR family, pyrin domain containing 3; NSAID's: Nonsteroidal anti-inflammatory drugs; P2X4R: P2X4 receptor; P2X7R: P2X7 receptor; P2YR: P2Y receptor; Et: Physiological buffer; PG: Prostaglandins; PGE2: Prostaglandin E2; (RT)-PCR: Reverse transcriptase-PCR; TX: Thromboxane.

Abstract

Prostaglandins (PG) are important lipid mediators involved in the development of inflammatory associated pain and fever. PGE2 is a well-established endogenous pyrogen activated by pro-inflammatory cytokine interleukin (IL)-1β. P2X7 receptors (P2X7R) expressed by inflammatory cells are stimulated by the danger signal extracellular ATP to activate the inflammasome and release IL-1β. Here we show that P2X7R activation is required for the release of PGE2 and other autacoids independent of inflammasome activation, with an ATP EC_{50} for PGE2 and IL-1β release of 1.58 and 1.23 mM, respectively. Furthermore, lack of P2X7R or specific antagonism of P2X7R decreased the febrile response in mice triggered after intra peritoneal (i.p.) LPS or IL-1β inoculation. Accordingly, LPS inoculation caused intraperitoneal ATP accumulation. Therefore, P2X7R antagonists emerge as novel therapeutics for the treatment for acute inflammation, pain and fever, with wider anti-inflammatory activity than currently used cyclooxygenase inhibitors.

Key words: Inflammation; Pyrogen; Macrophages; Purinergic receptors; *In vivo* bioluminescence.

Introduction

Metabolites of arachidonic acid derived from cyclooxygenase-2 (COX-2) activity, such as prostaglandins (PG), are critical for numerous pathophysiological processes, including inflammation, pain and fever (1-5). Pro-inflammatory cytokines of the interleukin (IL)-1 family are considered endogenous pyrogen because they activate the inflammatory cascade, regulate the biosynthesis of COX-2, leading to the production of PG, and cause fever (2, 6). PGE2 is the main PG during inflammation, is produced by different cell types, including antigen-presenting cells, and has various functions in the regulation of the immune response (7, 8). During inflammation PGE2 is responsible for the activation of the thermoregulatory area of the hypothalamus and the ensuing increase of body temperature (2). Pain receptors (nociceptors) are also sensitized by PGE2 causing increased pain sensation (5, 7). Drugs targeting COX-2 activity, including aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), are actually the most used therapeutics worldwide to treat inflammation, pain and fever (1, 5).

Extracellular nucleotides are emerging as key players in inflammation. Notably, *in vivo* data show that during inflammation ATP is released into the extracellular space and activates the P2X7 receptor (P2X7R) (9-12). This supports the view that extracellular ATP is a *bona fide* danger signal, or even the prototypic danger signal. P2X7R is widely expressed on many immune cells where it controls key signaling pathways (9-11, 13-17). In particular, P2X7R is the most potent plasma membrane receptor responsible for inflammasome activation and release of pro-inflammatory cytokines of the IL-1 family (12, 13). P2X7R activation also increases generation of reactive oxygen species, induces release of cathepsins, promotes

antigen-driven T lymphocyte proliferation and facilitates intracellular pathogens killing (15, 16, 18, 19). Experiments performed in P2X7R deficient mice and with selective drug-like P2X7R antagonists, have demonstrated a role for P2X7R in the progression of rheumatoid arthritis, lung inflammation and fibrosis, graft-versus-host disease, irritable Bowel syndrome, contact hypersensitivity and inflammatory and neuropathic pain (9-11, 14, 17, 20, 21). Therefore, P2X7R is a promising therapeutic target in the management of inflammation and pain, as witnessed by the large number of selective P2X7R antagonists developed by several drug companies and currently under clinical trials (21, 22).

Different previous studies suggest that P2 receptors, in particular P2X7R, couple to the release of different autacoids (23-28) and P2 receptor inhibition reduces fever induced by LPS in rats (29). Here, we investigate the specific role of P2X7R in PG generation during inflammation and its relevance to the febrile response. We demonstrate with pharmacological and genetic tools that P2X7R enhances release of PGE2 in primed mouse and primary human macrophages through an inflammasome-independent mechanism. P2X7R-stimulated PGE2 release was mediated through intracellular calcium increase and mitogen-activated protein kinase (MAPK) signaling. Besides PGE2, P2X7R activation stimulated release of other important lipid mediators, such as thromboxane (TX) B2 and leukotriene (LT) B4. Drug-like P2X7R antagonists were potent inhibitors of eicosanoids as well as IL-1β release. Finally, P2X7R deficiency or pharmacological blockage resulted in a reduced febrile response *in vivo* upon challenge with exogenous or endogenous pyrogens.

Materials and methods

Reagents and buffers

Key reagents and their sources were as follows: *Escherichia coli* LPS serotype 055:B5, LPS from *Salmonella enterica* serotype typhimurium, ATP, 3'-O-(4-benzoyl)benzoy-ATP (BzATP), adenosine, ADP, UDP, CTP, 5,8,11,14-eicosatetraynoic acid (ETYA), recombinant IL-1β, ivermectin, U73122, SB202190, SP600125 and U0126 were purchased from Sigma-Aldrich (Madrid, Spain); the reagents FR122047, SC-791 and the cell permeant irreversible caspase-1 inhibitor IV (Ac-YVAD-AOM) from Merck (Madrid, Spain); A438079 from Tocris (Bristol, UK). The composition of the physiological buffer (Et) used in all experiments was (in mM) NaCl 147, HEPES 10, D-glucose 13, KCl 2, $CaCl_2$ 2 and $MgCl_2$ 1; pH 7.4. In some experiments, an Et buffer containing no added $CaCl_2$ and 1 mM EGTA (Et-EGTA buffer) was used. The Fura 2-AM loading buffer for calcium measurements had the following composition (in mM): 136 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 5 $NaHCO_3$, 1.2 $MgSO_4$, 20 HEPES, 5.5 D-Glucose and 1 EGTA.

In vivo experiments

The Local Ethical Committee refined and approved all animal experiments. C57 BL/6 (wild-type, $P2X7R^{+/+}$) and P2X7R-deficient ($P2X7R^{-/-}$) mice in C57 BL/6 background (30) were injected i.p. with 200 µl of *Salmonella* or *E. coli* LPS (0.01 mg/kg), recombinant IL-1β (0.01 mg/kg) or saline vehicle. 100 µmol/kg of A438079 was injected i.p. 1 h before *Salmonella* LPS injection (0.01 mg/kg). The rectal temperature of mice was measured with an electronic thermometer (Panlab Harvard Apparatus, Barcelona, Spain) immediately before injections, and 3 and/or 6 h post-injection. The

increase in temperature was calculated comparing at each time the LPS-injected group with the saline-injected group.

In vivo extracellular ATP levels were measured by bioluminescent imaging using HEK293-pmeLUC cells as previously described (31). Briefly, 2 h after *Salmonella* LPS injection (0.01 mg/kg), 2 x 10^6 HEK293-pmeLUC cells were injected i.p. per mice, followed 2 h later by i.p. injection of 150 mg/kg D-luciferin (Xenogen Caliper Life Sciences, Villepinte, France). Ten minutes after luciferin injection luminescence was captured for three minutes with large binning from mice ventral view using an IVIS 100 System^{TM} imaging chamber (Xenogen Caliper Life Sciences, Hopkinton, MA, USA). Average radiance (p/s/cm^2/sr) was quantified in saline- and LPS-injected mice, respectively.

Preparation of mouse bone marrow derived macrophages (BMDM)

Ex vivo experiments were performed with cells from wild-type C57 BL/6 (WT), P2X7R-deficient (P2X7R$^{-/-}$) or P2X4R-deficient mice in C57 BL/6 background (14, 32). Bone marrow-derived macrophages (BMDMs) were obtained from leg bones of mice (8–10 weeks of age) euthanized by CO_2 inhalation. Femurs and tibia were removed, the bone marrow was flushed out and resuspended in DMEM (Lonza, Verviers, Belgium) supplemented with 25% of L929 medium containing macrophage-stimulating factor, 15% FCS (Invitrogen Life Technologies, Madrid, Spain), 100 U/ml penicillin-streptomycin (Lonza), and 1% L-glutamine (Lonza), plated onto 150-mm dishes, and cultured at 37˚C in the presence of 5% CO_2. After 6-7 days, the resulting BMDMs were detached with cold PBS, replated into 96, 24, 12 or 6-well plates at a confluence of 0.42 x 10^6 cells/cm^2, and used the following day. The macrophage purity of these preparations was usually higher than 90% and was

routinely checked by flow cytometry with the murine macrophage antigen specific Ab F4/80 in a FACScalibur flow cytometer (Beckton-Dickinson Biosciences, Madrid, Spain).

Human monocyte and macrophage culture

Primary human peritoneal macrophages were obtained upon patient consent in the "Virgen de la Arrixaca" Hospital (Murcia, Spain) from peritoneal lavage performed with 6 ml of sterile saline buffer before laparoscopy for benign ovarian cyst removal. Blood-contaminated peritoneal fluids were excluded. Human peripheral blood monocytes (HPBM) were isolated from healthy donors following standard procedure using Limphoprep (Axis-Shield, Oslo, Norway). Peritoneal cells and blood leukocytes were resuspended in RPMI 1640 medium (Lonza) with 10% FCS, 1% L-glutamine and 100 U/ml penicillin-streptomycin, and plated into 12-well plates for 16 h at 37˚C with 5% CO_2. Blood monocytes and peritoneal macrophages were isolated by adherence. Purity was checked by flow cytometry using CD163-staining using a FACScalibur flow cytometer (Beckton-Dickinson Biosciences). Peritoneal samples with an initial percentage (before adherence) of macrophages from 25 to 35 % were used for experiments.

***In vitro* macrophage stimulation**

Macrophage medium was replaced with fresh medium, and cells were either primed with 1 μg/ml of *E. coli* LPS for 4 h at 37°C, or left in medium alone. Cells were then rinsed with Et-buffer and incubated in this same medium at 37°C for different times and with various concentrations of nucleotides as stated in the text. In other experiments cells were stimulated with ATP on the top of the LPS-priming media

without washing the LPS. In some experiments, cells were pretreated with various pharmacological inhibitors 10 min before and during nucleotide stimulation. The effect of extracellular Ca^{2+} depletion was investigated in Et-EGTA buffer. To terminate the release reaction, the entire volume of extracellular medium was transferred to a tube on ice and then samples were centrifuged at 16,000 x g for 30 s to remove floating cells, followed by supernatant withdrawal and analysis.

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) release was measured using the Cytotoxicity Detection kit (Roche, Barcelona, Spain) following the manufacturer's instructions, and expressed as percent of total cell LDH content.

IL-1β release assay

IL-1β release was measured by ELISA for human or mouse IL-1β (R&D System, Abingdon, UK) following the manufacturer's instructions and read in Synergy Mx plate reader (BioTek, Winooski, VT, USA). For human macrophages and monocytes the concentration of IL-1β was normalized to the total amount of cellular LDH (pg/ml/LDH) to allow for differences in cell concentration among the different samples.

PGE2, TXA2 and LTB4 assay

After appropriate dilution of extracellular samples, PGE2, TXA2 and LTB4 concentration was measured in duplicate using specific metabolite ELISA competition system from different commercial sources (Cayman Chemical, Ann Arbor, MI, USA; Assaydesigns, Ann Arbor, MI, USA; and Arbor Assay, Ann Arbor,

MI, USA) according to the manufacturer's instructions. We ensure reproducibility of the results by comparing the different brands (Supplemental Fig. 1*B*). Autacoid release from human macrophages and monocytes was normalized to total cellular LDH content as described for IL-1β measurements.

Intracellular calcium assay

BMDMs were plated in 96-well plates with black walls and clear bottom (Costar Corning Life Sciences, Lowell, MA, USA), incubated at 37˚C for 40 min in loading buffer with 4 μM Fura 2-AM (Invitrogen Life Technologies) supplemented with 0.02% pluronic acid (Merck). At the end of this incubation, Fura 2-AM was removed and replaced with Et-buffer. Fluorescence was recorded by an automatic fluorescence plate reader (Synergy Mx, BioTek) for 200 s at 4 s intervals at a wavelength emission couple 340/380 nm, emission 510 nm. The different nucleotides were automatically injected into the wells at the designated time points. Intracellular calcium level was expressed as the ratio of the emission intensities at 340 and 380 nm, and the value was normalized to the fluorescence at time 0 (F/F_0). The data was fitted using KaleidaGraph 4.0 for Mac (Synergy Software) by applying a Stineman function and the output was then a geometrically weighted to the current point and ± 10 % of the data range to achieve a smoothed curve, which is the one presented in the results.

Quantitative reverse transcriptase-PCR analysis

BMDMs and human macrophages or monocytes plated in 6 or 12-well plates, respectively, were stimulated as described above. Reverse transcriptase (RT)-PCR experiments were done according to standard protocols (33). Total RNA extraction was performed using the RNeasy Mini kit (Qiagen, Madrid, Spain), followed by

reverse transcription using iScript cDNA Synthesis (BioRad, Madrid, Spain) with oligo(dT). The mix SYBR Premix ExTaq (Takara, Shiga, Japan) was used for quantitative PCR in iCycler MyiQ thermocycler (BioRad, Hercules, CA, USA). Specific primers were purchased from Qiagen (QuantiTech Primer Assays). Only a single product was seen on melt curve analysis and for each primer set the efficiency was > 95%, been for human P2X7R and P2X4R 97% and 96.9% respectively. For the relative expression of human P2X7R and P2X4R Cts were normalized to the housekeeping gene GAPDH using the $2^{-\Delta Ct}$ method. To study COX2 and PGE synthase expression, Cts were also normalized to GAPDH and compared to unprimed cells acting as a reference point with the $2^{-\Delta\Delta Ct}$ method and values were expressed in fold change.

Statistical analysis

All data shown are mean values and error bars represent standard error (s.e.m.) from the number of assays indicated in the figure legend (experiments were performed more than 3 times to ensure reproducibility). For statistical comparisons, data were analyzed by an unpaired two-tailed Student's t-test or with a paired one-sample t-test to determine difference between groups using Prism software (Graph-Pad Software, Inc.). *p* value is indicated as ***p < 0.001; **p>0.001 <0.01; *p>0.01 <0.05; p>0.05 not significant (ns).

Results

P2X7R activation induces PGE2 release

Short treatment (30 min) with 3 mM of ATP increased the levels of PGE2 in the supernatants of LPS-primed mouse macrophages by four to eight-fold (Fig. 1*A*). Similar increase was found when ATP was applied over cells without washing LPS stimulation media (Supplemental Fig. 1*A*). The release of PGE2 in response to ATP occurred at the same time as release of IL-1β (Fig. 1*C*), a well characterized response due to P2X7R-dependent NLRP3 inflammasome and caspase-1 activation (13). ATP was also a stimulus for release of PGE2 and IL-1β from primary human peritoneal macrophages or blood monocytes (Fig. 1*E*, *F*). Cell Treatment with an irreversible caspase-1 inhibitor (Ac-YVAD-AOM) prior to ATP stimulation blocked IL-1β but not PGE2 release (Fig. 1*A*, *C*), suggesting separate signaling pathways downstream to P2X7R for inflammasome-dependent IL-1β release and PGE2 production, respectively. Un-primed macrophages failed to release PGE2 or IL-1β in response to extracellular ATP (Fig. 1*A*, *C*). The use of BzATP, a specific P2X7R agonist, also induced PGE2 and IL-1β release from LPS-primed macrophages (Fig. 1*B*, *D*). Furthermore, a selective P2X7R antagonist (A438079) reduced release of both PGE2 and IL-1β in response to ATP or BzATP (Fig. 1*A-D*).

Dose response curves revealed an ATP EC_{50} for PGE2 and IL-1β release of 1.58 mM (Fig. 2*A*) and 1.23 mM (Fig. 2*B*), respectively. Both EC_{50} are in line with the ATP EC_{50} for the activation of ion currents through P2X7R (34). The P2X7R antagonist A438079 potently inhibited ATP-mediated PGE2 release at all ATP concentrations used (Fig. 2*A*). Because prolonged P2X7R stimulation is known to cause cell death (35), LDH was also measured in cell supernatants during PGE2

release experiments. Under no condition did LDH levels increase above 20% of total cellular LDH content (Fig. 2*C*), suggesting that PGE2 release was not a consequence of cell death.

P2X7R and P2X4R differentially regulate PGE2 release from macrophages

ATP was unable to trigger PGE2 release from macrophages derived from P2X7R-deficient mice, while on the contrary ATP-stimulated PGE2 release was unaffected by lack of P2X4R (Fig. 3*A*), thus confirming that P2X7R is the main purinergic receptor coupled to a robust PGE2 release in macrophages. Furthermore, A438079 decreased ATP-induced PGE2 release in both wild type and P2X4R-deficient macrophages, again suggesting a negligible role for P2X4R in PGE2 release in response to high ATP concentrations. On the other hand, as previously shown by Rassendren and co-workers (26), a low ATP dose (100 μM), subthreshold for P2X7 activation, was able to trigger a modest PGE2 release but to a level three fold lower than that caused by an ATP dose fully stimulatory for P2X7R (Fig. 3*B*). To further characterize the involvement of P2X4R in our experiments, we used ivermectin, a drug which specifically potentiates P2X4R responses (36). Treatment of macrophages with 100 μM ATP plus ivermectin caused a two-fold increase in PGE2 release compared to 100 μM ATP alone (Fig. 3*B*), confirming a role for P2X4R in PGE2 release in response to low ATP concentrations. Ivermectin treatment did not enhance PGE2 release in response to 3 mM of ATP (data not shown). As already reported (26), treatment with 100 μM of ATP plus ivermectin was ineffective to increase PGE2 release from P2X4R-deficient macrophages (Fig. 3*B*). As a control, IL-1β release was monitored in parallel and found to occur to the same level in response to ATP stimulation in wild type and P2X4R-deficient macrophages, being in both cases

sensitive to A438079 (Fig. 3*C*). On the contrary, ATP was unable to trigger IL-1β release from P2X7R-deficient macrophages at any concentrations tested (Fig. 3*C*), and when used at low concentration (100 μM) from wild type or P2X4R-deficient macrophages, whether alone or in the presence of ivermectin (Supplemental Fig. 1*C*). P2X4R and P2X7R functional responses were further validated by checking intracellular calcium changes in response to different ATP concentrations in wild type and genetically-deleted mice (Fig. 3*D*, *E*). At variance with mouse macrophages, human peritoneal macrophages and blood monocytes released negligible PGE2 levels in response to 100 μM ATP, and this response was not potentiated by ivermectin (Table 1), although in both primary human macrophages and monocytes expression of P2X4R was significantly higher than that of P2X7R (Fig. 3*F*).

Adenosine (physiological agonist of P1 receptors) and various nucleotides selective for P2YR, all at 100 μM, lacked PGE2-releasing activity and did not increase intracellular calcium (Supplemental Fig. 2*A*, *B*), ruling out the involvement of adenosine or P2YR or receptors in ATP-induced PGE2 release from macrophages.

Sustained intracellular calcium mediates PGE2 release after ATP stimulation

Levels of PGE2 release in response to different extracellular ATP concentrations correlated with the P2X7-dependent sustained rise in intracellular calcium, as also shown by the selective effect of the P2X7R antagonist A438079 on the late, sustained phase of calcium increase (Fig. 3*D*). A sustained increase in intracellular calcium is also likely to play a role in response to P2X4 stimulation, as ivermectin potentiated and prolonged the increase in intracellular calcium to low ATP concentrations (Fig. 3*E*) and the initial transient calcium rise occurring in the presence of P2X7R blockage is probably due to activation of phospholipase C via activation of

G-protein–coupled P2YR (37) as it was abolished by the phospholipase C inhibitor U73122 (Supplemental Fig. 2*B*). ATP treatment in Et-EGTA buffer significantly decreased PGE2 release (Fig. 4*A*), and accordingly the P2X7R-dependent sustained increase in intracellular calcium was also obliterated (Fig. 4*B*). To confirm that P2Y receptors were not involved in ATP-dependent PGE2 release, no significant differences were observed in in the presence of the phospholipase C inhibitor U73122 (Fig. 4*A*), which fully blocked P2YR dependent release of calcium from intracellular stores but did not affect the sustained increase in intracellular calcium (Fig. 4*B*). This data strongly suggest that calcium influx through P2X7R is the intracellular signal responsible for PGE2 release.

MAPK and COX-2 meditates PGE2 release after P2X7R stimulation

PGE2 release in response to P2X7R stimulation was blocked by a selective COX-2 inhibitor (SC-791), but was unaffected by a COX-1 selective inhibitor (FR122047) (Fig. 4*C*). ETYA, a non-metabolizable analogue of arachidonic acid which blocks generation of all arachidonic acid metabolites, also reduced PGE2 release in response to P2X7R stimulation (Fig. 4*C*), suggesting that the site of action of P2X7R is upstream to arachidonic acid, probably at the level of calcium-dependent cytosolic phospholipase A2 (cPLA2).

MAPK are largely involved in PGE2 release by modulating arachidonic acid release and by increasing the expression levels of COX-2 (38, 39). It is known that P2X7R activates MAPK signaling and participation of this pathway in P2X7-dependent PGE2 release was confirmed by using specific JNK (SP600125) or ERK (U0126) inhibitors (Fig. 4*D*). On the contrary, a p38 selective antagonist (SB202190) had no effect on ATP-stimulated PGE2 release (Fig. 4*D*). JNK mediates the synthesis

of COX-2 both *in vitro* and *in vivo* (38, 39), thus it might be possible that ATP effects on PGE2 release were at least in part due to induction of COX-2 synthesis, however ATP stimulation of unprimed- or primed-macrophages did not change expression levels of COX-2 or PGE synthase (Fig. 4*E*, *F*).

P2X7R is important for the development of fever

IL-1β and PGE2 are key mediators of the febrile response, and as such are also known as endogenous pyrogens (2, 6, 7). Since P2X7R is a key trigger for release of both pyrogens, we investigated its involvement in the pathogenesis of the febrile response. P2X7R$^{-/-}$ mice presented the same temperature profile as their wild type counterparts at three different times of the day (Table 2). These time points were then used to measure the temperature before and after LPS injection. The temperature of both wild type and P2X7$^{-/-}$ mice injected with sterile vehicle saline solution significantly decreased during the day (Table 2). After i.p. LPS injection, P2X7R$^{-/-}$ mice developed an attenuated febrile response compared to their wild type counterparts (Fig. 5*A*). Treatment with A438079 in wild type mice before LPS injection impaired the increase of temperature associated to LPS inoculation (Fig. 5*B*).

We next analyzed by *in vivo* bioluminescence imaging whether ATP was released in the peritoneum of mice after injection of LPS. Intraperitoneal LPS administration triggered a larger ATP release compared to saline injected mice, as detected by bioluminescence emission from HEK293-pmeLUC cells (Fig. 5*C*). These data show that injection of the exogenous pyrogen LPS causes release of the endogenous danger signal ATP, which in turn activates P2X7R to cause fever.

We next explored the role of PGE2 and IL-1β in the reduced febrile response in P2X7R$^{-/-}$ mice. We failed to detect an increase of PGE2 in serum or peritoneal lavage fluid of mice injected with LPS compared to mice injected with saline (data not shown). On the contrary, a robust increase in IL-1β was easily and reproducibly detected in both serum and peritoneal lavage fluid of LPS-injected mice (Fig. 5*D*). IL-1β levels were substantially reduced in LPS-challenged P2X7R$^{-/-}$ mice (Fig. 5*D*), suggesting that the lack of IL-1β release in P2X7R$^{-/-}$ mice could explain the reduced febrile response.

To asses if P2X7R-derived PGE2 release was an important mediator for the development of the febrile response *in vivo*, we challenged mice with recombinant IL-1β and monitored body temperature at different time points. In contrast to LPS-induced fever, IL-1β was a powerful pyrogen in both wild type and P2X7R$^{-/-}$ mice (Fig. 5*E*). However, the increase in body temperature in P2X7R$^{-/-}$ mice was significantly shorter that in the wild-type animals (Fig. 5*E*, *F*).

P2X7R activation induces thromboxane and leukotriene release

Since the effect of P2X7R stimulation on PGE2 release was mainly due to an increase of free arachidonic acid, we asked if P2X7R could also couple to the production of other arachidonic acid metabolites. Treatment of macrophages with 3 mM ATP caused accumulation of the COX-2-dependent TXB2 and to a lesser extent the amount of COX-2-independent LTB4 (Fig. 6*C*, *D*). Primary human peritoneal macrophages and blood monocytes also released TXB2 and LTB4 after ATP application (Fig. 6*E*, *F*). The P2X7R antagonist A438079 powerfully blocked macrophage release of TXB2, LTB4, PGE2 and IL-1β (Fig. 6*A-D*). The selective COX-2 inhibitor (SC-791) blocked TXB2 and PGE2, but not LTB4 or IL-1β release

(Fig. 6*A*-*D*), indicating that P2X7R antagonism offers a wider anti-inflammatory range than the blocking spectra of the currently used NSAID.

Discussion

In this study, we have shown that P2X7R stimulation in primary human and murine macrophages released different arachidonic acid mediators, including PGE2, TXB2 and LTB4. Deficiency of P2X7R reduced febrile response after LPS or IL-1β challenge, mediated by the release of the endogenous danger signal ATP and production of IL-1β. Finally, P2X7R antagonism lead to a wider anti-inflammatory range when compared with COX-2 or caspase-1 inhibitors.

The release of the pro-inflammatory cytokine IL-1β is tightly regulated by a two-step process, which comprises an initial priming step (signal 1, usually bacterial endotoxins) and a second inflammasome formation step (signal 2, usually a danger signal) (12). The nature of signal 2 differs from crystals to pathogens, but extracellular ATP is the most studied endogenous danger signal acting through purinergic P2X7R (12, 13). Pathophysiological production of PGE2 is tightly couple to COX-2 expression levels, and COX-2 expression is a well-established marker of inflammation (1). COX-2 expression is negligible in unprimed cells, but after endotoxin (classically LPS) priming there is a rapidly induction of the levels of COX-2 (40). Long endotoxin exposure lead macrophages to the release of COX-2-dependent PGE2 compared with un-primed cells (40) and here we found that short time (4 h) of LPS-priming, induces in macrophages a 600-times fold increase in COX-2 expression levels and a four-times increase of PGE2 release. However, LPS-induced PGE2 release was small if we compared with the release after P2X7R stimulation, which resulted in a further up to eight-times increase in PGE2 release. Therefore, similar to IL-1β release, we found that the lipidic mediator PGE2 is also subjected to a two-step release mechanism, where signal 1 induces the synthesis of

COX-2 and signal 2 increase the enzyme substrate: the arachidonic acid. Here we have found that signal 2 is mediated by P2X7R activation, but recently it has been described that other inflammasome activators such as silica crystals or aluminum salts can also regulate the production of PGE2 acting as signal 2 (41). Surprisingly, PGE2 release induced by signal 2 is independent of the inflammasome activation and the use of a casapse-1 inhibitor (present study) or macrophages deficient in the inflammasome adapter protein ASC or NLRP3 (41) have no altered release of PGE2.

Therefore, the mechanism underlying P2X7R activation and PGE2 release differs from the inflammasome pathway, and we found that was strongly dependent on the sustained rise of intracellular calcium, permeating directly through P2X7R pores. This calcium-dependent signaling pathway for P2X7R inducing PGE2 release is similar to the recently described pathway for P2X7R inducing cathepsin release or T-cell proliferation (15, 19); and in line with P2X7R inflammasome activation, which is a calcium-independent process (42, 43). P2X7R strongly activates MAPK signaling (44-46) and we found that JNK activity was required for P2X7R dependent PGE2 release. It is well known that JNK modulates COX-2 expression (38, 39), but ATP did not alter COX-2 or PGE-synthase transcript levels and ruling out a synthesis step in the P2X7R-PGE2 release pathway. A non-metabolizing analogue of arachidonic acid blocked the release of PGE2 after P2X7R stimulation, suggesting that the rise in intracellular calcium and activation of MAPK leads to an increase of free arachidonic acid through cPLA2 activation, since the enzymatic activity of cPLA2 is highly controlled by intracellular calcium and phosphorylation (26, 41, 47, 48). Also, intracellular calcium rise after P2X7R stimulation is known to activate cPLA2 in rat submandibular gland (49) and our data validate this model in macrophages. The modulation of arachidonic acid by P2X7R leads us to study if ATP and P2X7R could

also induce the release of other important lipidic mediators. We found for first time, that P2X7R could also couple to the release of other arachidonic acid mediators such as thromboxanes and leukotriens from primed macrophages and monocytes.

In this study we used primary human macrophages and monocytes to corroborate mice data, it has been reported that *in vitro* maturation of human macrophages from monocytes increase the expression of P2X7R (50). Our data using human fresh blood monocytes and primary mature peritoneal macrophages from a cohort of different individuals show similar expression of P2X7R gene, but monocytes presented a higher release rate (from 10 to 100 fold times) of IL-1β and autacoids in response to P2X7R activation. This is in agreement with previous data supporting that P2X7R is more active in monocytes than in macrophages and that the endogenous ATP release from monocytes is able to activate P2X7R induced IL-1β release (51). Our mouse macrophage *in vitro* data support previous observations for a role of P2X4R in PGE2 release due to low extracellular ATP concentrations (26), but P2X4R contribution to PGE2 release was negligible when P2X7R was activated. Despite primary human monocytes and peritoneal macrophages presented higher expression of P2X4R compared to P2X7R, low ATP concentrations alone or in combination with ivermectin could not potentiate the release of PGE2, suggesting that P2X4R could be arrested in intracellular compartments in human monocytes and macrophages and be no functional at plasma membrane level, as P2X4R present a potent internalization sequence (52).

All these finding have enormous implications, since autacoids control key signaling steps in many physiological and pathophysiological processes, where purinergic signaling and P2X7R can now be novel master regulators. For example, it is well known that PGE2 sensitize nociceptors during inflammation, and therefore

increase pain sensation (5, 7). Experiments with P2X7R-deficient mice and with selective P2X7R antagonist have revealed P2X7R as a key mediator of pain sensation in models of neuropathic pain, thermal hyperalgesia and hypersensitivity post-inflammatory bowel disease (14, 20, 53, 54). The anti-nociceptive effects of P2X7R inhibition, has been attributed to an impairment of IL-1β release, since IL-1β has been described as a mediator of pain (55). However, after P2X7R blockage there is a reduced noxious evoked activity of neurons (20, 56), which directly correlate with the effect of PGE2 promoting nociceptor sensitization and hyperexcitability (5, 7).

Here we focus on the involvement of P2X7R on the febrile response, one of the five classical signs associated to the inflammatory process. Fever is a primary systemic response to infection, coordinated by the induction of different endogenous pyrogens (57). Actually, it is well characterized that IL-1β is one of the intermediate endogenous pyrogens transducing pathogen-derived signals. IL-1β signaling activate the production of COX-2 and therefore increase the levels of PGE2, which has been postulated as one of the final local endogenous pyrogen which acts on the thermoregulatory area of the hypothalamus increasing body temperature (2, 57, 58). It is known that P2 receptor blockade attenuates fever induced by LPS in rats (29). Among P2 receptors, P2X7R controls the release of both pyrogens IL-1β and PGE2, and here we found that its deficiency or specific blockage with A438079 decrease the febrile response in mice in response to LPS. However, such decrease could be due to either, an impairment of IL-1β and/or PGE2 production. We successfully found that P2X7R deficiency was related to impairment on local and systemic IL-1β levels; however, we fail to detect a reliable increase on PGE2 *in vivo* after LPS challenge. This could be due to the very short half-life of PGE2 *in vivo* (approximately 20 seconds) and to local changes in PGE2 production during fever, which are unlikely to

be detected systemically (4, 58-60). We therefore used IL-1β to induce fever, and found that the increase in body temperature was significantly shorter in P2X7R deficient animals. We can suggest either that P2X7R deficiency alter IL-1β positive feedback loop or alter PGE2 release by exogenous IL-1β. We also found that ATP, the endogenous ligand for P2X7R, was accumulated *in vivo* after i.p. LPS administration. This result is in accordance with recent publications where ATP has been found to be release as a danger molecule *in vivo* during graft-*vs*-host disease and contact hypersensitivity (10, 11). All together we can speculate that bacterial endotoxin causes release of the endogenous danger signal ATP, which in turns activate P2X7R to cause fever.

Different pharmaceutical companies are actively producing drug-like P2X7R antagonists, some of them have been extensively used in animal models of inflammation and pain, and some of them are under clinical trials (22, 61). These molecules have successfully passed phase I clinical trials, and some of them are in phase II/III for rheumatoid arthritis, osteoarthritis, Crohn's disease and chronic obstructive pulmonary disease (22). We simultaneously compared the inhibitory effects of P2X7R antagonism with caspase-1 inhibitors (inflammasome blockers) and with inhibitors of COX-2 as representative of NSAIDs. As expected caspase-1 inhibition only decreased pro-inflammatory cytokine IL-1β release, without affecting the release of lipidic mediators. COX-2 inhibitors belong to the NSAIDs family and comprise one of the largest used drugs mainly due to their potent anti-inflammatory and pain killer actions (62). However, COX-2 inhibitor was only effective blocking the release of PGE2 and TXB4, without affecting LTB2 or IL-1β release. P2X7R antagonism was able to block autacoids and IL-1β release, conferring to these novel drug-like antagonists a wider anti-inflammatory and anti-pain spectra than classical

NSAIDs, emerging as important therapeutics for inflammation, pain and fever.

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Author contribution

M.B-C. execute and analyze experiments from all Figures. A.B-M. performed flow cytometry experiments with macrophages and A.I.G. plated human macrophages, performed some of the cell stimulation and IL-1β ELISAs. F.M. collected human peritoneal lavage. F.D.V. provided pmeLUC-HEK probe and helped with *in vivo* ATP determination experiments. P.P. conceived, designed and supervised this study, analyzed and interpreted experiments and wrote the final manuscript with contributions from the other authors.

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Figure legends

Figure 1. ATP and BzATP induce release of PGE2 and IL-1β from human and murine macrophage and monocyte. *A, B*) ELISA for released PGE2 from mouse BMDM primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP (*A*) or 300 μM of BzATP (*B*). The caspase-1 inhibitor (Ac-YVAD-AOM, 100 μM) or the P2X7R selective antagonist (A438079, 10 μM) were applied 10 min before and during ATP or BzATP treatment. For *A-D* $n = 4$ to 8 independent experiments. *C, D*) ELISA for released IL-1β from mouse BMDM treated as in *A* and *B*. *E, F*) ELISA for released PGE2 (*E*) or IL-1β (*F*) from primary human peritoneal macrophages or blood monocytes primed with LPS and subsequently stimulated with 3 mM of ATP, $n = 4$ to 7 different individuals.

Figure 2. ATP dose-response relations for PGE2, IL-1β and LDH release. *A*) ELISA for released PGE2 from mouse BMDM primed with LPS and subsequently stimulated for 30 min with different concentrations of ATP in the presence or absence of the P2X7R selective antagonist (A438079, 10 μM) 10 min before and during ATP stimulation ($n = 5$ to 8 independent experiments for ATP and $n = 3$ to 6 independent experiments for ATP+A438079). ATP EC_{50} for PGE2 release was 1.58 mM and maximum concentration of PGE2 released was 2.05 ± 0.4 ng/ml ($n = 7$). *B*) ELISA for released IL-1β from mouse BMDM treated as in *A* ($n = 4$ independent experiments for each ATP concentration). ATP EC_{50} for IL-1β release was 1.23 mM and maximum concentration of IL-1β released was 5.17 ± 0.8 ng/ml ($n = 4$). *C*) Presence of LDH in the supernatants of mouse BMDM treated as in *A* as a test for cell

death. In any conditions the levels of LDH were over 20 % of the total cellular LDH content (n = 6 independent experiments for each ATP concentration).

Figure 3. P2X7R and P2X4R differentially regulate PGE2 release from macrophages in response to extracellular ATP. *A*) ELISA for released PGE2 from BMDM wild type (WT), P2X4R-deficient ($P2X4^{-/-}$) or P2X7R-deficient ($P2X7^{-/-}$) mice primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP in the presence or absence of the P2X7R selective antagonist (A438079, 10 μM) 10 min before and during ATP stimulation (n = 6 to 12 for WT and n = 4 for $P2X4^{-/-}$ and $P2X7^{-/-}$ independent experiments). Basal PGE2 release from the different genotypes (n = 4 independent experiments) used to calculate the fold-increase showed in the figure were (in pg/ml) 99.0 ± 15.9 for WT, 79.2 ± 17.3 for $P2X4^{-/-}$ and 80.5 ± 16.2 for $P2X7^{-/-}$. *B*) ELISA for released PGE2 from BMDM from WT or $P2X4^{-/-}$ mice primed as in *A* and stimulated for 30 min with 0.1 mM of ATP in the presence or absence of 3 μM of ivermectin (n = 4 independent experiments). *C*) IL-1β release was monitored in parallel to PGE2 release as explained in *A* (n = 4 to 6 independent experiments). *D, E*) Representative trace out of 4 to 11 independent experiments for intracellular calcium rise in BMDM derived from WT, $P2X4^{-/-}$ or $P2X7^{-/-}$ mice stimulated as in *A* and *B*. *F*) Relative gene expression for P2X4R and P2X7R determined by quantitative RT-PCR in different individual samples of primary human macrophages (n = 10) and blood monocytes (n = 4).

Figure 4. MAPK and COX-2 meditates PGE2 release after P2X7R stimulation. *A*) ELISA for released PGE2 from BMDM primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP in the presence or absence of extracellular

calcium (EGTA) or PLC inhibitor U73122 (10 μM). *B*) Representative trace out of 2 to 5 independent experiments for intracellular calcium rise in BMDM stimulated as in *A*. *C, D*) ELISA for released PGE2 from BMDM stimulated as in *A* in the presence or absence of COX1 selective inhibitor (FR122047, 2 μM), selective COX2 inhibitor (SC-791, 2 μM), P2X7R selective antagonist (A438079, 10 μM), non-metabolizable analogue of arachidonic acid (ETYA, 10 μM), a specific JNK inhibitor (SP600125, 10 μM), specific ERK inhibitor (U0126, 10 μM) or p38 selective inhibitor (SB202190, 10 μM) 10 min before and during ATP stimulation. n = 4 to 7 independent experiments for PGE2 determination showed in *A*, *C* and *D*. *E, F*) Relative gene expression for COX2 and PGE synthase determined by quantitative RT-PCR in unprimed- or LPS primed-BMDM and subsequently stimulated with ATP for 30 min (n = 3 independent experiments).

Figure 5. P2X7R is important for the development of fever. *A*) C57 BL/6 (wild-type, $P2X7R^{+/+}$) and P2X7R-deficient ($P2X7R^{-/-}$) mice were i.p. injected with *Salmonella* or *E. coli* LPS (n = 6 to 10 mice/group). The rectal temperature of mice was measured with an electronic thermometer immediately before injections and after 3 and 6 h post-injection; asterisks mark significant differences between WT and $P2X7R^{-/-}$ group. *B*) C57 BL/6 (wild-type) mice were i.p. injected with A438079 or vehicle, and one hour later with *Salmonella* LPS (n = 4 mice/group). The rectal temperature of mice was measured as in *A*. *C*) ATP release in the peritoneum of mice after 4 h injection with saline or *Salmonella* LPS. Mice received ATP-dependent luciferase-expressing HEK293 cells into the peritoneum after 2 h saline or LPS injection and luciferin was injected i.p. before 10 min of imaging. One representative experiment is shown on the left panel and bioluminescence was quantified as average radiance

(p/s/cm^2/sr) from three different experiments (right panel); asterisk marks significant differences between saline and LPS group (n = 3 mice per group). *D*) ELISA for IL-1β in the serum or peritoneal lavage of WT or P2X7R$^{-/-}$ mice after 4 h i.p. injection with saline or *Salmonella* LPS (n = 3 to 12 mice/group). *E*) WT or P2X7R$^{-/-}$ mice were i.p. injected with recombinant IL-1β and rectal temperature was monitored as in *A* (n = 6 mice). *F*) Temperature decrease in WT and P2X7R$^{-/-}$ mice from 3 to 6 h after IL-1β injection (n = 6 mice/group).

Figure 6. P2X7R activation couple to thromboxane and leukotriene release. *A-D*) ELISA for PGE2 (*A*), IL-1β (*B*), TXB2 (*C*) and LTB4 (*D*) release from BMDM primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP in the presence or absence of P2X7R selective antagonist (A438079, 10 μM) or the selective COX-2 inhibitor (SC-791, 1 μM) 10 min before and during ATP stimulation. n = 3 to 5 independent experiments. *E, F*) ELISA for TXB2 and LTB4 from primary human peritoneal macrophages (*E*; n = 3 different individuals) and blood monocytes (*F*; n = 4 different individuals) treated as in *A*.

Figure 1

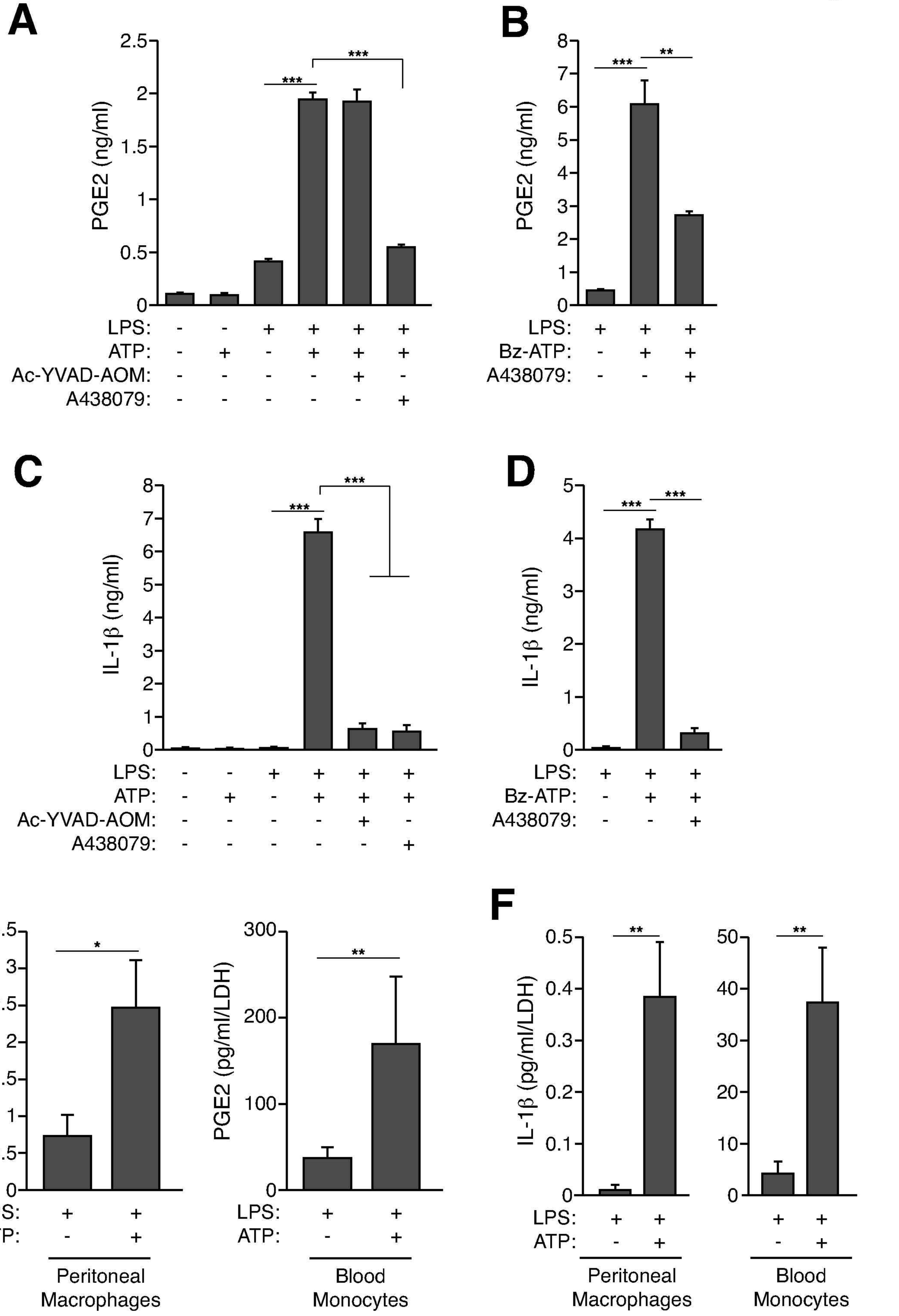

A
PGE2 (ng/ml)
2.5
2
1.5
1
0.5
0
***
***
LPS: - - + + + +
ATP: - + - + + +
Ac-YVAD-AOM: - - - - + -
A438079: - - - - - +
B
PGE2 (ng/ml)
8
7
6
5
4
3
2
1
0
***
**
LPS: + + +
Bz-ATP: - + +
A438079: - - +
C
IL-1β (ng/ml)
8
7
6
5
4
3
2
1
0
***
***
LPS: - - + + + +
ATP: - + - + + +
Ac-YVAD-AOM: - - - - + -
A438079: - - - - - +
D
IL-1β (ng/ml)
5
4
3
2
1
0
***
***
LPS: + + +
Bz-ATP: - + +
A438079: - - +
E
PGE2 (pg/ml/LDH)
3.5
3
2.5
2
1.5
1
0.5
0
*
LPS: + +
ATP: - +
Peritoneal Macrophages
PGE2 (pg/ml/LDH)
300
200
100
0
**
LPS: + +
ATP: - +
Blood Monocytes
F
IL-1β (pg/ml/LDH)
0.5
0.4
0.3
0.2
0.1
0
**
LPS: + +
ATP: - +
Peritoneal Macrophages
50
40
30
20
10
0
**
+ +
- +
Blood Monocytes

Figure 2

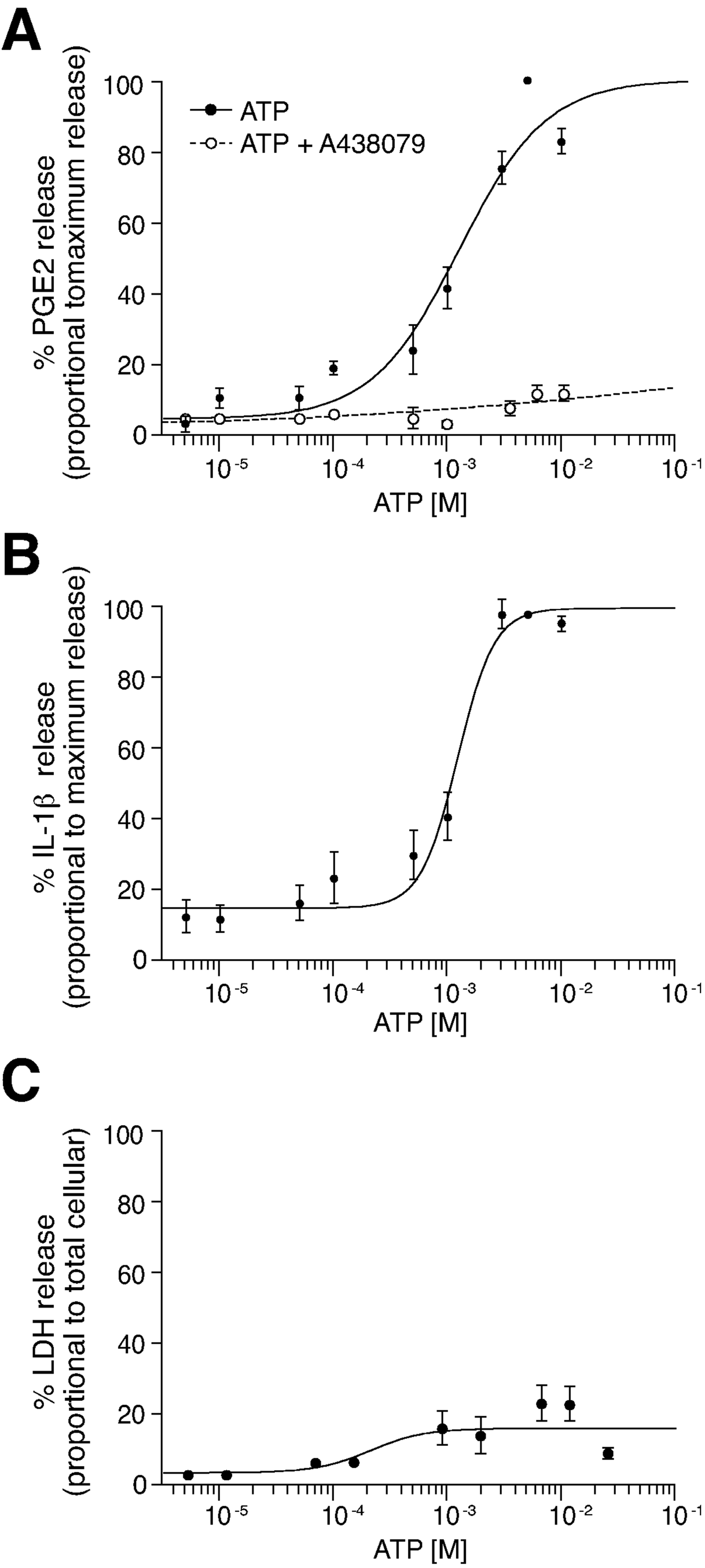
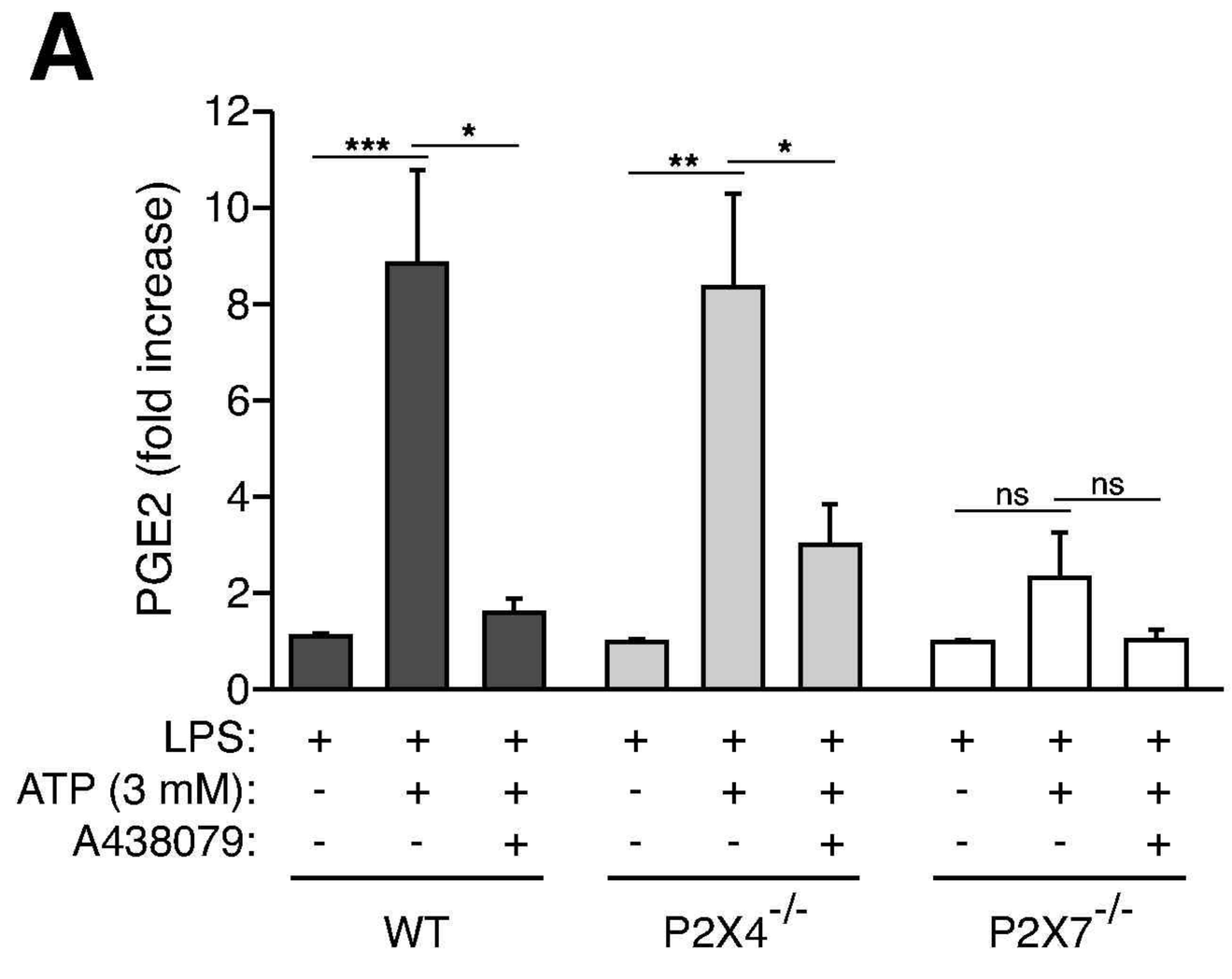
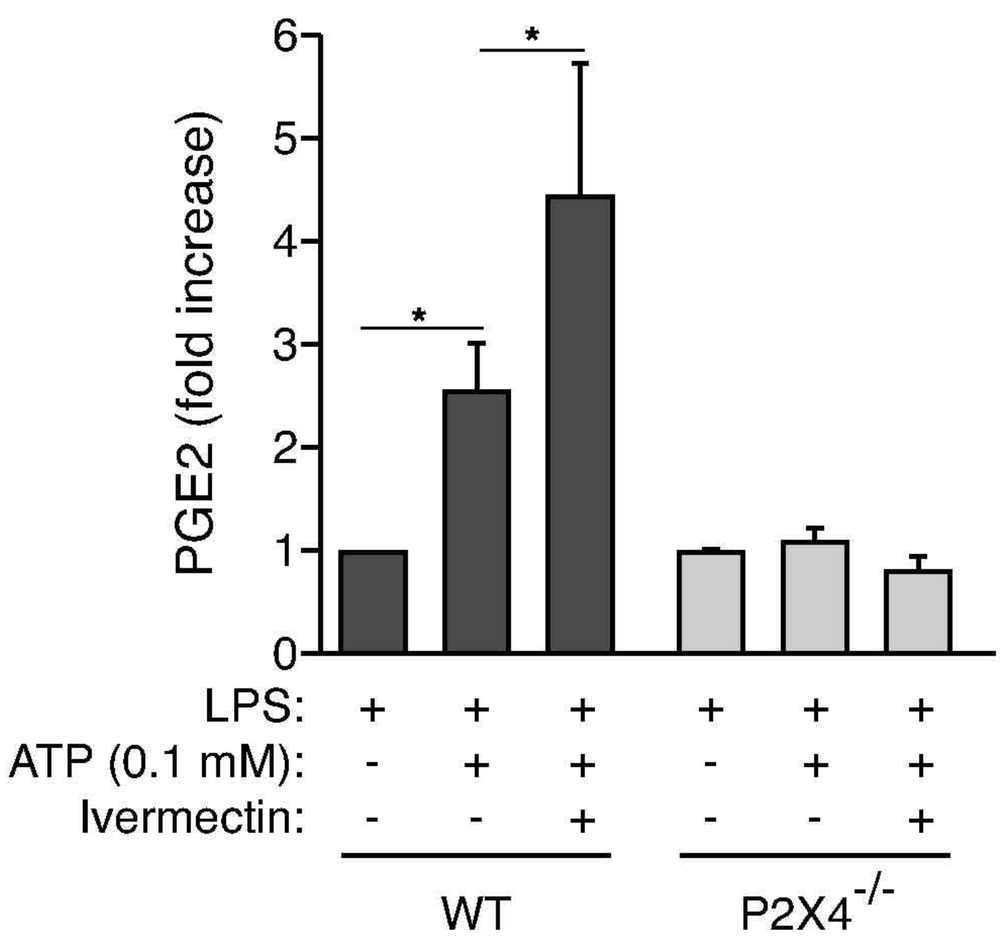
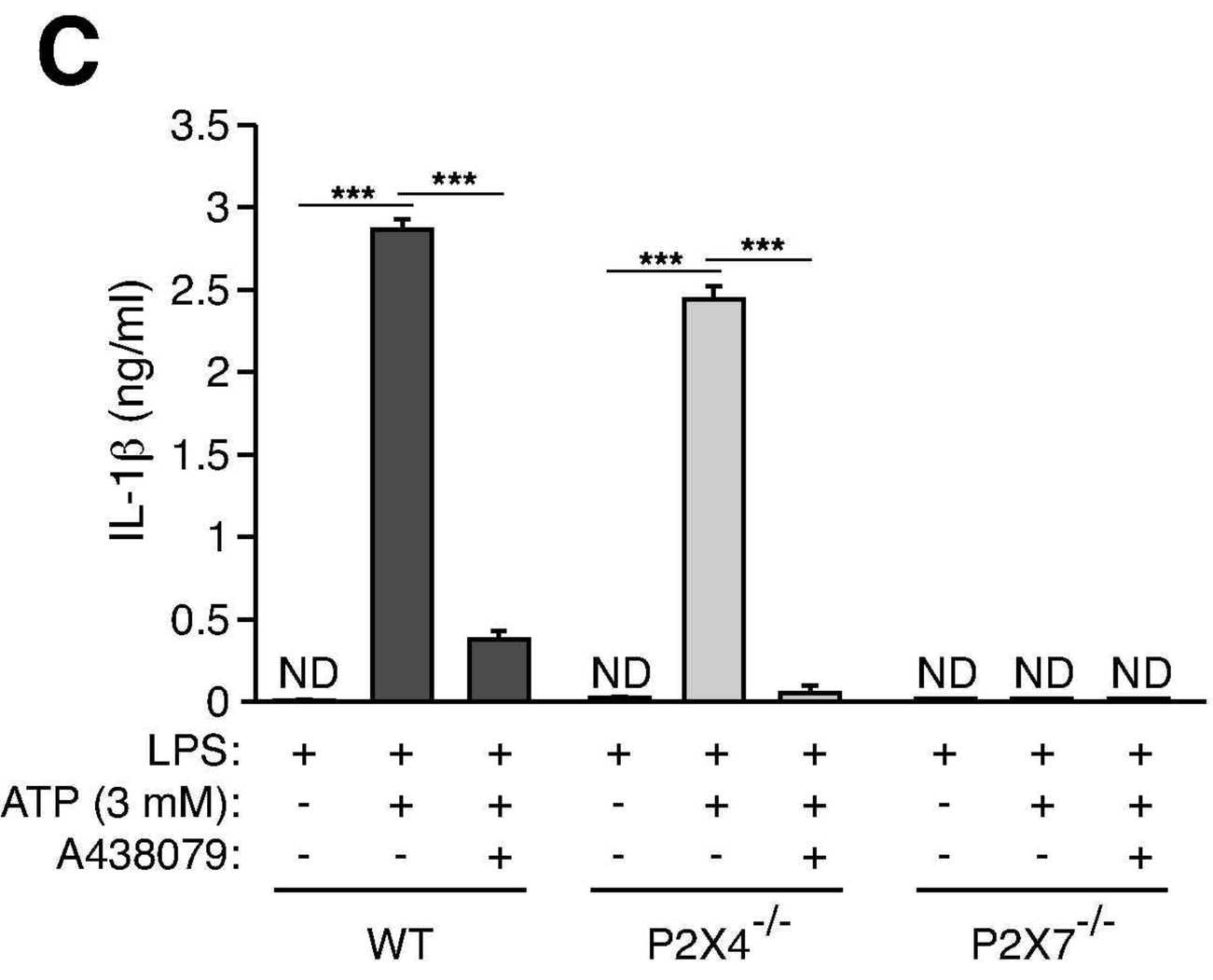
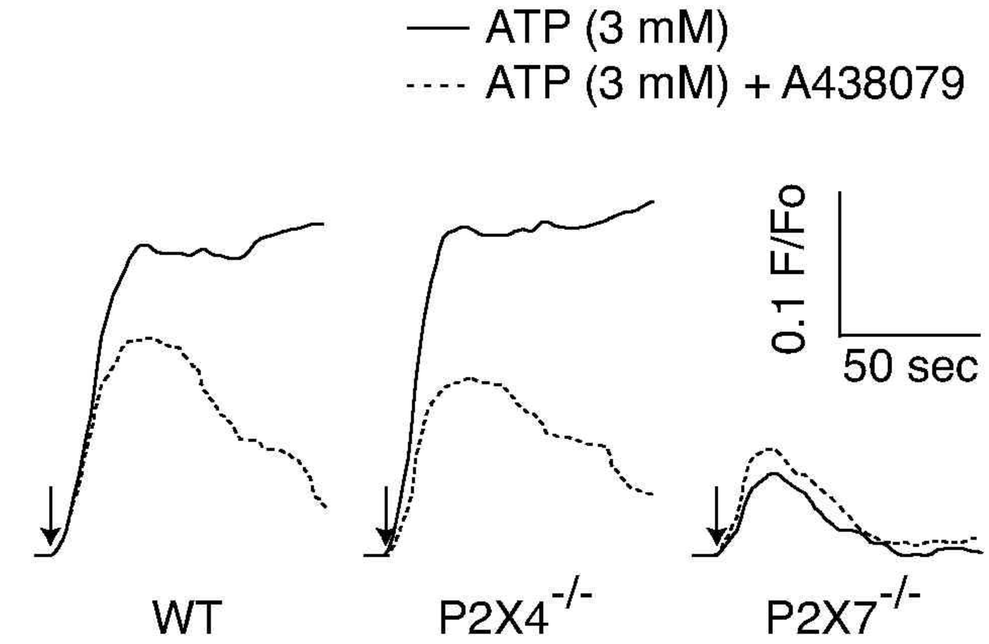

A
% PGE2 release
(proportional to maximum release)
100
80
60
40
20
0
ATP
ATP + A438079
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
ATP [M]
B
% IL-1β release
(proportional to maximum release)
100
80
60
40
20
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
ATP [M]
C
% LDH release
(proportional to total cellular)
100
80
60
40
20
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
ATP [M]

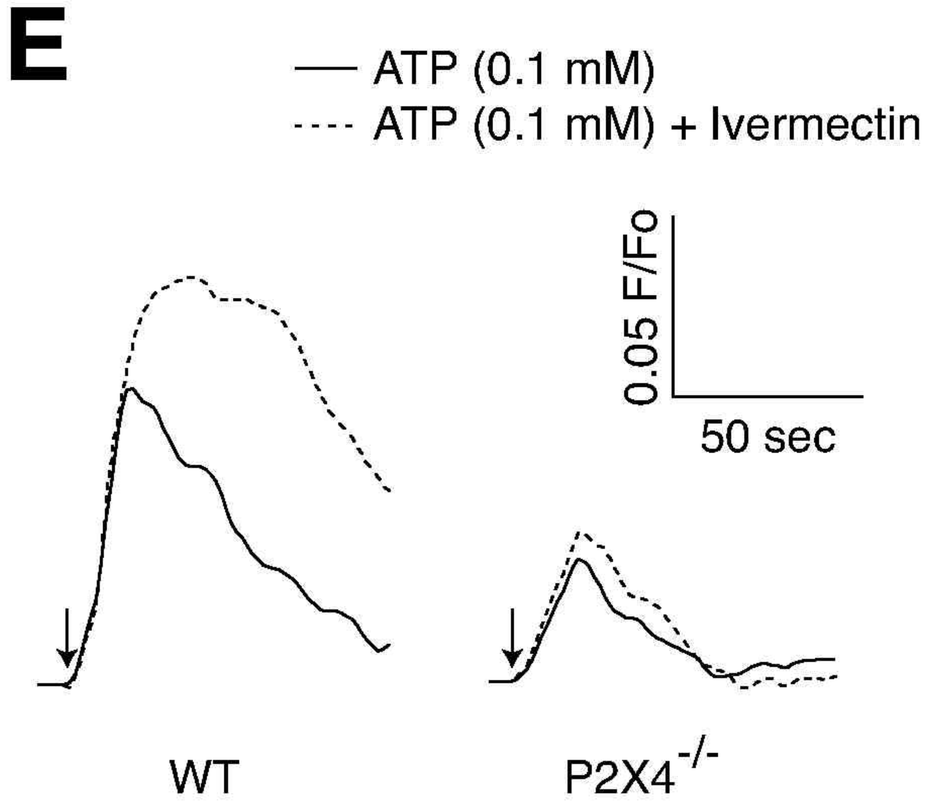
Figure 3


A
PGE2 (fold increase)
12
10
8
6
4
2
0
***
*
**
*
ns
ns
LPS: + + + + + + + + +
ATP (3 mM): - + + - + + - + +
A438079: - - + - - + - - +
WT
P2X4-/-
P2X7-/-


B
PGE2 (fold increase)
6
5
4
3
2
1
0
*
*
LPS: + + + + + +
ATP (0.1 mM): - + + - + +
Ivermectin: - - + - - +
WT
P2X4-/-


C
IL-1β (ng/ml)
3.5
3
2.5
2
1.5
1
0.5
0
***
***
***
***
ND
ND
ND ND ND
LPS: + + + + + + + + +
ATP (3 mM): - + + - + + - + +
A438079: - - + - - + - - +
WT
P2X4-/-
P2X7-/-


D
ATP (3 mM)
ATP (3 mM) + A438079
0.1 F/Fo
50 sec
WT
P2X4-/-
P2X7-/-


E
ATP (0.1 mM)
ATP (0.1 mM) + Ivermectin
0.05 F/Fo
50 sec
WT
P2X4-/-

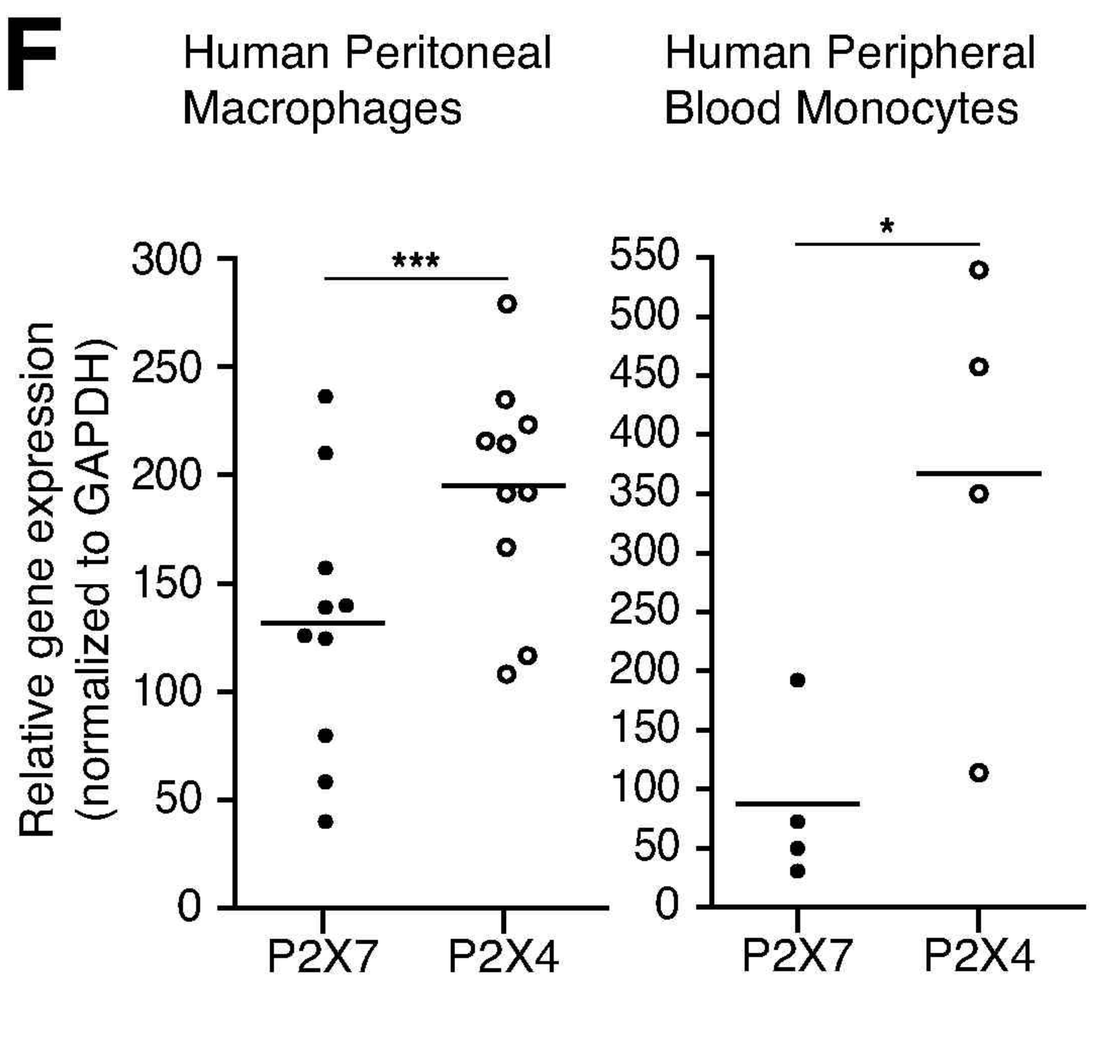
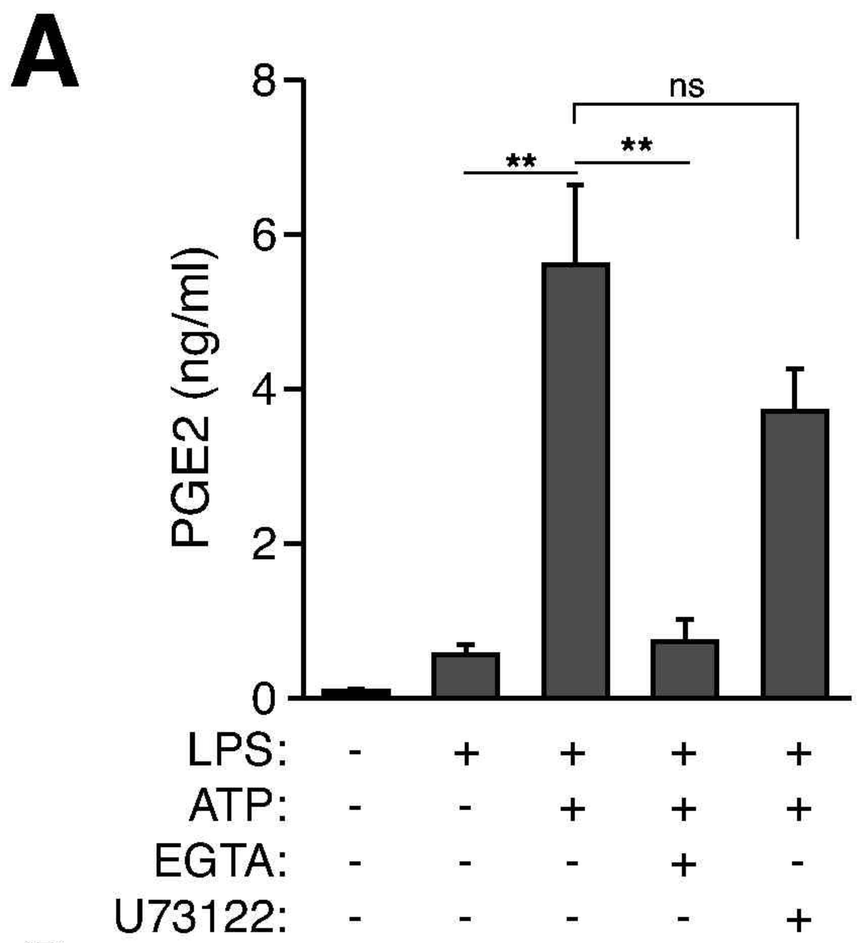
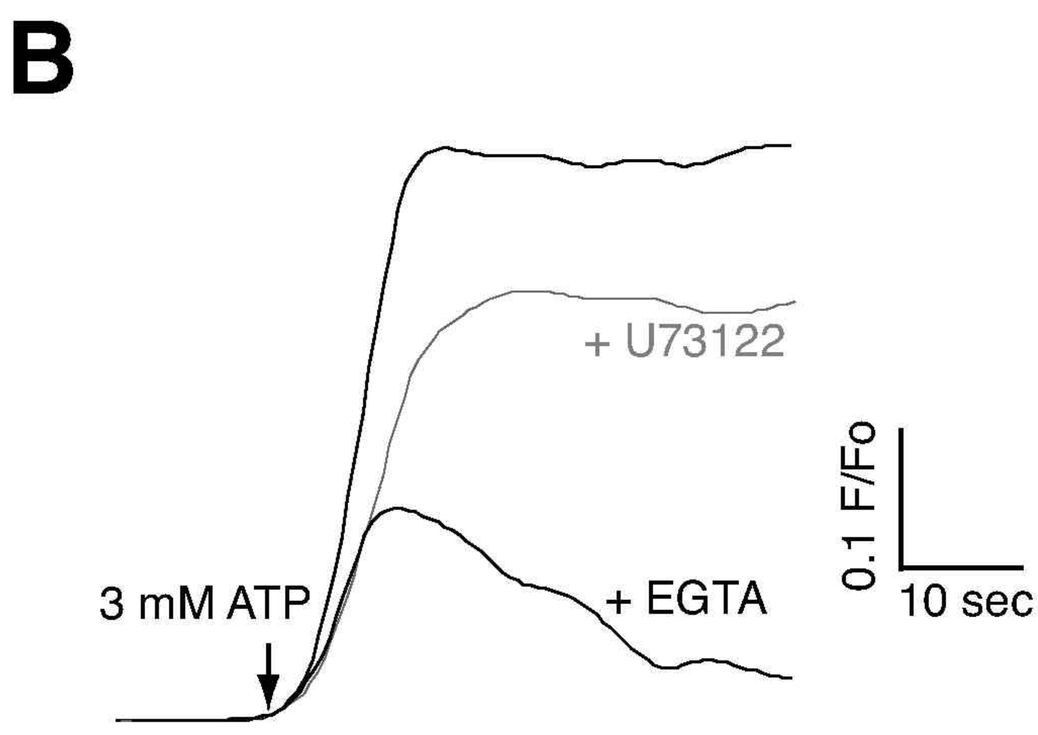
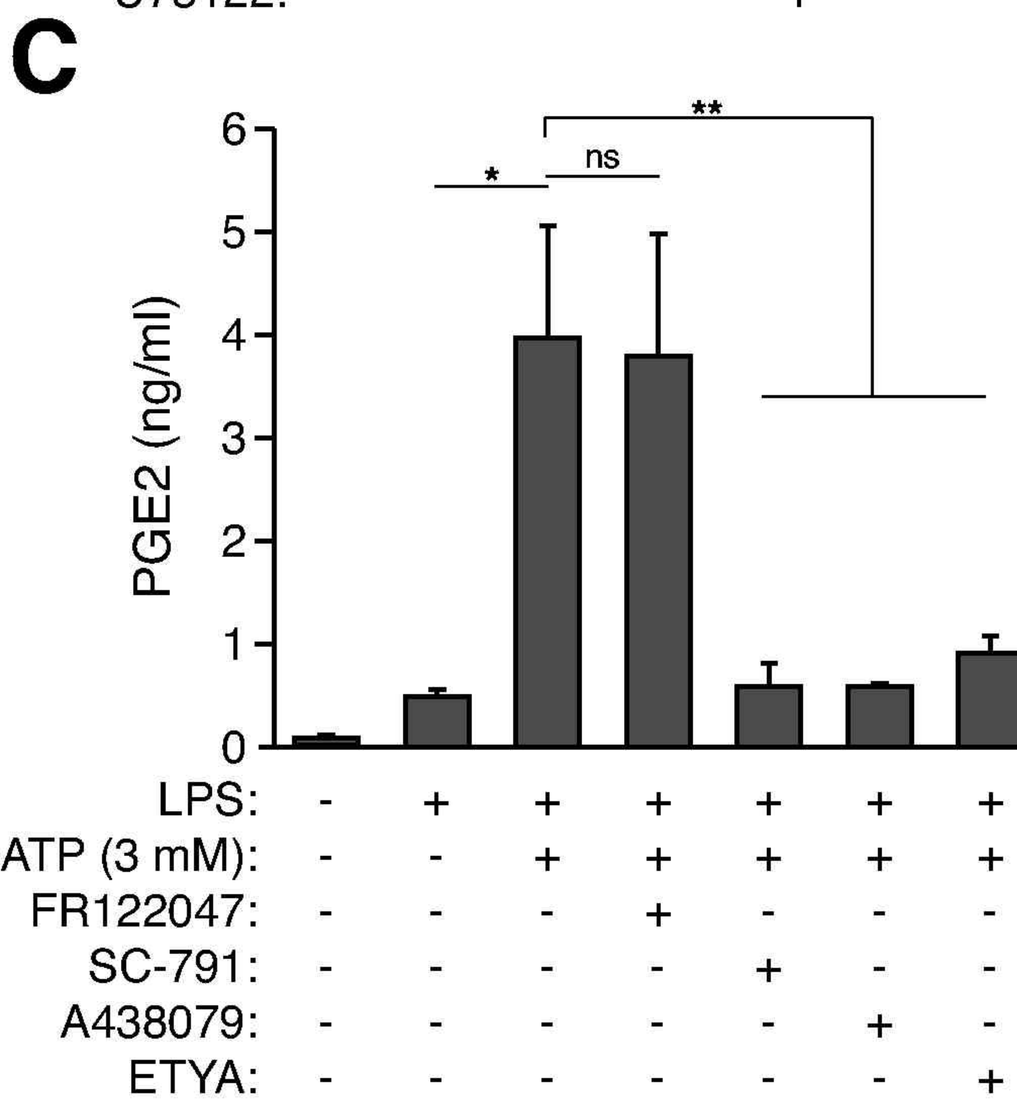
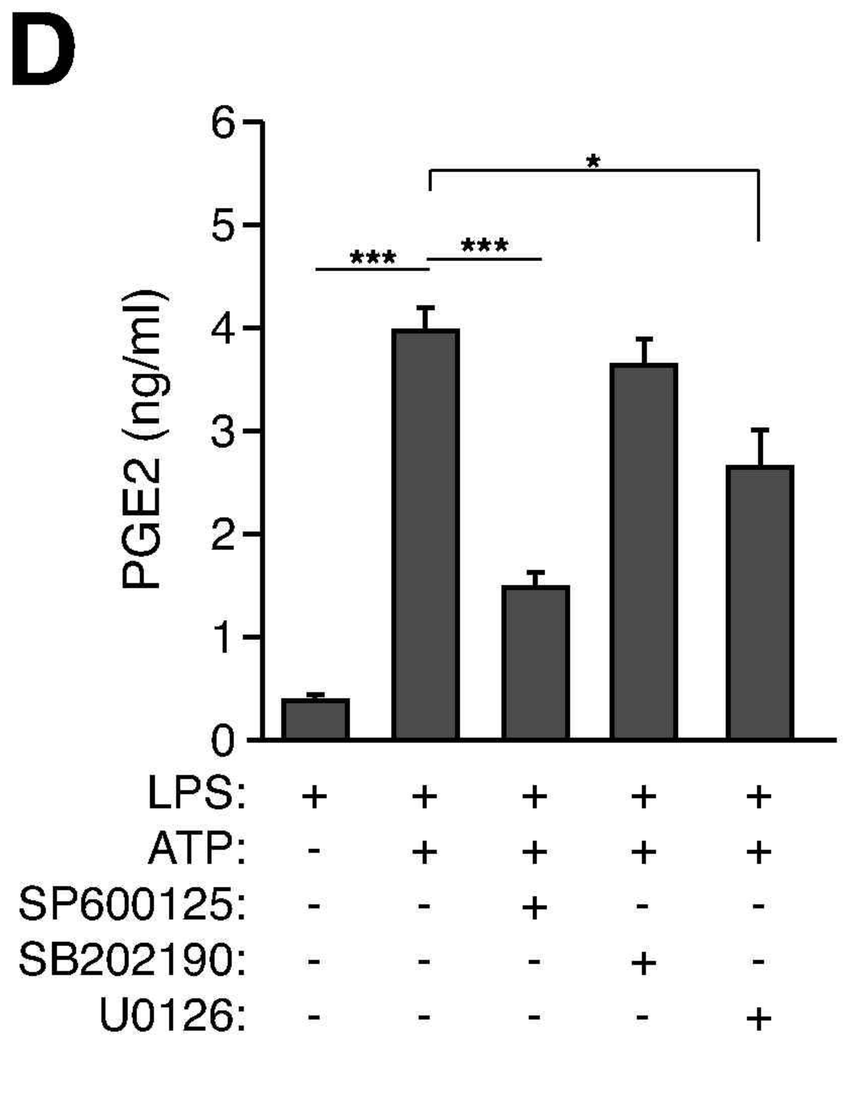

F
Human Peritoneal Macrophages
Human Peripheral Blood Monocytes
Relative gene expression (normalized to GAPDH)
300
250
200
150
100
50
0
***
550
500
450
400
350
300
250
200
150
100
50
0
*
P2X7
P2X4
P2X7
P2X4

Figure 4


A
PGE2 (ng/ml)
8
6
4
2
0
ns
**
**
LPS: - + + + +
ATP: - - + + +
EGTA: - - - + -
U73122: - - - - +


B
3 mM ATP
+ U73122
+ EGTA
0.1 F/Fo
10 sec


C
PGE2 (ng/ml)
6
5
4
3
2
1
0
*
ns
**
LPS: - + + + + + +
ATP (3 mM): - - + + + + +
FR122047: - - - + - - -
SC-791: - - - - + - -
A438079: - - - - - + -
ETYA: - - - - - - +


D
PGE2 (ng/ml)
6
5
4
3
2
1
0
***
***
*
LPS: + + + + +
ATP: - + + + +
SP600125: - - + - -
SB202190: - - - + -
U0126: - - - - +

E
COX2 fold change gene expression to unprimed cells (normalized to GAPDH)
800
600
400
6
4
2
0
ns
ns
LPS: - - + +
ATP (3 mM): - + - +

F
PGE synthase fold change gene expression to unprimed cells (normalized to GAPDH)
400
300
200
100
6
4
2
0
ns
ns
LPS: - - + +
ATP (3 mM): - + - +

Figure 5

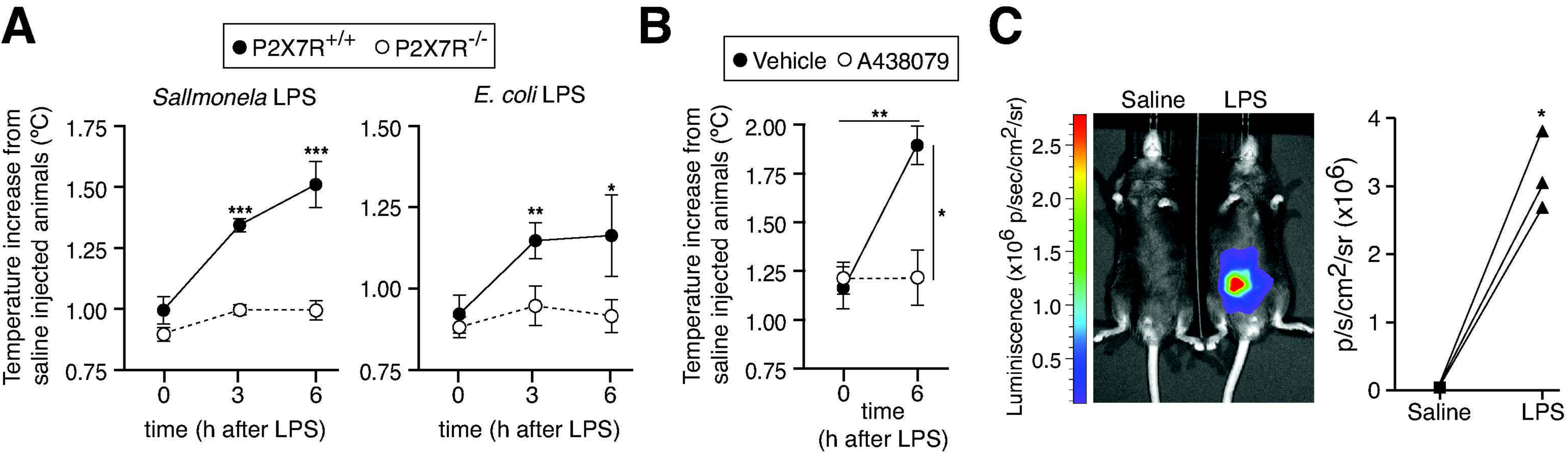

A
P2X7R+/+ P2X7R-/-
Sallmonela LPS
E. coli LPS
Temperature increase from saline injected animals (ºC)
time (h after LPS)
B
Vehicle A438079
time (h after LPS)
C
Saline LPS
Luminiscence (x10^6 p/sec/cm^2/sr)
p/s/cm^2/sr (x10^6)
Saline LPS
D
Serum IL-1β (pg/ml)
Peritoneal IL-1β (pg/ml)
LPS:
E
Temperature increase from saline injected animals (ºC)
ns
time (h after IL-1β)
F
Temperature decrease from 3 to 6 h after IL-1β injection (ºC)

Figure 6

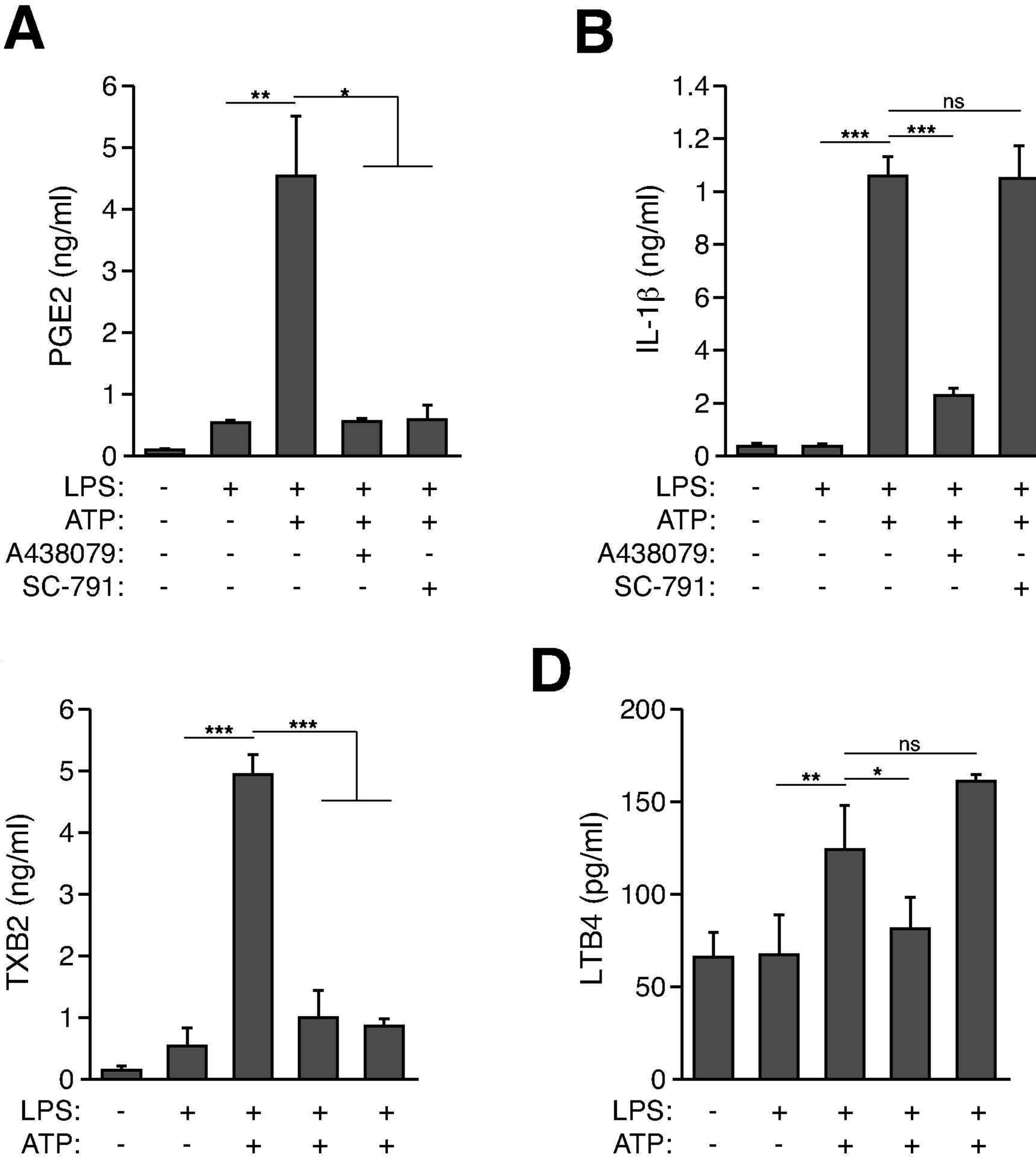

A
PGE2 (ng/ml)
0
1
2
3
4
5
6
**
*
LPS: - + + + +
ATP: - - + + +
A438079: - - - + -
SC-791: - - - - +
B
IL-1β (ng/ml)
0
2
4
6
8
1
1.2
1.4
***
***
ns
LPS: - + + + +
ATP: - - + + +
A438079: - - - + -
SC-791: - - - - +
C
TXB2 (ng/ml)
0
1
2
3
4
5
6
***
***
LPS: - + + + +
ATP: - - + + +
A438079: - - - + -
SC-791: - - - - +
D
LTB4 (pg/ml)
0
50
100
150
200
**
*
ns
LPS: - + + + +
ATP: - - + + +
A438079: - - - + -
SC-791: - - - - +
E
Human Peritoneal Macrophgaes
TXB2 (pg/ml/LDH)
0
1
2
3
4
5
6
7
8
9
*
LPS: + +
ATP: - +
LTB4 (pg/ml/LDH)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
*
LPS: + +
ATP: - +
F
Human Peripheral Blood Monocytes
TXB2 (pg/ml/LDH)
0
25
50
75
100
125
150
175
*
LPS: + +
ATP: - +
LTB4 (pg/ml/LDH)
0
1
2
3
4
5
6
7
8
*
LPS: + +
ATP: - +

TABLE 1. *PGE2 release from primary human peritoneal macrophages (pm) and blood monocytes (hbm) stimulated with ATP from four different individuals.*

	PGE2 (pg/ml*)			
	LPS	ATP[†] (F.I.[¶])	ATP[†] + IVM (F.I.[¶])	ATP[‡] (F.I.[¶])
pm 01	3.05	4.73 (1.55)	3.62 (1.19)	5.64 (1.85)
pm 02	1.06	0.65 (0.61)	0.78 (0.73)	1.79 (1.68)
pm 03	0.36	0.73 (2.00)	0.84 (2.32)	1.17 (3.21)
pm 04	0.54	0.97 (1.82)	0.63 (1.18)	1.07 (1.99)
Av F.I.[¶¶]	-	1.49±0.31 (p=0.21)	1.35±0.34 (p=0.37)	2.18±0.35 (**p=0.04**)
hbm 05	43.38	65.24 (1.50)	90.34 (1.39)	85.17 (1.96)
hbm 06	75.75	221.01 (2.92)	313.48 (1.42)	295.34 (3.90)
hbm 07	3.503	3.94 (1.12)	11.94 (3.03)	10.56 (3.01)
hbm 08	38.28	30.76 (0.80)	48.64 (1.58)	187.22 (4.89)
Av F.I.[¶¶]	-	1.58±0.46 (p=0.29)	1.85±0.39 (p=0.12)	3.44±0.62 (**p=0.02**)

LPS (1 mg/ml, 4h) and then 30 min of ATP (100 μM[†] or 3 mM[‡]) in the presence or absence of IVM (ivermectin 3 μM).

*pg/ml is normalized to the total amount of cellular LDH content as detailed in Methods.

[¶]F.I.: fold increase *vs*. LPS

[¶¶] Average of F.I. values *vs*. LPS

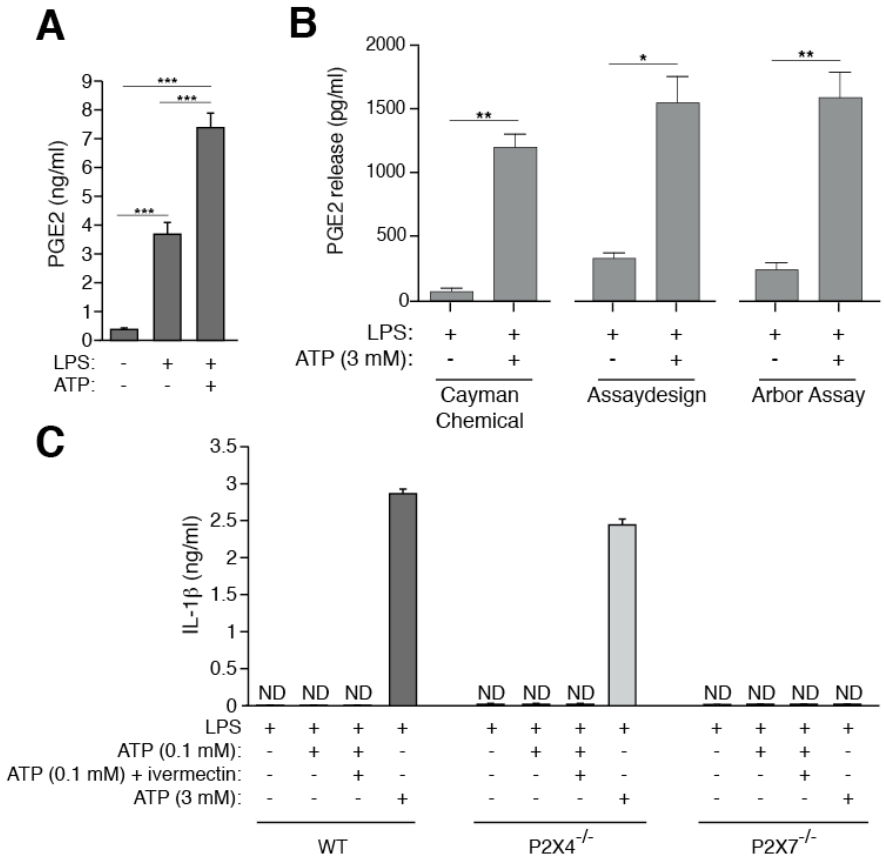
TABLE 2. *Rectal temperature of C57 BL/6 (wild type, P2X7R$^{+/+}$) and P2X7R-deficient (P2X7R$^{-/-}$) mice at different day times.*

	9.30		13.30		16.30	
	P2X7R$^{+/+}$	P2X7R$^{-/-}$	P2X7R$^{+/+}$	P2X7R$^{-/-}$	P2X7R$^{+/+}$	P2X7R$^{-/-}$
Mean temperature	37.08	36.96	36.54***	36.47**	36.42***	36.79
s.m.e	0.04	0,06	0,12	0,15	0,12	0,15
n	146	89	18	12	18	12

There were no differences in basal temperatures between P2X7R$^{+/+}$ and P2X7R$^{-/-}$ genotypes at any time point measured.

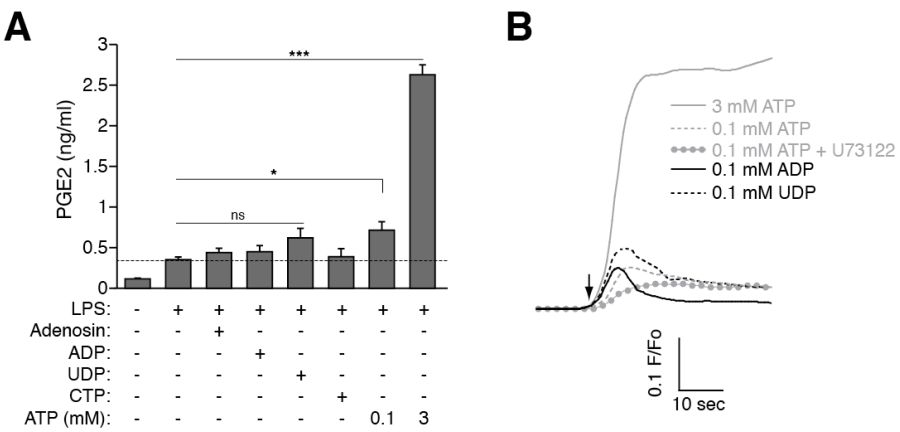
*** and ** statistically significant with 9.30 am group.

Supplemental Figure 1.



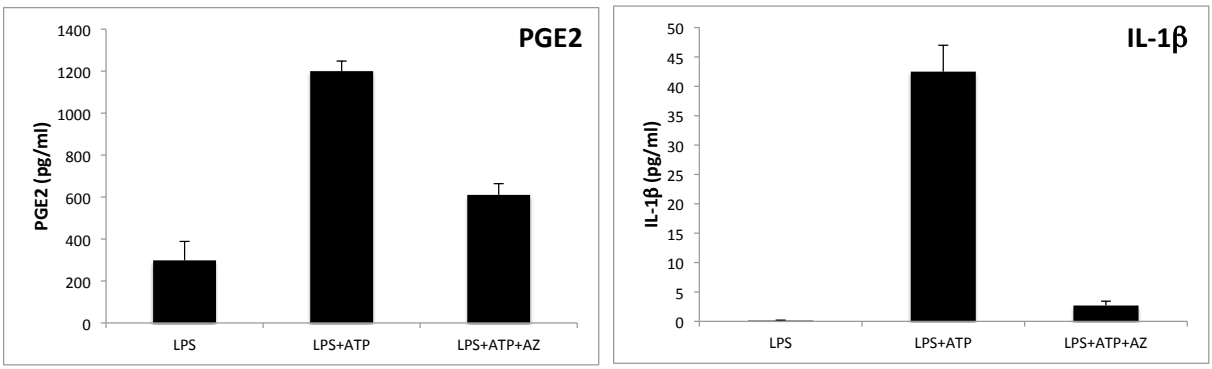
Supplemental Figure 1. *A*) ELISA for released PGE2 from mouse BMDM primed with LPS and subsequently stimulated for 30 min with 3 mM of ATP on the top of LPS (without washing LPS stimulation medium). *B*) ELISA for PGE2 release from BMDM primed with LPS and subsequently washed with Et-buffer and stimulated for 30 min with 3 mM of ATP. The PGE2 determination was performed using three different ELISA kit brands; n = 4 independent experiments for each ELISA kit (samples were not the same among the different ELISA kit). *C*) ELISA for IL-1β release from BMDM wild type (WT), P2X4R-deficient ($P2X4^{-/-}$) or P2X7R-deficient ($P2X7^{-/-}$) mice primed with LPS and subsequently stimulated for 30 min with 0.1 mM ATP or 3 mM of ATP in the presence or absence of ivermectin (3 μM) 10 min before and during ATP stimulation (n = 4 independent experiments for each genotype); ND: not detected.

Supplemental Figure 2.



Supplemental Figure 2. *A*) ELISA for PGE2 release from BMDM primed with LPS and subsequently stimulated for 30 min with 0.1 mM of different nucleotides or 3 mM of ATP. *B*) Representative trace out of 3 independent experiments for intracellular calcium rise in BMDM stimulated as in A in the presence or absence of the PLC inhibitor U73122 (10 μM). Asterisks represent significance compared with LPS treated group.

SUPPLEMENTAL MATERIAL FOR REVIEWER 1 (FOR REVISION PROPOSE)



PGE2 and IL-1b release from human peritoneal macrophages primed with LPS (1 ug/ml, 4h) and treated with or without ATP (3 mM, 30 min) in the presence or absence of P2X7 antagonist AZ 11645373 (AZ, 10uM); n=1 individual.