Transient P2X<sub>7</sub> Receptor Activation Triggers Macrophage Death Independent of Toll-like Receptors 2 and 4, Caspase-1, and Pannexin-1 Proteins

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Background: P2X<sub>7</sub> receptors are thought to be primarily involved in inflammatory signaling.

Results: Transient (1–4 min) high ATP induced delayed (hours) cell death in macrophages from WT and TLR2/4, Casp1, or Panx1 knock-out mice.

Conclusion: Transient P2X<sub>7</sub> receptor activation triggers macrophage-selective apoptotic cell death independent of TLR signaling, Casp1, and Panx1.

Significance: P2X<sub>7</sub> receptors function foremost as death triggers in macrophages.

The function of P2X<sub>7</sub> receptors (ATP-gated ion channels) in innate immune cells is unclear. In the setting of Toll-like receptor (TLR) stimulation, secondary activation of P2X<sub>7</sub> ion channels has been linked to pro-caspase-1 cleavage and cell death. Here we show that cell death is a surprisingly early triggered event. We show using live-cell imaging that transient (1–4 min) stimulation of mouse macrophages with high extracellular ATP ([ATP]<sub>e</sub>) triggers delayed (hours) cell death, indexed as DEV- influx, a signature of P2X<sub>7</sub>-ligation, was highly protective, whereas no protection was conferred in macrophages lacking caspase-1 or TLR2 and TLR4. Furthermore, pannexin-1 (Panx1) deficiency had no effect on transient ATP-induced delayed cell death or ATP-induced Yo-Pro-1 uptake (an index of large pore pathway formation). Thus, “transient” P2X<sub>7</sub> receptor activation and Ca<sup>2+</sup> overload act as a death trigger for native mouse macrophages independent of Panx1 and pro-inflammatory caspase-1 and TLR signaling.

P2X receptors are cation-selective channels, and seven genes encoding the subunits P2X<sub>1</sub> to P2X<sub>7</sub> have been identified (1, 2). The P2X<sub>7</sub> receptor stands out from the others because it is resistant to desensitization (3), and prolonged activation induces membrane permeability to large molecules (∼1 kDa) such as the 375-Da fluorescent probe Yo-Pro-1 (2, 4, 5). Pannexin-1, a putative hemichannel, has been implicated as the P2X<sub>7</sub>-receptor-activated large pore pathway (6, 7). The P2X<sub>7</sub>-receptor is predominantly expressed in immune cells (8). It was previously denoted as the pro-cytolytic P2Z receptor, but cloning in 1996 revealed that it belonged to the P2X family (9). Since that time, P2X<sub>7</sub> has emerged as a potentially important second stimulus for Toll-like receptor 4 (TLR4)-dependent IL-1β and IL-18 secretion. First, priming of macrophages with the TLR4 ligand lipopolysaccharide (LPS) induces intracellular accumulation of pro-IL-1β and pro-IL-18. Second, P2X<sub>7</sub> receptor activation promotes assembly of the “inflammasome” and caspase-1-dependent cleavage and release of biologically active IL-1β and IL-18. This scenario is well established in both in vivo (10) and in vitro models (11–20).

Typically, pro-inflammatory IL-1β and IL-18 cytokine processing and release can be detected after about 20–30 min of P2X<sub>7</sub>-receptor stimulation, and cell death, commonly indexed as lactate dehydrogenase release, is moderately low under such conditions (10–12, 15). In contrast, prolonged (>30 min) P2X<sub>7</sub>-receptor stimulation is well known to be lethal (4, 11, 15). Hence, cell death is generally assumed to be a late event in relation to inflammatory cytokine processing. However, the extracellular [ATP] is tightly controlled by ectonucleotidases (21), and it is difficult to imagine a situation in which macro-

2 The abbreviations used are: TLR, Toll-like receptor; dKO, double knock-out; DMPS, dimethyl sulfoxide; TMRE, tetramethylrhodamine, ethyl ester; DIC, differential interference contrast; z-VAD-fmk, carbobenzoxy-Val-alanyl-aspartyl-[O-methylfluoromethylketone.
phage P2X receptors are stimulated by high [ATP]e for a long duration (15–20 min) after sensing bacteria via TLR4. Brief (5 min) stimulation of LPS-primed macrophages with millimolar ATP concentrations is a more likely signaling scenario. Indeed, brief stimulation of P2X receptors is sufficient to initiate the processing of IL-1β in macrophages (16, 18).

The aim of this study was to elucidate the role of P2X receptors in innate immune cells. We used resident macrophages from mice deficient in various genes (Casp1, P2rx7, Tlr2, Tlr4, P2ry2, and Panx1) and real-time single-cell imaging to elucidate, in particular, a link between transient P2X receptor activation and cell death.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were obtained from Sigma-Aldrich unless stated otherwise. ATP was added from a 100 mM stock solution in Dulbecco’s phosphate-buffered saline (PBS; pH 7.4). The fluoroergic substrate for caspase-3 (and caspase-7) DEVD-ForView488 was added from a 1 mM stock solution in dimethyl sulfoxide (DMSO) (BioTrend, Germany). z-VAD-fmk (20 mM stock in DMSO) was obtained from R&D Systems, and both tetramethylrhodamine, ethyl ester (TMRE) and fluo-3/AM were from Invitrogen. Calpeptin (Tocris Bioscience) was added from a 100 mM stock in DMSO. Yo-Pro-1 iodide (M, 629) was added from a 1 mM stock in DMSO (Invitrogen). Yo-Pro-1 is a monomeric cyanine dye with a cationic side chain.

**Knock-out Mice—**P2X7−/−, P2Y2−/−, Casp1−/−, Panx1−/−, and TLR2/4 double knock-out (dKO) mice were backcrossed to C57BL/6 mice. Casp1−/− mice were provided by GlaxoSmithKline (M, 629). For selected experiments, macrophage colony-stimulating factor (R&D Systems), which is also called macrophage-stimulating factor 1 (macrophage), was added from a 20 mM stock in DMSO. Bone marrow-derived macrophages were used to produce glass bottom WillCo dishes (40-mm glass diameter and 0.17-mm thickness) with highly confluent macrophages. The femurs of mice were cleared of tissue and completely fractured in the middle of the shaft (diaphysis) using a surgical scalpel blade (number 21). Bone marrow cells were flushed out of each bone fragment using ~5 ml of Dulbecco’s modified Eagle’s medium (DMEM), injected via a 20-gauge needle. The cell suspension, collected in a 50-ml Falcon tube, was centrifuged (1200 rpm for 8 min) and resuspended in 10 ml of RPMI 1640 medium (Biochrom AG) containing 10% heat-inactivated fetal calf serum, 10 µg/ml LPS from *Escherichia coli* 0111:B4 (L3012, Sigma) for 4 h.

**Bone Marrow-derived Neutrophils**—Bone marrow cells were flushed from the hind leg femurs of mice using Hank’s buffered salt solution (Invitrogen) containing 10% FCS and Hepes (pH 7.4). After filtration via a cell strainer with 70-µm pores (BD Falcon, BD Biosciences), the cell suspension was centrifuged at 1200 rpm for 10 min. During this time, a density gradient was prepared in a round bottom 14-ml tube (BD Falcon, BD Biosciences) by layering 4-ml Histopaque-1119 underneath a 4-ml Histopaque-1077 via a long syringe needle. The bone marrow cell pellet was resuspended in 1 ml of solution, layered onto the Histopaque density gradient, and centrifuged at 1800 rpm (without using brakes) for 30 min at room temperature. The granulocyte layer, sandwiched between the Histopaque-1077 and -1119, was removed using a pipette, washed once, and resuspended in 10 ml of conditioned medium containing RPMI 1640 medium (Biochrom AG), 20% heat-inactivated fetal calf serum, 10% culture supernatant from WEHI-3B cells (mouse myelomonocytic leukemia cell line; ATCC TIB-68), 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured overnight at 37 °C (5% CO2). The following morning, cells were centrifuged at 1200 rpm for 8 min and resuspended in 10 ml of Heps-Ringer solution. Subsequently, cells were seeded into µ-Slide I chambers (Ibidi) freshly coated with fibronectin. After allowing 5–10 min for adherence, the medium was replaced with bicarbonate-free RPMI 1640 containing 20 mM Hepes (Biochrom AG), but no fetal calf serum.

**Bone Marrow-derived Macrophages**—For selected experiments, bone marrow-derived macrophages were used to produce glass bottom WillCo dishes (40-mm glass diameter and 0.17-mm thickness) with highly confluent macrophages. The femurs of mice were cleared of tissue and completely fractured in the middle of the shaft (diaphysis) using a surgical scalpel blade (number 21). Bone marrow cells were flushed out of each bone fragment using ~5 ml of Dulbecco’s modified Eagle’s medium (DMEM), injected via a 90° bent 23-gauge needle. The cell suspension, collected in a 50-ml Falcon tube, was centrifuged for 8 min at 1100 rpm and 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of lysis buffer for 5 min (before recentrifugation) to induce hemolysis. The lysis buffer contained: 155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA (pH 7.4). The suspension was then centrifuged for 10 min at 300 × g and room temperature. The supernatant was aspirated, and the cells were washed once using 10 ml of Dulbecco’s PBS and centrifuged (300 × g for 8 min at room temperature). Next, the pellet was resuspended in 30 ml of incubation medium, which consisted of DMEM, 2% glutamine, 1% kanamycin, 1% nonessential amino acids, 10% heat-inactivated FCS, and 20 ng/ml recombinant mouse colony-stimulating factor 1 (macrophage), which is also called macrophage colony-stimulating factor (R&D Systems). The suspended cells were incubated at 37 °C, 5% CO2 in 30-ml Teflon bags for 6 days. After this period, the cells were resuspended after placement on ice for 15 min and transferred to glass-bottomed WillCo dishes. After 2 h (to allow cell adhesion), the dishes were washed with RPMI 1640 medium (Biochrom) containing 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin. After incubating with 155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA (pH 7.4). After titrating the pH back to 7.4, the cells were centrifuged at 1200 rpm for 8 min. After centrifugation, the supernatant was aspirated, and the cells were washed once using 10 ml of Dulbecco’s PBS and centrifuged (300 × g for 8 min at room temperature). Next, the supernatant was resuspended in 30 ml of incubation medium, which consisted of DMEM, 2% glutamine, 1% kanamycin, 1% nonessential amino acids, 10% heat-inactivated FCS, and 20 ng/ml recombinant mouse colony-stimulating factor 1 (macrophage), which is also called macrophage colony-stimulating factor (R&D Systems). The suspended cells were incubated at 37 °C, 5% CO2 in 30-ml Teflon bags for 6 days. After this period, the cells were resuspended after placement on ice for 15 min and transferred to glass-bottomed WillCo dishes. After 2 h (to allow cell adhesion), the dishes were washed with RPMI 1640 medium (Biochrom) containing 10% heat-inactivated FCS, 100 units/ml penicillin, and
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100 μg/ml streptomycin. Before use, the cells were incubated overnight.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analyses—Living macrophages were labeled with Alexa Fluor 488-conjugated anti-mouse F4/80 antibodies (clone CI:A3-1; catalogue number MCA497A488, AbD Serotec, Oxford, UK), and F4/80<sup>+</sup> cells (~30% of total) were isolated using a BD FACSAria II cell sorter (BD Biosciences). Bone marrow-derived granulocytes were labeled with Alexa Fluor 488-conjugated anti-Gr-1 and phycoerythrin-conjugated anti-CD11b antibodies, and Gr-1<sup>hi</sup>/CD11b<sup>hi</sup> cells (neutrophils) were purified by cell sorting. The anti-mouse Gr-1 (Ly-6G) antibody (clone RB6-8C5) was obtained from eBioscience (catalogue number 53-5931-82). RNA was isolated using the RNeasy mini kit and protocol from Qiagen (Hilden, Germany). cDNA was synthesized using SuperScript III reverse transcriptase. In the case of panexins, the thermocycling protocol for the PCR was as follows: 94 °C for 4 min, then 29 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. The following primers were used:

- P2X7 primer pair for P2X7 (195 bp): forward, CCCTGCACAGTG-CACCTG; reverse, TAAAACTCGTCACTA; reverse, TACGTCACACAAGAATG-GAATAA.

- TGA; and Panx3 (268 bp), forward, CCTCACAAGGCTCTTCATACCCGCCAC; reverse, GGGTGAGCAGACATGGAA-CATTGACCCCATGCTACTCC; reverse, TCAGCCACA.

Lows: 94 °C for 4 min, then 29 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The following primers were used:

- 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1% Nonidet P-40, 10% glycerol, 5 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> (sodium orthovanadate), the protease inhibitors leupeptin, aprotinin, and Pefabloc (each at 10 μg/ml), and 50 mM Tris-HCl (pH 7.4). Proteins were separated by 6–15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Roche Applied Science, Mannheim, Germany). Membranes were blocked for 1 h at room temperature in TBS containing 5% nonfat dry milk and 0.05% Tween 20 followed by overnight incubation with anti-cleaved caspase-3 (17-kDa fragment) antibodies (catalogue number 9661), diluted 1:1000 (obtained from Cell Signaling Technology via New England Biolabs). For detection, horseradish peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany) were used in combination with SuperSignal West Pico chemiluminescence substrate (Perbio, Bonn, Germany). Blots using anti-β-actin antibody (Sigma-Aldrich) were performed to control sample loading.

Nuclear Staining and Cytochrome c Labeling—Macrophages were fixed with 4% paraformaldehyde in Dulbecco’s PBS for 15 min at 37 °C followed by permeabilization with 0.1% Triton X-100 in PBS, containing 5% normal goat serum, for 10 min. Cells were incubated with Alexa Fluor 555-conjugated anti-cytochrome c antibodies (BD Pharmingen) for 30 min. Subsequently, the nuclei were stained by incubating cells with 280 μM 4',6-diamidino-2-phenylindole (DAPI), a nucleic acid stain, for 90 s. Note that NucView488, similar to DAPI, is a nucleic acid stain, which allows assessment of nuclear morphology (28).

Single-cell Cytosolic [Ca<sup>2+</sup>] Measurements—A glass coverslip seeded with macrophages was sealed onto the bottom of a Perspex bath (volume, 100 μl) using silicone lubricant (Fine Science Tools). Macrophages were imaged via a 40 ×/1.4 oil objective lens and superfused at 1 ml/min. To monitor intracellular [Ca<sup>2+</sup>], cells were incubated for 15 min with 10 μM fluo-3/AM. To reduce the rate of fluo-3 loss, solutions contained 0.8 mM probenecid, and experiments were performed at room temperature (20–23 °C). In each experiment, a single macrophage (selected with a bilateral iris) was excited at 488 nm, whereas fluorescence was detected at 530 ± 15 nm using a microscope-based spectrofluorometer system (Photon Technology International, Seefeld, Germany). Fluorescence signals were normalized with respect to the resting fluorescent intensity (I<sub>0</sub>) and expressed as F/I<sub>0</sub>. Solutions were rapidly switched by means of miniature three-way valves (The Lee Co., Westbrook, CT).

Statistical Analysis—Normality and homoscedasticity were tested using the Kolmogorov-Smirnov and Levene tests, respectively. A one-way analysis of variance was performed using an α value of 0.05. The post hoc Scheffé test was used to compare groups. In the case of two independent groups, an unpaired t test was used to test for statistical significance. Statistical analyses were performed using SPSS software, and data are presented as means ± S.E.

RESULTS

Continuous ATP Application—We initially tested whether prolonged P2X<sub>7</sub>-receptor stimulation activated caspase-3/7 and induced cell death in resident peritoneal macrophages using live-cell time-lapse imaging. After preloading with TMRE to monitor mitochondrial membrane potential, macrophages were continuously incubated in medium containing the cell-permeable and nonfluorescent probe DEVD-NucView488 to detect caspase-3/7 activity. Application of high [ATP]<sub>e</sub> (3 mM), required to activate P2X<sub>7</sub> receptors, induced microblebbing of wild-type (WT) and Casp1<sup>−/−</sup> macrophages (Fig. 1A) within 60 s and dissipated the mitochondrial membrane potential in all cells within 2–3 min. Subsequently, between 10 and 60 min, one cell after the other became caspase-3/7-positive (Fig. 1B), indicated by the appearance of green fluorescence; the nonfluorescent probe DEVD-NucView488 is cleaved by caspase-3/7.
and the NucView488 moiety becomes fluorescent upon binding to RNA and DNA (28).

In contrast to macrophages, prolonged application of ATP (3 mM) did not induce microblebbing, activation of caspase-3/7, or cell death in bone marrow-derived neutrophils (Gr-1<sup>−/−</sup> cells) (Fig. 1C). This could be explained by a lack of P2X<sub>7</sub> receptors in this cell type. Indeed, we could not detect P2X<sub>7</sub> mRNA in Gr-1<sup>high</sup>/CD11b<sup>high</sup> cells (neutrophils) purified by cell sorting. Macrophages isolated from P2X<sub>7</sub>-deficient mice were similarly insensitive to prolonged ATP application (Fig. 1D). Hence, prolonged stimulation with high [ATP]<sub>e</sub> induces the activation of caspase-3/7 and cell death in WT and Casp1<sup>−/−</sup>/H11002/macrophages, but has no effect on the viability of P2X<sub>7</sub><sup>−/−</sup>/H11002/macrophages and neutrophils (summarized in Fig. 1D). Thus, in principle, a modest time window is available for P2X<sub>7</sub>-mediated pro-IL-1β processing before caspase-3/7 activity and cell destruction

FIGURE 1. Continuous ATP application. A, simultaneous measurement of mitochondrial membrane potential (mitoΔΨ; red fluorescence) and caspase-3/7 activity (green fluorescence) in WT and caspase-1 deficient (Casp1<sup>−/−</sup>) macrophages challenged with 3 mM ATP (P2X<sub>7</sub> receptor ligand). Images are 70 × 70 μm. B, cumulative plot of WT and Casp1<sup>−/−</sup> macrophage cell death, indexed as caspase-3 and caspase-7 (Casp-3/7) activity, during continuous stimulation with 3 mM ATP in medium containing the cell-permeable and nonfluorescent caspase-3/7 substrate DEVD-NucView488. Following caspase activation, the cleavage product NucView488 binds to DNA and becomes highly fluorescent. C, prolonged ATP application has no effect on the viability of neutrophils (Gr-1<sup>−/−</sup> cells). D, summary of data obtained using macrophages isolated from WT, Casp1<sup>−/−</sup>/H11002, or P2X<sub>7</sub><sup>−/−</sup>/H11002 mice (n = 5 independent experiments (56–73 cells) per group) or neutrophils (n = 4 independent experiments; 61 cells).

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(MARCH 23, 2012•VOLUME 287•NUMBER 13 JOURNAL OF BIOLOGICAL CHEMISTRY 10653)
manifest. However, it is unlikely that macrophages are confronted with high [ATP] for prolonged periods. Other unidentified factors may increase the sensitivity of P2X7 ion channels to ATP. Alternatively, transient increases of ATP above 500 μM may be physiologically more relevant.

**Transient ATP Application**—Next, we tested whether transient (1–4 min) P2X7 receptor stimulation is sufficient to induce cell death in macrophages. Macrophages were stimulated with 3 mM ATP for 1, 2, 3, or 4 min. Following washout of ATP, macrophages were incubated in medium containing DEVD-NucView488, and fluorescent and DIC images were taken every 1 min for 6 h. After application of ATP for 4 min and washout, the macrophages had “fuzzy” edges (probably due to cell swelling) and a paucity of lamellipodia (Fig. 2A and supplemental Video 1). However, within 1–2 h, the cells resumed a normal morphology characterized by broad lamellipodial membrane extensions and peripheral ruffling (Fig. 2A and supplemental Video 1). This pseudo-recovery period was interrupted by abrupt cell contraction and dynamic membrane blebbing, characteristic of apoptosis, followed by concurrent caspase-3/7 activity and massive membrane blebbing, indicating secondary necrosis (Fig. 2, A–C, and supplemental Video 1; for comparison, see the supplemental Video accompanying the review by Taylor et al. (29)). The kinetics of caspase-3/7 activation in individual cells is shown in Fig. 2B.

The sequence of events described above (and shown in Fig. 2, A–C) was also observed in bone marrow-derived macrophage plated at high (>80%) confluency (supplemental Fig. 1). The
macrophage purity was high, as indicated by anti-F4/80 labeling of living macrophages (supplemental Fig. 1A). At such high confluency, individual macrophages are in close contact with neighboring cells. However, following transient stimulation with high [ATP]e, confluency is decreased due to the retraction of lamellipodia (supplemental Fig. 1, B and C).

Activation of DEVDase (caspase-3/7) activity and massive blebbing consistently coincided, as shown in the example in Fig. 2C. Note that the cell becomes diffusely green due to the binding of NucView488 to RNA, and then NucView488 redistributes to DNA (28). Thus, in addition to reporting DEVDase activity, NucView488 provides a “view” of nuclear morphology. The nucleus of macrophages appeared condensed or fragmented, indicative of apoptosis, during delayed cell death (supplemental Video 1). As in the case of prolonged P2X7 receptor stimulation, delayed caspase-3/7 activation and massive blebbing following transient high [ATP]e were end-stage apoptotic events. To confirm that transient high [ATP]e leads to caspase-3 activation, we performed Western blot (Fig. 2D). Fully cleaved caspase-3 (17-kDa fragment) could be detected 4 h after transient high [ATP]e. Susceptibility to delayed caspase-3/7 activation and cell death increased steeply as a function of the duration of ATP application (Fig. 2E).

Loss of Cytochrome c and Nuclear Contraction—Cytochrome c was localized to the mitochondria in fixed and permeabilized macrophages labeled with Alexa Fluor 555-conjugated anti-cytochrome c antibodies (supplemental Fig. 2A). Apoptotic macrophages fixed 3 h after transient (4 min) ATP challenge were characterized by loss of mitochondrial cytochrome c and contracted nuclei (supplemental Fig. 2, B and C).

Absence of Delayed Cell Death in P2X7−/− Macrophages—No apoptotic changes, such as microblebbing and caspase-3/7 activation, could be detected in P2X7−/− macrophages following 4 min of stimulation with millimolar ATP (Fig. 3, A–C, and supplemental Video 2). Instead of forming microblebs, P2X7−/− macrophages generated large lamellipodial membrane protrusions in response to high [ATP]e, such that cell area increased by ~50% (Fig. 3, D and E). These observations confirm that transient millimolar ATP induces delayed cell death specifically via the activation of P2X7 receptors.

FIGURE 3. Transient high [ATP]e induces cell spreading but not delayed cell death in P2X7−/− macrophages. A, lack of delayed caspase-3/7 (Casp-3/7) activation in P2X7−/− macrophages challenged with 3 mM ATP for 4 min. Images are 100 × 100 μm. B, P2X7−/− macrophages 12 h after transient challenge with 3 mM ATP. Image is 100 × 150 μm. C, summary data (n = 4 independent experiments (29 cells) in the WT group; n = 4 independent experiments (35 cells) in the P2X7−/− group). D, high ATP (3 mM) induces lamellipodial membrane protrusions in P2X7−/− but not WT macrophages. E, mean cell areas of WT (n = 30) and P2X7−/− (n = 22) macrophages before and 1 min after application of 3 mM ATP. *, p value < 0.05.
Transient ATP-induced Cell Death Is Independent of Caspase-1 and TLR Signaling—P2X7 receptor stimulation has been shown to activate caspase-1, and we speculated that this pro-inflammatory and pro-apoptotic enzyme could play a role in delayed ATP-induced cell death. However, Casp1−/− macrophages were clearly not protected from delayed P2X7-dependent cell death (Fig. 4A and supplemental Video 3). In addition, we found that macrophages isolated from TLR2 and TLR4 double knock-out (TLR2/4 dKO) mice were not protected from ATP-induced cell death (Fig. 4B). The cumulative death rates of individual WT, Casp1−/−, and TLR2/4 dKO macrophages (solid symbols) are plotted in Fig. 4C. It can be seen that one cell after the other becomes caspase-3/7-positive (cell death end point) starting at around 1 h after washout of ATP. For comparison, the data from Fig. 1B, showing the cumulative death rates during continuous ATP application, are superimposed...
Most cells die within 1 h of continuous high [ATP]e. As in the case of WT cells, the nucleus of Casp1−/− and TLR2/4 dKO macrophages became fragmented during delayed cell death (for example, Fig. 4D), and by 12 h after washout, less than ~20% of WT, Casp1−/−, and TLR2/4 dKO cells survived (Fig. 4E). Thus, the data in Fig. 4 indicate that TLR signaling and cleavage of pro-caspase-1 to active caspase-1 are not required in the P2X7-activated signaling cascade culminating in caspase-3/7 activation and cell death.

Effects of LPS Priming on Transient ATP-induced Cell Death—Primin with LPS (1 μg/ml for 2 h) has previously been reported to render WT macrophages susceptible to cell death (indexed as lactate dehydrogenase release) within 30 min of a brief ATP (5 mM for 5 min) challenge, whereas Casp1−/− cells are protected (15). We therefore tested whether LPS priming (1 μg/ml for 4 h) potentiated transient (4 min) ATP-induced caspase-3/7 activation and cell death. Similar to unprimed macrophages (Fig. 4), transient ATP-induced delayed cell death was observed in both WT and Casp1−/− macrophages primed with LPS (Fig. 5). However, ~18% of LPS-primed WT macrophages became caspase-3/7-positive within 30 min (Fig. 5C) of ATP washout, as compared with 0% in unprimed WT cells (Fig. 4C). In the case of Casp1−/− macrophages, ATP-induced delayed caspase-3/7 activation and cell death were similar in unprimed (Fig. 4C) and LPS-primed (Fig. 5C) cells.

Massive Ca2⁺ Influx Is Critical Determinant of P2X7-mediated Delayed Cell Death—Superfusion of macrophages with ATP concentrations up to 300 μM has previously been reported to induce a large Ca2⁺ transient attributable to P2Y2 receptor activation and a small Ca2⁺ influx signal via P2X7 and P2X4 receptors (30). In single-cell Ca2⁺ recordings, we found that high [ATP]e (3 mM) induced a sustained increase in cytosolic [Ca2⁺] following the initial Ca2⁺ spike in P2X7−/− macrophages superfused with high [ATP]e (Fig. 6A). The elevated Ca2⁺ plateau could be interrupted by superfusing the cell with Ca2⁺-free medium. We presumed that the sustained Ca2⁺ component was solely due to P2X7 receptor activation. Indeed, there was no Ca2⁺ plateau in P2X7−/− macrophages superfused with high [ATP]e (Fig. 6B). Instead, ATP induced a single Ca2⁺ spike in P2X7−/− macrophages, consistent with P2Y2 receptor activation. The lack of sustained Ca2⁺ influx in P2X7−/− macrophages is good evidence, together with the lack of microblebbing and cell death, that the functional P2X7 splice variant found in some tissues (31) is not (functionally) expressed in macrophages. Using P2Y2−/− macrophages, we could “isolate” the P2X7-mediated sustained Ca2⁺ signal (Fig. 6C). An overlay of the P2X7 and P2X7−/− mediated Ca2⁺ signaling components is shown in Fig. 6D.

Ca2⁺ has been recognized as a death trigger for a long time (32), and we speculated that the striking Ca2⁺ influx and Ca2⁺ overload mediated by P2X7 receptor activation may play a role in transient high [ATP]e-induced cell death. Consistent with
this notion, application of millimolar ATP in Ca\(^{2+}\)-free (0 Ca\(^{2+}\)) medium protected macrophages from delayed cell death (Fig. 6E). That is, the transient (4 min) application of ATP was bracketed with Ca\(^{2+}\)-free medium, and under these conditions, a single Ca\(^{2+}\) spike, similar to that seen in P2X\(_7^{-/-}\) cells (Fig. 6B), is evoked. Hence, the duration of P2X\(_7\), ligation and accompanying sustained Ca\(^{2+}\) influx are important determinants of death signaling.

Pan-caspase and Calpain Inhibitors Attenuate Transient ATP-induced Delayed Cell Death—In the presence of the cell-permeable pan-caspase inhibitor z-VAD-fmk (40 \(\mu M\)), macrophages were partially protected from delayed cell death induced by 4 min of exposure to 3 mM ATP (Fig. 7A). Cells were similarly protected by calpeptin (100 \(\mu M\)), an inhibitor of the calpain family of Ca\(^{2+}\)-activated proteases (Fig. 7B). These inhibitor experiments indicate that calpain and caspases contribute to transient ATP-induced cell death signaling cascade.

**Pannexin-1 Is Not P2X\(_7\)-dependent Large Pore Pathway**—In addition to the ions Na\(^+\) (23 Da), K\(^+\) (39 Da), and Ca\(^{2+}\) (40 Da), activated P2X\(_7\) receptors become permeable to molecules up to a size of \(\sim 900\) Da. Pannexin-1 has been implicated as the molecular correlate of this P2X\(_7\)-dependent large pore pathway and has been implicated in cell death (6, 33–36). In purified F4/80\(^\text{+}\) cells (macrophages), we could detect mRNA for Panx1, but not Panx2 or Panx3 (Fig. 8A). To investigate whether Panx1 is the large pore pathway accompanying P2X\(_7\), receptor ligation,
we compared the rates of ATP-induced dye (Yo-Pro-1; M<sub>f</sub> 375) uptake in single WT and Panx<sup>−/−</sup> macrophages using time-lapse microscopy. Yo-Pro-1 is a green fluorescent DNA and RNA stain that is normally cell-impermeable. The mean rates of ATP-induced Yo-Pro-1 uptake by WT and Panx<sup>−/−</sup> macrophages are shown in Fig. 8B. These results indicate that Panx1 is not the large pore pathway in native macrophages.

The kinetics of Yo-Pro-1 uptake in individual cells is shown in Fig. 8 (C–F). During the initial 20-min incubation period with 2 μM Yo-Pro-1, there was no constitutive uptake of Yo-Pro-1 in WT macrophages (Fig. 8, C and D). However, application of 3 mM ATP induced Yo-Pro-1 uptake, consistent with the opening of a large pore (Fig. 8C and supplemental Video 4). Yo-Pro-1 uptake was not impaired in Panx<sup>−/−</sup> macrophages. The time course of Yo-Pro-1 uptake by Panx<sup>−/−</sup> macrophages is shown in Fig. 8, E and F (see also supplemental Video 5).

We tested whether stimulation of P2X<sub>7</sub> receptors with a lower concentration of ligand, 500 μM instead of 3 mM ATP, was sufficient to induce Yo-Pro-1 uptake (supplemental Fig. 3). There was no obvious change in cell morphology after 20 min (supplemental Fig. 3A), or even 60 min, of continuous stimulation with 500 μM ATP. However, a weak increase in Yo-Pro-1 uptake could be detected (supplemental Fig. 3, A and B). The magnitude of Yo-Pro-1 fluorescence induced by 500 μM ATP was much less than that evoked by 3 mM (compare supplemental Fig. 3B with Fig. 8D). A similar weak increase in Yo-Pro-1 uptake was measured in Panx<sup>−/−</sup> macrophages challenged with 500 μM ATP (supplemental Fig. 3, C and D). Thus, Panx1 is not responsible for the weak dye uptake response to 500 μM ATP. As in the case for WT macrophages, continuous stimulation of Panx1-deficient cells with 500 μM ATP also did not induce cell death.

**Lack of ATP-induced Yo-Pro-1 Uptake in P2X<sub>7</sub>−/− Macrophages**—We speculated that the stimulation of P2Y<sub>2</sub> receptors may cause weak (low capacity) Yo-Pro-1 uptake, which is otherwise masked by the dominant effect of P2X<sub>7</sub>. However, this was not the case because no Yo-Pro-1 uptake could be detected when P2X<sub>7</sub>−/− macrophages were challenged with ATP (Fig. 9, A and B, and supplemental Video 6). These findings indicate that P2X<sub>7</sub> receptors mediate the weak Yo-Pro-1 uptake induced by 500 μM ATP, as well as the robust dye uptake evoked by 3 mM ATP.

**Panx1 Deficiency Does Not Protect Macrophages from Transient ATP-induced Cell Death**—Panx1 activation has been implicated in cell death in response to P2X<sub>7</sub> receptor ligands (33, 35). Therefore, we investigated whether Panx1−/− macrophages were protected from cell death (Fig. 9, C and D). Delayed transient ATP-induced cell death was clearly not reduced in Panx1−/− macrophages (Fig. 9, C and D). Thus, the pro-death signaling triggered by P2X<sub>7</sub> receptor activation in native macrophages is not dependent on Panx1.

**DISCUSSION**

In the innate immune system, P2X<sub>7</sub> receptors have emerged as potentially important modulators of inflammation on the basis that moderately prolonged (typically 15–20 min) stimulation of these ion channels induces caspase-1-dependent processing of pro-inflammatory cytokines (11–20). Here we identify a new paradigm in which transient (1–4 min) stimulation of P2X<sub>7</sub> receptors triggers delayed (hours) macrophage death independent of caspase-1, TLR signaling, and pannexin-1. It was already known that prolonged (>30 min) stimulation of P2X<sub>7</sub> receptors was lethal to cells, presumably due to massive perturbations of Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> homeostasis and large pore formation (6, 18). The surprising finding that transient (1–4 min) P2X<sub>7</sub> receptor activation triggers delayed effector caspase (caspase-3/7) activity and cell death suggests that ATP-gated P2X<sub>7</sub> ion channels are essentially macrophage death receptors.
High ATP (acting at P2X<sub>7</sub> receptors) is one of many “danger signals” known to activate the inflammasome, a complex formed by the assembly of subsets of NLR (nucleotide-binding domain and leucine-rich repeat containing) proteins, such as NLRP3 (also called NALP3) (37), and the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), encoded by the gene Pycard (38, 39). Inflammasomes bind to pro-caspase-1 and induce its proteolytic-dependent activation. Thus, the inflammasome, which activates pro-inflammatory caspase-1, can be considered as the inflammatory equivalent of the “apoptosome,” a protein complex that activates pro-apoptotic caspase-9 (40).

High ATP (acting at P2X<sub>7</sub> receptors) can also induce macrophage-specific cell death, and our data indicate that activation of P2X<sub>7</sub> receptors for 1–4 min is sufficient to trigger apoptotic cell death. What was already known about the effects of transient P2X<sub>7</sub> receptor stimulation on cell fate? In 2002, Le Feuvre et al. (15) reported that “a brief pulse of ATP (5 mM for 5 min) had no effect on basal lactate dehydrogenase release (measured in supernatant samples taken 0.5 and 2 h after stimulation) from control WT (mouse peritoneal) macrophages, and the cells completely excluded trypan blue (data not shown).” In light of our findings, revealed by live-cell imaging, that transient high ATP leads to caspase-3/7 activation and cell death delayed by hours (Figs. 2 and 4), we presume that Le Feuvre et al. (15) would have detected loss of cell integrity (secondary necrosis) if they had followed the fate of the cells for a longer period. The authors, however, found that a brief pulse (5 min) of ATP variably induced massive lactate dehydrogenase release (>100% of total release measured in two separate experiments, respectively) within 30 min from WT, but not caspase-1-deficient, macrophages that had been primed with LPS. In contrast, Pelegrin et al. (20) reported that application of 5 mM ATP for 20 min caused negligible lactate dehydrogenase release (2–4% of total) in mouse peritoneal macrophages primed with LPS for 4 h. Thus, the extent to which LPS...

FIGURE 8. ATP-induced dye (Yo-Pro-1) uptake in WT and Panx1<sup>−/−</sup> macrophages. A, purified mouse peritoneal macrophages (F4/80<sup>+</sup> cells) express Panx1, but not Panx2 or Panx3. B, summary of high ATP (3 mM)-induced Yo-Pro-1 uptake data obtained using macrophages isolated from WT (n = 30 cells; 3 independent experiments) or Panx1<sup>−/−</sup> mice (n = 30 cells; 3 independent experiments). C, time-lapse Yo-Pro-1 fluorescence (green) and DIC images of WT macrophages challenged with 3 mM ATP. Yo-Pro-1 (2 μM), which becomes fluorescent upon binding to DNA and RNA, was present throughout the experiment. Images are 70 × 70 μm. D, kinetics of Yo-Pro-1 uptake in individual WT macrophages. After a 20-min baseline period, a high ATP (3 mM) was added. E, time-lapse Yo-Pro-1 fluorescence and DIC images of Panx1<sup>−/−</sup> macrophages challenged with 3 mM ATP. Images are 70 × 70 μm. F, kinetics of Yo-Pro-1 uptake in individual Panx1<sup>−/−</sup> macrophages.
priming sensitizes macrophages to ATP-induced cell death is unclear. In our study, we found that transient ATP similarly induced delayed cell death in WT, Casp1−/−, TLR2/4 dKO, and Panx1−/− macrophages, as well as in LPS-primed (4 h) WT and Casp1−/− cells. Cell death, indexed as caspase-3/7 activity, was negligible in the first 60 min after transient ATP application, except in LPS-primed WT macrophages, 20% of which became caspase-3/7-positive within 30 min. Thus, priming with LPS, and possibly other pathogen-associated molecular patterns or even macrophage infection, may shorten the delay between transient ATP stimulation and cell death in a caspase-1-dependent fashion.

Live-cell imaging revealed that the fate of macrophages after transient P2X7 receptor stimulation follows a distinct sequence of events. After washout of ATP, the cells appear to recover from the initial microblebbing and loss of lamellipodial membrane protrusive activity, but in the following hours, suddenly one cell after the other tightly contracts and further dynamic microblebbing manifests, followed by the formation of large, expansive blebs. The nuclei become contracted or fragmented, features typical for apoptosis (29). A consistent feature of delayed death in individual cells is that massive bleb formation coincides with the emergence of caspase-3/7 activity. We assume that the effector caspases caspase-3 and caspase-7 contribute to the massive blebbing. These effector caspases target multiple proteins of the cytoskeleton, including key components of actin filaments, intermediate filaments, and microtubules (29). The pan-caspase inhibitor z-VAD-fmk did not completely abrogate transient ATP-induced delayed cell death, indicating that the mode of death signaling is only partially caspase-dependent.

What is the function of P2X7 receptors expressed on macrophages? The major differences between P2X7 receptors and other members of the P2X family are (i) activation by high [ATP]e (0.5–3 mM rather than 0.1–100 μM range), (ii) lack of desensitization (P2X receptors typically desensitize within seconds), and (iii) the permeability transition to larger molecules upon stimulation. The requirement for high [ATP]e suggests that P2X7 ion channels are activated under extreme conditions, such as massive tissue injury. Our data indicate that the Ca2+ overload induced by several minutes of P2X7 receptor stimulation serves as a death trigger. Identifying pathophysiological scenarios in which [ATP]e and cytosolic [Ca2+] are increased, even for several minutes, remains a challenge in the fields of purinergic signaling and immunology. Unidentified endogenous or pathogen-associated factors may come into play to increase the affinity of P2X7 receptors for ATP. The contribution of P2X7 receptor-induced large pore formation to transient high [ATP]e-induced cell death remains unclear. Genetic deletion of pannexin-1, putative P2X7-mediated large pore pathway, did not affect the kinetics of ATP-induced Yo-Pro-1 uptake, and transient ATP-induced delayed cell death was unaffected. We speculate that instead of recruiting another transport pathway, the P2X7 channel protein probably changes configuration to allow the passage of larger molecules, although to date single-channel current recordings have not been able to reveal such dramatic changes in size selectivity (41).
In summary, there is considerable evidence linking P2X7 receptor function with caspase-1 and TLR4 signaling pathways, and prolonged P2X7 ion channel stimulation is known to induce cytolytic cell death. We now show, surprisingly, that death is an early triggered event, such that transient (1–4 min) high [ATP]e leads to delayed (hours) cell death. Moreover, our results clearly show that death signaling depends on the duration of trigger ATP and Ca2+ overload, but it is independent of pro-inflammatory caspase-1 activation and TLR signaling. Finally, we also show that pannexin-1 is not involved in P2X7-dependent Yo-Pro-1 uptake or transient high [ATP]e-induced delayed cell death.

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