Priming of Macrophages with Lipopolysaccharide Potentiates P2X7-mediated Cell Death via a Caspase-1-dependent Mechanism, Independently of Cytokine Production*

Rosalind A. Le Feuvre‡, David Brough‡, Yoichiro Iwakura¶, Kiyoshi Takeda§, and Nancy J. Rothwell‡

From the ‡School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom, the ¶Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan, and the §§Laboratory of Molecular Medicine, Osaka University, Suita, Osaka 565-0871, Japan

ATP stimulation of cell surface P2X7 receptors results in cytolsis and cell death of macrophages. Activation of this receptor in bacterial lipopolysaccharide (LPS)-activated macrophages or monocytes also stimulates cytokine processing and release of the cytokine interleukin-1β (IL-1β) through activation of caspase-1. The cytokine interleukin 18 (IL-18) is also cleaved by caspase-1 and shares pro-inflammatory characteristics with IL-1β. The objective of the present study was to test the hypothesis that IL-1β, IL-18, and/or caspase-1 activation contribute directly to macrophage cell death induced by LPS and ATP. Macrophages were cultured from normal mice or those in which genes for the P2X7 receptor, IL-1β, IL-1α, IL-18, or caspase-1 had been deleted. Our data confirm the importance of the P2X7 receptor in ATP-stimulated cell death and IL-1β release from LPS-primed macrophages. We demonstrate that prolonged stimulation with ATP leads to cell death, which is partly dependent on LPS priming and caspase-1, but independent of cytokine processing and release. We also provide evidence that LPS priming of macrophages makes them highly susceptible to the toxic effects of brief exposure to ATP, which leads to rapid cell death by a mechanism that is dependent on caspase-1 but, again, independent of cytokine processing and release.

The cytokine interleukin-1 (IL-1)† is a key mediator of host immune and acute phase responses, inflammation, and tissue injury and is produced abundantly by macrophages and monocytes (1). IL-1β, the predominant form of IL-1 in many cell types, is expressed as an inactive precursor (pro-IL-1β), which must be cleaved by the enzyme caspase-1 (interleukin-1-converting enzyme) to produce active mature IL-1β (2–5). Expression of pro-IL-1β is regulated by inflammatory stimuli (e.g. bacterial lipopolysaccharide (LPS)) (6), but cleavage to the active form is regulated independently and linked to cellular release through a largely unknown mechanism (3, 4). In vitro, post-translational processing of pro-IL-1β depends on LPS-activated monocytes or macrophages encountering a secondary stimulus (e.g. ATP), which stimulates caspase-1 activation, cleavage of pro-IL-1β, and subsequent release of the mature cytokine (7, 8). Indeed, exposure of macrophages to ATP alone causes only modest release of IL-1β but, together with LPS, induces marked release of IL-1β and cell death (9).

The cellular release of IL-1β can be regulated by activation of plasma membrane purinergic receptors of the P2X7 subtype (9–11), for which extracellular ATP is thought to be the major endogenous ligand (12, 13). ATP-induced IL-1β release in LPS-treated monocytes and microglia (14) can be prevented by inhibition of caspase-1. P2X7 receptor stimulation by ATP probably causes the activation of caspase-1 (15, 16) through depletion of intracellular K+ (17, 18). However, the relationship between P2X7 receptor stimulation, pro-IL-1β cleavage by caspase-1, cellular release, and cell death has not been confirmed. Reduced cell death has been reported in monocytes and microglia from animals lacking caspase-1 or exposed to pan-caspase inhibitors (18, 19). Other reports have demonstrated that apoptosis in macrophages is not affected by caspase-1 inhibition (11, 20) or report a switch from apoptosis to necrosis (18). IL-1β appears to have a functional role in caspase-mediated cell death in HeLa cells (21), but its contribution to macrophage cell death has not been well characterized. The pro-inflammatory cytokines IL-18 (also processed by caspase-1) and IL-1α are also regulated by ATP stimulation of cells (22) (11) and may therefore contribute to cell death.

The objective of the present study was to test the hypothesis that activation of caspase-1 and release of active IL-1β and/or IL-18 contribute directly to the rapid cytolytic macrophage cell death induced by either prolonged (30 min) or brief (5 min) exposure to ATP. To test this, macrophages were isolated from mice lacking genes (knockout (KO)) for the P2X7 receptor, IL-1β, IL-1α, IL-18, or caspase-1. We present evidence that prolonged ATP stimulation leads to cell death via LPS-dependent and LPS-independent mechanisms, the former requiring the presence of caspase-1. Perhaps more importantly, we present evidence that LPS priming can render macrophages highly sensitive to a short pulse of ATP, which leads to almost complete cell death through a mechanism dependent on the presence of caspase-1 but is independent of mature cytokine production.
Caspase-1 Mediates P2X7-induced Toxicity in LPS-primed Macrophages

EXPERIMENTAL PROCEDURES

Animals

P2X7 receptor knockout (KO) and wild type (WT, B6D2 (C57 BL/6 × DBA/2 F1)) mice were kindly supplied by Solle et al. (10) (Pfizer) and bred at the University of Manchester. The absence of the P2X7 receptor was confirmed in KO mice using PCR (primer sequences used for WT: 5’-GCA GCC CAG CCC TGA TAC AGA CAT T3’ and 5’-CTG GGA CAG CAG GGT CTT ATG GA-3’, for the KO: 5’-GAC AGC CCG AGT TCG TGC CAG TGT G-3’ and 5’-GCT GGT CCG GCT GCG ATT AGA T-3’) and immunoblot analysis (see below). C57BL/6 wild type (WT) mice, the background for all other transgenic strains, were supplied by Charles River (UK). The genetic status of the mice was confirmed using PCR (IL-1 β KO (23); primers: 5’-CTG TGT CTT TTC CGT GGA CC-3’ and 5’-CAG CTC ATG TAG TTG TTC CGA CA-3’ (24); IL-18 KO (25); primers: A 5’-AAC TCG TGT CTT TTC CGTC CTA TCA CTG TGT B 5’-GGA AAA GAA CTA CGT GGC TTG GGT TTC C and C 5’-ATC GGC TTC TAT CGC CTT TCC CGT CTT CTT GAC GAG (primers A and B used for WT, B and C for KO); and caspase-1 KO (5) (Supplied by Dr. W. Wong, BSAF); primers: 5’-CTG GAG GGC AAA GAG GAA GC and 3’-GAG CAA GAA GC and 5’-GAG CAG AAA CCT GAG GGC AAA GAG GAA G).

Peritoneal Macrophages

Adult WT and KO male and female mice were sacrificed, and the peritoneal cavity was lavaged with 8 ml of RPMI 1640 medium (containing 25 mM HEPES, pH 7.3, 2 mM glutamine, 5% fetal calf serum, 100 units of penicillin, and 100 μg/ml streptomycin (Invitrogen, UK). The medium recovered from four to five mice was pooled. The cells were collected by centrifugation (800 x g, 10 min) and plated onto 12- or 24-well plates at a density of 1 x 10^5 cells/ml. Macrophages were allowed to adhere for 2 h (37° C, 5% CO₂), washed with fresh medium to remove unattached cells, and incubated overnight.

Sustained Stimulation with ATP—Cells were primed for 2 h with 1 μg/ml LPS (Escherichia coli 026:B6, Sigma, UK) or vehicle (sterile PBS), and then treated with ATP (Sigma, UK, 1 or 5 mM), vehicle (PBS), nigericin (Sigma, UK, 20 μM), or lysis buffer (100 μg/ml Triton X-100 (9% v/v, final concentration) to measure total cell lysate) for a further 30 min. At the time of LPS incubation, some cells were also treated with PBS or the P2X7 receptor antagonist aATP (26) (Sigma, UK, 100 μM). In other experiments some cells received PBS, IL-1 receptor antagonist (IL-1ra, Dr. S. Poole, National Institute for Biological Standards and Control (NIBSC), 100 ng/ml), recombinant rat IL-1β (100 ng/ml, Dr. S. Poole), or cycloheximide (50 μM, Sigma, UK) 5 min before ATP treatment, whereas zVAD-fmk (Bachem, UK, 50 μM) was administered 30 min before ATP (1.5 h after the start of LPS incubation). At the end of the experiments, the medium was collected for LDH and cytokine analysis, or immunoblot analysis. In selected experiments cell/medium lysates were prepared by the addition of Triton X-100 to the wells for total cytokine measurements and immunoblot analysis.

Detection of Pro and Mature IL-1β—Macrophages were incubated as above, primed with LPS or PBS for 2 h, and then briefly stimulated with either PBS or ATP (5 mM) for 5 min then washed with fresh medium. Samples of medium were taken at 0.5, 2, and 4 h after addition of ATP for analysis of LDH and IL-1β release, and the ability of the cells to exclude trypan blue was examined.

Immunoblot Analysis

The protein concentration of the medium or medium/cell lysates was determined by the Bradford method (Bio-Rad, Germany). Samples (20 μg of protein per lane) were loaded on an 8% SDS acrylamide gel, and the proteins were resolved by gel electrophoresis. The proteins were then transferred onto a nitrocellulose membrane (Amersham Biosciences, Inc., UK). The membrane was briefly exposed to Ponceau S (Sigma, UK) to visualize the protein bands. To reduce nonspecific antibody binding, the membrane was washed in 5% milk (Marvel, UK; 5% in PBS Tween 20, Invitrogen) for 1 h at room temperature.

P2X7 Receptor Expression—The nitrocellulose membrane was incubated with a polyclonal antibody raised to a peptide sequence from the C terminus of the rat P2X7 receptor (1:200 dilution in 5% milk, Alomone) 1 h at 1.5 μg/ml, and an anti-actin polyclonal antibody (Sigma, UK; 1:10,000 dilution in 5% milk) 1 h. The membrane was then finally stained with a polyclonal antibody raised against rabbit IgG, conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA), which was detected by ECL (Amersham Biosciences, Inc.).

Detection of Pro and Mature IL-1β—Proteins were resolved on a 12% acrylamide gel by the above protocol. The membrane was probed with a polyclonal antibody raised to mouse IL-1β (1:1000 dilution of stock in 5% milk, S239, NIBSC, UK) followed by staining with a polyclonal antibody raised against sheep IgG conjugated to horseradish peroxidase (1:4000 dilution of stock in 5% milk, NIBSC, UK), which was detected by ECL (Amersham Biosciences, Inc.).

Cytotoxicity (LDH Release)

Cell death was assessed by the CytoTox-96 assay (Promega), which measures release of lactate dehydrogenase (LDH) from dying cells, according to the manufacturer’s instructions. Total LDH release was achieved by adding Triton X-100 (100 μM/well of a 9% solution, Sigma, UK) to untreated control cells. Treatment values were then expressed as a percent of the total LDH release.

Trypan Blue Exclusion

Trypan blue exclusion was used to quantify the number of viable cells. At the end of the brief ATP stimulation experiments, 150 μl of medium was removed for measurements of LDH and IL-1β release. Trypan blue (10 μl/100 μl medium, Sigma, UK) was added to the remaining medium, and the macrophages were observed under a light microscope. The number of trypan blue-positive cells per 50 cells was counted in triplicate and expressed as a percentage.

Detection of IL-1 by ELISA

The concentrations of IL-1α and IL-1β (both pro and mature) released into the medium, or in lysates containing medium and cells (medium/cell lysates for total IL-1), were quantified by specific mouse IL-1α and β sandwich ELISAs. ELISA reagents were kindly supplied by Dr. S. Poole (NIBSC, UK). The detection limits were <20 pg/ml, and internal quality controls (recombinant cytokines at concentrations of 200 and 1000 pg/ml) were included in each assay. To test the ability of the ELISA to distinguish between the pro and mature forms of IL-1β, samples of pro-IL-1β (as determined by Western blotting) were cleaved by incubation with activated caspase-1 (Nancy Thornberry, Merck Research Laboratory, 100 units/ml sample + 10 mM dithiothreitol) and the un-cleaved and cleaved samples (confirmed by immunoblot analysis) were compared with the ELISA.

Statistical Analysis

All data are presented as the means ± S.E. of measurements in three or more independent cultures (each culture assessed in triplicate). Statistical analysis was performed using analysis of variance (ANOVA) followed by Neuman-Keuls post hoc test. In some cases (e.g. nVAD) the data presented are the result of one experiment performed in triplicate, which was replicated on a further two occasions due to the variability of cytokine levels between independent cultures.

RESULTS

Prolonged (30 min) P2X7 Receptor Stimulation Leads to Cell Death and IL-1 Release in LPS-primed Macrophages—In control WT macrophages, spontaneous LDH release was ~12% of maximal death (Fig. 1A). Cell lysis was not affected by 2 h incubation with LPS (1 μg/ml), although cells exhibited an activated state with morphological changes, including elongation of processes. Stimulation of control macrophages with 1 mM ATP (30 min) increased LDH release (90%, but not significantly), whereas exposure to 5 mM ATP elicited a significant (p < 0.05) 3-fold increase in cell death. ATP-induced LDH release was significantly greater in macrophages primed with LPS (2 h before the ATP) with 3.2- and 4-fold increases observed in cells exposed to 1 or 5 mM ATP, respectively (p < 0.05 and p < 0.01 versus LPS alone). Incubation of the macrophages with the potassium ionophore nigericin (20 μM), for 30 min in LPS-primed cells resulted in 80% LDH release.

WT macrophages exposed to vehicle (PBS) or LPS released only small amounts of IL-1β into the culture medium (<150 pg/ml). Treatment with ATP alone resulted in a small (NS) increase in IL-1β release. However, combined exposure to ATP and LPS (ATP/LPS) resulted in dramatic cytokine release (3500 ± 826 and 5300 ± 1790 pg/ml after 1 and 5 mM ATP, respectively Fig. 1B). The cytokolytic actions of nigericin (Fig. 1A) were also accompanied by dramatic release of IL-1β (8250 ± 1746 pg/ml, Fig. 1B) from LPS-primed macrophages. ATP-stimulated LDH and IL-1β release in LPS-primed macroph-
phages were completely prevented by pretreatment of the cells with the P2X7 receptor antagonist oATP (100 μM at the time of LPS treatment, Fig. 1, A and B).

Spontaneous LDH release from P2X7 KO vehicle-treated macrophages was slightly higher (21 ± 4% total LDH release, NS) than from WT cells (12 ± 2% total). Incubation of P2X7 KO cells with ATP (1 or 5 mM) in the presence or absence of LPS priming failed to affect LDH or IL-1β release (Fig. 2). However, treatment of LPS-primed P2X7 KO macrophages with nigericin (20 μM) dramatically induced the release of both LDH (81 ± 8% total) and IL-1β (7100 ± 200 pg/ml) to a similar extent to that observed in WT macrophages (82 ± 4% total and 8250 ± 1750 pg/ml, respectively).

To investigate cleavage of IL-1β, Western blots were performed on aliquots of culture medium. When stimulated with ATP (5 mM), WT macrophages released pro-IL-1β and a very small quantity of mature IL-1β into the culture medium accompanying cell lysis (Fig. 3A). However, in the presence of LPS and ATP IL-1β was again released, but was found predominantly in the mature form following 1 min ATP, and in greater quantities following 5 min ATP.

To distinguish between the cytolytic effects of ATP and IL-1 release, IL-1β and IL-1α were measured in medium/cell lysates, giving a measure of total IL-1 within the cells and medium. Priming of WT and P2X7 KO macrophages with LPS resulted in a significant increase in IL-1β (5500 ± 460 and 4270 ± 210 pg/ml, respectively, Fig. 4A), which was exclusively pro-IL-1β (Fig. 3B). In WT macrophages the addition of ATP (5 mM) increased total IL-1β by 68% (9280 ± 430 pg/ml, Fig. 4A), with ~50% cleaved to the mature form (Fig. 3B). In P2X7 KO macrophages, ATP had no effect on either the production (Fig. 4A, 4270 ± 90 versus 4900 ± 90) or the cleavage of IL-1β (Fig. 3B).

Cleavage of pro-IL-1β samples (LPS-primed WT macrophage samples) with activated caspase-1 did not alter the ELISA reading of the samples (3980 ± 250 versus 3350 ± 370 pg/ml pre- and post-caspase-1 incubation, respectively).

To examine the role of protein synthesis in the increase in total IL-1β following ATP stimulation of LPS-treated WT cells, separate cultures were incubated with cycloheximide (50 μM). Although cycloheximide did not affect LDH release (data not shown), it reduced total IL-1β production in LPS-stimulated control cells (33%, 4830 ± 490 versus 3240 ± 20), but did not significantly alter total IL-1β in LPS-primed cells treated with ATP (5 mM, 5332 ± 80 versus 4550 ± 350).

In response to LPS and ATP (5 mM), macrophages from WT mice also released IL-1α (4000 ± 300 pg/ml) in a similar pattern to IL-1β (data not shown). This release of IL-1α was completely absent from macrophages from P2X7 KO mice. In WT and P2X7 KO macrophages, LPS increased the level of total IL-1α in cells/medium (5480 ± 270 and 4800 ± 450 pg/ml, respectively) compared with vehicle (<150 pg/ml). However, stimulation with ATP (5 mM) did not significantly affect total IL-1α in either WT (5306 ± 68 pg/ml) or P2X7 KO macrophages (5400 ± 460 pg/ml, Fig. 3B).

Expression of the P2X7 receptor was not altered by incubation with LPS in WT macrophages, but the receptor was completely absent in P2X7 KO animals (data not shown).

Role of Caspases in P2X7-mediated Cell Death—Treatment of WT (C57BL6) cultures with the pan-caspase inhibitor zVAD-fmk (50 μM) did not affect basal cell death of vehicle or LPS-primed cells (Fig. 5A). However, zVAD-fmk significantly inhibited cell death induced by 5 mM ATP in LPS-primed cells (47% reduction, p < 0.001), bringing it to the level seen in control ATP-treated cells. zVAD-fmk also dramatically reduced total IL-1β production from LPS-primed ATP stimulated cells (measured in cell/medium lysates; 58% reduction, p < 0.001, Fig. 5B, and prevented its cleavage to the mature form (Fig. 6).

Macrophage cultures from caspase-1 KO mice showed levels of basal cell death in the absence and presence of LPS similar to WT cultures. Stimulation with ATP (5 mM, 30 min) again resulted in significant cell death in vehicle-treated macrophages (2.0-fold increase, p < 0.01, Fig. 7A), but this was not significantly altered by LPS incubation (2.8-fold increase). LDH release from LPS-primed ATP (5 mM) treated caspase-1 KO macrophages was reduced (25% total) relative to WT macrophages (approximately 50% total). Vehicle-treated macrophages from caspase-1 KO mice, however, exhibited high basal IL-1β as measured in cell/medium lysates (1290 ± 220 pg/ml compared with WT macrophages, 210 ± 40 pg/ml). Incubation of cultures with LPS for 2 h dramatically increased total IL-1β production (5-fold increase, 7850 ± 735, p < 0.001), which was all detected as the pro form (Fig. 6). Addition of ATP (5 mM) reduced IL-1β production (25%, p < 0.001) in caspase-1 KO macrophages (Fig. 7B) and failed to induce cleavage of IL-1β to the mature 17-kDa form (Fig. 6).

IL-1 is not involved in ATP-induced cell death—P2X7 receptor expression was similar in macrophages from WT (C57/BL6) and P2X7 KO mice (data not shown). To examine the role of IL-1 in ATP-induced toxicity, WT macrophages were incubated with LPS and ATP (5 mM) and the IL-1α (4000 ± 300 pg/ml) and IL-1β (5480 ± 270 and 4800 ± 450 pg/ml, respectively) levels were measured (Fig. 2). Addition of neutralizing anti-IL-1α antibody did not affect ATP-induced apoptosis (Fig. 7A, B). However, neutralizing anti-IL-1β antibody significantly reduced ATP-induced apoptosis (Fig. 7A, B). This finding was confirmed using macrophages from caspase-1 KO mice, which exhibited high basal IL-1β as measured in cell/medium lysates (1290 ± 220 pg/ml compared with WT macrophages, 210 ± 40 pg/ml). Incubation of cultures with LPS for 2 h dramatically increased total IL-1β production (5-fold increase, 7850 ± 735, p < 0.001), which was all detected as the pro form (Fig. 6). Addition of ATP (5 mM) reduced IL-1β production (25%, p < 0.001) in caspase-1 KO macrophages (Fig. 7B) and failed to induce cleavage of IL-1β to the mature 17-kDa form (Fig. 6).
FIG. 2. ATP-induced LDH and IL-1β release is absent in macrophages from P2X7 KO mice. Peritoneal macrophages from P2X7 KO mice were incubated with LPS (1 µg/ml) or PBS for 2 h (37 °C), then stimulated with ATP (1 or 5 mM) or nigericin (20 µM) for a further 30 min. Supernatants were collected and assayed for (A) LDH (expressed as % total LDH release, Triton X-100) and (B) IL-1β (mouse IL-1β-specific ELISA, pg/ml). (***, p < 0.001 versus control cells, one-way ANOVA.) Data shown are means ± S.E. for triplicate determinations repeated on at least three separate cultures.

FIG. 3. Stimulation of WT, but not P2X7 KO macrophages, with LPS and ATP induces IL-1β cleavage. Macrophage cultures from WT and P2X7 KO mice were prepared and incubated for 2 h with PBS or LPS (1 µg/ml) followed by stimulation with PBS or ATP (5 mM) for 30 min. Samples of (A) medium and (B) medium/cell lysates (prepared by the addition of Triton X-100) from WT and KO cultures were fractioned by SDS-PAGE, and the polypeptides were transferred to a nitrocellulose membrane. The blot was then probed for IL-1β with a mouse-specific antibody to reveal pro- (31 kDa) and mature (17 kDa) IL-1β.

FIG. 4. Prolonged (30 min) P2X7 receptor stimulation with ATP increases the total production of IL-1β but not IL-1α. Macrophage cultures from WT and P2X7 KO mice were incubated for 2 h with PBS or LPS (1 µg/ml) followed by stimulation with PBS or ATP (5 mM) for 30 min. Medium/cell lysates were prepared by the addition of Triton X-100. IL-1β (A) and IL-1α (B) were measured using mouse-specific IL-1 ELISAs (picograms/ml). Data shown are means ± S.E. for triplicate determinations repeated on at least three separate cultures.
Caspase-1 Mediates P2X7-induced Toxicity in LPS-primed Macrophages

**Fig. 5.** The pan-caspase inhibitor zVAD-fmk reduces cell death (LDH release) and total IL-1β induced by prolonged stimulation with ATP (30 min) in LPS-primed macrophages. WT (C57/BL6 mice) peritoneal macrophages were primed with LPS (1 μg/ml) or PBS for 2 h (37°C). After 1.5 h cells were treated with zVAD-fmk (50 μM) or PBS for 30 min then incubated with PBS or ATP (1 or 5 mM) for a further 30 min. A, medium was collected and assayed for LDH (expressed as % total LDH release, Triton X-100 Cytotox-96 assay); B, medium/cell lysates were prepared (by addition of Triton X-100) and IL-1β measured using a mouse IL-1β-specific ELISA. (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ‡, p < 0.001 versus control cells; ++++, p < 0.001 ATP versus ATP + zVAD-treated cells, one-way ANOVA.) Data shown are means ± S.E. for triplicate determinations repeated on at least three separate cultures.

**Fig. 6.** Cleavage of IL-1β induced by prolonged (30 min) ATP stimulation in LPS-primed macrophages is inhibited by pre-treatment of cells with zVAD-fmk (WT, C57/BL6) and is absent in caspase-1 KO macrophages. Peritoneal macrophages from WT (C57/BL6) and caspase-1 KO mice were primed with LPS (1 μg/ml) or PBS for 2 h (37°C). After 1.5 h cells were treated with zVAD-fmk (50 μM) or PBS for 30 min, then stimulated with PBS or ATP (1 and 5 mM) for a further 30 min. Medium/cell lysates were prepared (by addition of Triton X-100) and fractioned by SDS-PAGE, and the polypeptides were transferred to nitrocellulose. The blot was then probed for IL-1β using a mouse-specific antibody to reveal pro (34 kDa) and mature (17 kDa) IL-1β.

BL6) and IL-1β KO mice and was not affected by exposure to LPS (data not shown). Spontaneous LDH release was similar in vehicle and LPS-stimulated macrophages cultured from WT (Fig. 8A) and IL-1β KO (Fig. 8B) mice. Addition of 1 mM ATP did not affect LDH release in either control WT or IL-1β KO mice, whereas exposure to 5 mM ATP induced similar increases in LDH release (127% in cells from WT (NS versus vehicle) and IL-1β KO (126%, p < 0.05) mice. In LPS-primed macrophages, ATP dramatically increased LDH release from both WT and IL-1β KO cells (64 ± 11% and 53 ± 7% after 5 mM ATP, respectively, p < 0.001 versus vehicle). Treatment of LPS-stimulated macrophages, from either strain of mice, with nigericin resulted in similar levels of LDH release (increases of 84 ± 11% and 61 ± 18% in WT and IL-1β KO mice, respectively, p < 0.001), which were comparable to those observed in macrophages from P2X7 WT and KO mice (Figs. 1 and 2). Treatment of macrophages with IL-1ra (100 ng/ml) at the time of vehicle/LPS incubation did not affect basal LDH release or that induced by 5 mM ATP in LPS-treated macrophages (LPS + 5 mM ATP: 38 ± 9% versus IL-1ra + LPS + 5 mM ATP: 37 ± 6%, NS). Treatment of WT macrophages with IL-1β (100 ng/ml) at the same time as ATP did not affect either control or ATP-induced LDH release in LPS-primed macrophages (37 ± 7% versus 29 ± 1% total LDH release, ATP + LPS versus ATP + LPS + IL-1β respectively, NS).

To investigate whether the actions of ATP on LDH release in IL-1β KO macrophages were due to compensatory effects of IL-1α, the release of this cytokine was measured. LPS-primed WT macrophages stimulated with ATP (5 mM) released a large quantity of IL-1α (2902 ± 653 pg/ml). However, IL-1β KO macrophages showed very low levels of IL-1α release in response to LPS/ATP (5 mM, 385 ± 190 pg/ml), despite similar levels of cell death. Indeed, further evidence that IL-1 is not required for ATP-induced cell death was provided by the cytotoxic actions of ATP in LPS-primed macrophages isolated from IL-1α KO (animals with both IL-1α and IL-1β genes deleted) KO mice (LPS: 13 ± 3%, LPS plus 5 mM ATP: 47 ± 6% cell death, p < 0.001).

IL-18 Is Not Involved in ATP-induced Cell Death—Macrophages from IL-18 KO mice showed a similar profile of LDH and IL-1β release in response to ATP/LPS (Fig. 9A) to WT cells. ATP (1 mM) induced a 2-fold increase in LDH release from LPS-primed IL-18 KO macrophages, compared with vehicle alone, but this response failed to reach statistical significance. However, 5 mM ATP stimulated LDH release to 50% of total (p < 0.001), and 4.0- and 8.8-fold increases seen in vehicle and LPS-primed cells, respectively. The cytotoxic actions of ATP in LPS-primed, IL-18 KO macrophages were accompanied by IL-1β release (LPS/ATP 5 mM: 7380 ± 320 pg/ml). Although the active forms of IL-1β and IL-18 are both dependent on cleavage by caspase-1, IL-1β cleavage stimulated by LPS/ATP was similar in WT and IL-18 KO macrophages (data not shown).

Cell Death Induced by a Brief (5-min) Pulse of ATP Requires Both LPS Priming and Caspase-1—A brief pulse of ATP (5 mM for 5 min) had no effect on basal LDH release from control WT macrophages, and the cells completely excluded trypan blue (data not shown). Similarly, treatment of LPS-primed cells with PBS or zVAD-fmk was without effect. However, in LPS-primed cells, a brief pulse of ATP resulted in a dramatic release of LDH within 30 min (Fig. 10A, 42 ± 4% total) and almost complete release after 2 h (96 ± 26% total). This was accompanied by a loss of membrane integrity as indicated by the uptake of trypan blue (42 ± 3 and 54 ± 2% at 0.5 and 2 h respectively, Fig. 10B) and release of IL-1β into the medium (LPS + ATP, 2570 ± 310 pg/ml).

Pre-treatment of LPS-primed cells with zVAD-fmk (30 min prior to ATP) almost completely prevented ATP-induced LDH...
Caspase-1 Mediates P2X7-induced Toxicity in LPS-primed Macrophages

The extracellular purinergic receptor P2X7 is implicated in signaling between macrophages and other cells involved in the immune response and target cells (13). Stimulation of the P2X7 receptor with high concentrations of ATP triggers massive transmembrane fluxes (particularly influx of Ca\(^{2+}\) and Na\(^{+}\)), and efflux of K\(^{+}\) and the formation of non-selective plasma membrane pores, resulting in cell swelling, vacuolization, and cell death by necrosis and apoptosis (27). Evidence from studies on macrophages, monocytes, and microglia indicates that P2X7 receptors also stimulate post-translational processing and release of the cytokine IL-1\(\beta\) (3, 9, 11, 28), through depletion of intracellular K\(^{+}\) and subsequent activation of caspase-1 (2). However, the relationship between activation of caspase-1, the subsequent cleavage/maturation of pro-IL-1\(\beta\) (and pro IL-18), and cell death has not been clearly defined and has been complicated by the use of different schedules of ATP stimulation and the use of multiple cell types and cell lines.

In the present study, prolonged stimulation (30 min) with ATP induced cell death in macrophages, and this was associated with the release of IL-1\(\beta\) and IL-1\(\alpha\). The release of these cytokines was accompanied by the activation of caspase-1, as evidenced by the cleavage of pro-caspase-1 to the active caspase-1 p17 subunit.

**DISCUSSION**

Cell Death Induced by a Brief (5-min) Pulse of ATP Is Independent of IL-1 and IL-18—Macrophages harvested from IL-1\(\alpha\) or IL-18 KO mice and primed with ATP showed an identical pattern of complete LDH release and trypan blue exclusion (~50%) following brief exposure to ATP at 0.5 and 2 h post ATP as seen in WT macrophages (Fig. 12, A and B). No effect of ATP was observed in cells that had not been primed with LPS.

**FIG. 7.** Prolonged (30 min) ATP stimulation increases LDH release but not IL-1\(\beta\) production in caspase-1 KO macrophages. Peritoneal macrophages prepared from caspase-1 KO mice were incubated with LPS (1 \(\mu\)g/ml) or PBS for 2 h (37 °C), then stimulated with ATP (5 mM) for a further 30 min. A, medium was collected and assayed for LDH (expressed as % total LDH release, Triton X-100, Cytotox-96 assay). B, medium/cell lysates were prepared (by addition of Triton X-100) and IL-1\(\beta\) was measured using a mouse IL-1\(\beta\)-specific ELISA. Data shown are means ± S.E. for triplicate determinations repeated on at least three separate cultures.

**FIG. 8.** Prolonged (30 min) ATP stimulation increases LDH release in peritoneal macrophages isolated from WT and IL-1\(\beta\) KO mice. Peritoneal macrophages recovered from (A) WT (C57/BL6) and (B) IL-1\(\beta\) KO mice were incubated with LPS (1 \(\mu\)g/ml) or PBS for 2 h (37 °C), then stimulated with ATP (1 or 5 mM) or nigericin (20 \(\mu\)M) for a further 30 min. Medium was collected and assayed for LDH (expressed as % total LDH release, Triton X-100, Cytotox-96 assay). Data shown are means ± S.E. for triplicate determinations repeated on at least three separate cultures.
ATP markedly increased IL-1 (α and β) release, cleavage of IL-1β to the mature 17-kDa form, and release of LDH from LPS-primed WT primary macrophages. Involvement of P2X7 receptors was demonstrated by the ability of the receptor antagonist oATP to block this response. However, oATP may exert actions through inhibition of other ATP-dependent enzymes or signaling proteins (29). We therefore confirmed the work of Solle et al. (10), showing that LPS-stimulated macrophages from P2X7 KO mice are resistant to ATP-induced cell death and IL-1 processing and release, despite normal responses to the potassium ionophore nigericin, which activates caspase-1 directly (30, 31). Immunoblot analysis confirmed that ATP-stimulated cleavage of pro-IL-1β required LPS incubation as a “priming event,” as reported by Mehta et al. (22). To assess whether the effects of ATP on IL-1 release were due to cytolysis, total IL-1α and IL-1β were measured in macrophage medium/cell lysates. In LPS-primed WT cells, ATP increased total (intra- and extracellular) production of IL-1β (but not IL-1α) by over 50%. The affinity of the IL-1β ELISA was similar for pro and mature forms of IL-1β, because almost identical data were obtained when samples of pro-IL-1β were cleaved with activated caspase-1 to the mature form. These results suggest that ATP acts not only at the level of IL-1β cleavage but also at the transcriptional and/or translational level. Indeed, P2X7-dependent induction of the transcriptional activator NFκB has been observed in microglia (17, 32, 33). Sanz and Di Virgilio (8) reported a similar phenomenon in a LPS-primed N13 microglial cell line and suggested that secretion of IL-1β could be a rate-limiting...
step in its de novo synthesis. In the present study, experiments with cycloheximide confirm that neo-synthesis was involved in some of the IL-1 production induced by LPS treatment but not the additional ATP/LPS-induced component.

The mechanisms involved in ATP-stimulated cleavage of pro-IL-1β are not fully understood but are likely to involve activation of caspase-1 (8, 16). Previous studies have reported that caspase-1 activation is not involved in ATP-induced cell death, because in microglia/macrophages, ATP-induced cell death is not influenced by inhibition of caspases (20) or altered in caspase-1-deficient mice (5, 18). However, our results show that treatment of macrophages with the pan-caspase inhibitor zVAD-fmk not only reduced the cell death induced by prolonged (30 min) stimulation with ATP in LPS-primed cells (to that observed in unprimed cells) but also the total production and cleavage of IL-1β. Ferrari et al. (18), also demonstrated that zVAD inhibited the ATP-induced DNA fragmentation and partially reduced the release of LDH. These actions of zVAD may reflect inhibition of multiple caspases involved in apoptosis (e.g. caspase 3 or 8) (18).

To determine the specific role of caspase-1, we investigated the effect of prolonged stimulation with ATP in LPS-primed macrophages harvested from caspase-1 KO mice. ATP induced cell death in macrophages from caspase-1 KO mice, although the response was smaller than in WT macrophages and was not increased by priming the cells with LPS. This shows that a proportion of the ATP-induced cell death is independent of caspase-1. Indeed, cell death was comparable to that seen in cells treated with zVAD. This is in agreement with Laliberte et al. (16), who showed that caspase-1 inhibitors do not prevent release...
of LDH, and Li et al. (5), who demonstrated that caspase-1 KO macrophages exposed to ATP for 30 min still undergo apoptosis as demonstrated by DNA fragmentation. Caspase-1 KO macrophages exhibited high basal IL-1β concentrations compared with WT macrophages possibly reflecting reduced clearance of pro-IL-1. Although LPS priming increased total IL-1 in these cells, ATP failed to stimulate synthesis and cleavage to the mature form. This is consistent with the report of Ferrari et al. (33), who demonstrated the involvement of caspase-1 proteolytic pathways in the activation of NFκB. The death of caspase-1 KO macrophages induced by prolonged exposure to ATP may reflect the induction of other death pathways, such as the activation of stress-activated protein kinases (17) or pathways involving the activation of other caspases (e.g., Refs. 3 and 9). This, together with the inhibitory action of zVAD on cell death, suggests that caspase-1 contributes at least in part to the cell death induced by prolonged ATP stimulation and that it is the LPS-induced component, which is altered in the absence of caspase-1.

To further investigate this altered pattern of ATP-induced cell death observed in caspase-1 KO cells, we examined the effects of a brief pulse (5 min) of ATP in WT and caspase-1 KO macrophages. In WT cells, brief stimulation with ATP caused a breakdown in membrane integrity, indicated by maximal release of LDH and uptake of trypan blue, and release of IL-1β within 30 min, only in LPS-primed cells. Thus, LPS in some way renders the cells susceptible to ATP. Although incubation with LPS did not alter P2X7 receptor expression in WT macrophages, LPS may alter the distribution of P2X7 receptors within the cell (34), their activation (35), or make the cell more susceptible to ATP-induced death by changing the intracellular ionic environment. This effect of LPS and ATP was almost completely inhibited by pre-treatment of cells with the pan-caspase inhibitor zVAD-fmk and was totally absent from caspase-1 KO macrophages. These results confirm that, in macrophages, the LPS priming effect is dependent on caspase-1. It is possible that LPS increases pro-caspase-1 expression or its availability for subsequent activation into the active fragment by ATP (8), which then leads to cell death and LDH release (18). Like WT macrophages, caspase-1 KO macrophages exhibited an activated morphology following priming with LPS and ATP-induced morphological changes, including cell swelling and a reduction in processes, demonstrating that some responses to ATP are still present in the absence of caspase-1.

The major function of caspase-1 is the processing and release of IL-1β. However, IL-1β is not required for the cell death induced by either prolonged or brief stimulation with ATP in LPS-primed cells, because this was not altered by application of IL-1ra or exogenous IL-1β, and was present in macrophages harvested from IL-1β or IL-1β KO mice. Some caution is required in interpreting results using gene knockout mice because up-regulation of related cytokines and altered sensitivity of receptors may occur. IL-18, like IL-1, requires processing by caspase-1, binds to a receptor of the IL-1 family, and exists in the cytosol without a classic signal sequence. Mehta et al. (22) demonstrated that IL-18 is released from monocytes following stimulation with ATP in a similar manner to IL-1β and that this required priming by LPS. Our results demonstrate that, in the absence of IL-18, the cytotoxic actions of prolonged ATP stimulation and the effects of a brief “pulse” of ATP were similar to those in WT cultures, and the release and processing of IL-1β was not altered. These results demonstrate that IL-18 release is not an essential requirement for the LPS priming event.

In conclusion, ATP can induce cell death in primary cultures of macrophages via activation of the P2X7 receptor via two different mechanisms, one of which is enhanced by LPS priming and is dependent on caspase-1. Although IL-1β and IL-18 release and synthesis are induced following stimulation of cells with ATP and this requires caspase-1, these cytokines are not involved in cell death induced by prolonged (30 min) or brief (5 min) exposure to ATP. The role of the P2X7 receptor in vivo remains to be studied in detail. However, it is possible that ATP may be released in high concentrations at sites of cell death or inflammation where it may regulate the release and maturation of IL-1β and thus play an important role in the actions of this cytokine (36). Indeed, inhibition of caspase-1 has been required to reduce LPS-induced lethality in rats without affecting cytokine responses, suggesting the importance of the link between LPS and caspase-1 (37).

Acknowledgments—ELISA reagents were generously supplied by the NIBSC. P2X7 knockout mice were kindly supplied by Dr. M. Solle (Pfizer) and caspase-1 knockout by Dr. W. Wong (BASF). We also thank Dr. R. Gibson for her help in the preparation of this manuscript.
Priming of Macrophages with Lipopolysaccharide Potentiates P2X7-mediated Cell Death via a Caspase-1-dependent Mechanism, Independently of Cytokine Production
Rosalind A. Le Feuvre, David Brough, Yoichiro Iwakura, Kiyoshi Takeda and Nancy J. Rothwell

doi: 10.1074/jbc.M104388200 originally published online November 12, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104388200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 23 of which can be accessed free at http://www.jbc.org/content/277/5/3210.full.html#ref-list-1