Altered Cytokine Production in Mice Lacking P2X<sub>7</sub> Receptors*

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Mike Solle‡, Jeff Labasi, David G. Perregaux, Ethan Stam, Nina Petrushova, Beverly H. Koller‡‡, Richard J. Griffiths, and Christopher A. Gabel¶

From the Department of Respiratory, Allergy, Immunology, Inflammation, and Infectious Diseases, Pfizer Inc., Groton, Connecticut 06340 and the Department of Medicine and Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, North Carolina 27599

The P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) is an ATP-gated ion channel expressed by monocytes and macrophages. To directly address the role of this receptor in interleukin (IL)-1β post-translational processing, we have generated a P2X<sub>7</sub>R-deficient mouse line. P2X<sub>7</sub>R<sup>−/−</sup> macrophages respond to lipopolysaccharide and produce levels of cyclooxygenase-2 and pro-IL-1β comparable with those generated by wild-type cells. In response to ATP, however, pro-IL-1β produced by the P2X<sub>7</sub>R<sup>−/−</sup> cells is not externalized or activated by caspase-1. Nigericin, an alternate secretion stimulus, promotes release of 17-kDa IL-1β from P2X<sub>7</sub>R<sup>−/−</sup> macrophages. In response to in vivo lipopolysaccharide injection, both wild-type and P2X<sub>7</sub>R<sup>−/−</sup> animals display increases in peritoneal lavage IL-6 levels but no detectable IL-1β. Subsequent ATP injection to wild-type animals promotes an increase in IL-1β, which in turn leads to additional IL-1β production; similar increases did not occur in ATP-treated, LPS-primed P2X<sub>7</sub>R<sup>−/−</sup> animals. Absence of the P2X<sub>7</sub>R thus leads to an inability of peritoneal macrophages to release IL-1β in response to ATP. As a result of the IL-1 deficiency, in vivo cytokine signaling cascades are impaired in P2X<sub>7</sub>R-deficient animals. Together these results demonstrate that P2X<sub>7</sub>R activation can provide a signal that leads to maturation and release of IL-1β and initiation of a cytokine cascade.

Interleukin (IL)-1 is a multipotential inflammatory mediator produced in abundance by activated monocytes and macrophages (1). When released from producing cells, IL-1 binds to receptors on target cells and elicits signaling cascades leading to the up-regulation of gene products that contribute to an inflammatory state including matrix metalloproteinases, cyclooxygenase-2 (Cox-2), IL-6, and cellular adhesion molecules (2–5). Two distinct gene products, IL-1α and IL-1β, contribute to IL-1 biological activity (6, 7). The amino acid sequences of IL-1α and IL-1β are &lt;30% identical yet these two polypeptides bind to the same receptors on target cells (8). Human IL-1α and IL-1β both are initially produced as 31-kDa procytokines containing amino-terminal extensions; these extensions subsequently are removed by proteolysis. In the case of pro-IL-1α, the propolypeptide and its 17-kDa cleavage product display equivalent signaling activity, indicating that proteolytic cleavage is not necessary to generate a receptor-competent ligand (9). In contrast, pro-IL-1β does not bind to the signaling IL-1 receptor (9), and cleavage by caspase-1 is necessary to generate the mature 17-kDa signaling-competent form of this cytokine (10, 11).

The two forms of IL-1 share another very unusual attribute; both pro-IL-1α and pro-IL-1β are synthesized without a signal sequence (7), the peptide epitope required to direct nascent polypeptides to the endoplasmic reticulum (12). As a result, newly synthesized pro-IL-1α and pro-IL-1β accumulate within the cytoplasmic compartment of producing cells (13) rather than being sequestered to the secretory apparatus. Caspase-1 also is produced as a cytosol-localized proenzyme; the 45-kDa propolypeptide must be proteolytically processed to generate the 20- and 10-kDa subunits that constitute the mature active protease (11, 14, 15). In activated monocytes and macrophages, therefore, pro-IL-1β and procaspase-1 co-exist within the cytoplasm. Mechanisms that control activation of procaspase-1, and in turn cleavage of pro-IL-1β, are not well understood. Recent studies, however, have provided evidence that proteolytic processing of IL-1β and release of the mature cytokine product extracellularly do not proceed constitutively. Rather, the post-translational processing of pro-IL-1 requires that lipopolysaccharide (LPS)-activated monocytes and/or macrophages encounter an external stimulus that promotes activation of procaspase-1, cleavage of pro-IL-1β, and release of the 17-kDa cytokine (16–18). Stimuli that function in vitro to promote IL-1 post-translational processing by LPS-activated monocytes and/or macrophages include ATP, nigericin, cytolytic T-cells, bacterial toxins, and hypotonic stress (19–24). This requirement for a secretion stimulus is not restricted to cells in culture; mouse peritoneal macrophages produce pro-IL-1β in response to intraperitoneal (ip) injection of LPS, but release little cytokine extracellularly (25). Subsequent ip injection of ATP, however, stimulates generation of large quantities of extracellular mature IL-1β (25).

The mechanism by which ATP activates IL-1β post-translational processing is believed to involve the P2X<sub>7</sub> purinergic receptor (17, 18, 26, 27). Like other members of the P2X receptor family, the P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) is an ATP-gated ion channel (28–30). The P2X<sub>7</sub>R, however, demonstrates attributes that clearly distinguish it from other members of the family. For example, the P2X<sub>7</sub>R requires levels of ATP in excess of 1 mM to achieve activation, whereas other P2X receptors activate at ATP concentrations of &lt;=100 μM (31, 32); the higher concentration requirement reflects, in part, the preference of the P2X<sub>7</sub>R for ATP<sup>4</sup> as its ligand and the relatively low abundance of this species in media containing physiological

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¶To whom correspondence should be addressed. Tel.: 860-441-5483; Fax: 860-715-2467; E-mail: christopher_a_gabel@groton.pfizer.com.
‡The abbreviations used are: IL, interleukin; LPS, lipopolysaccharide; P2X<sub>R</sub>, P2X receptor; ES, embryonic stem; ip, intraperitoneal; Cox-2, cyclooxygenase-2; LDH, lactate dehydrogenase; FSL, phosphospecific luminescence; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HEK, human embryonic kidney cells; ELISA, enzyme-linked immunosorbent assay.
concentrations of divalent cations (e.g., Ca\(^{2+}\) and Mg\(^{2+}\)). An additional unique feature of the P2X-R is found in its conductance properties. All P2X receptors demonstrate non-selective channel-like properties following ligation, but the channels formed by the P2X-R rapidly transform to pores that allow passage of solutes as large as 900 Da (32, 33). Molecular details of this transformation remain to be described, but domain swapping and deletion experiments have suggested that the carboxyl-terminal domain of the P2X-R participates in pore complex formation (28, 29); the carboxyl-terminal domain of the P2X-R is significantly longer than the comparable domains in the other P2X receptors (34). Possibly as a consequence of this pore-like activity, continuous ligation of the P2X-R receptor for times of >15 min can lead to cell death (35–37).

In this study we employ a genetic approach to inactivate the P2X-R. Mice lacking the P2X-R are healthy and fertile and demonstrate no overt phenotype. However, in contrast to their wild-type counterparts, LPS-activated peritoneal macrophages from P2X-R\(-/-\) animals fail to generate mature IL-1\(\beta\) when challenged with ATP. This defect is not because of an inability of the macrophages to produce pro-IL-1\(\beta\) but rather to an inability of the cytokine producing cells to respond to the purinoceptor agonist. As a consequence of their inability to produce mature IL-1\(\beta\) post-ATP challenge, P2X-R\(-/-\) animals generate reduced quantities of IL-6 relative to their wild-type controls. Therefore, the knockout animals establish that the P2X-R is a necessary component of ATP-induced IL-1 post-translational processing, and demonstrate that this receptor can serve as an important element of an inflammatory cascade mechanism.

**EXPERIMENTAL PROCEDURES**

**Construction of the P2X-R Targeting Vector and Generation of the Knockout Mice**—A CDNA probe specific to the mouse P2X-R gene was synthesized by reverse transcription-polymerase chain reaction using primers P2X-R1 (5'-CGGGGGTGCCTTGGCATCCT-3') and P2X-R2 (5'-AGGGGGCTGCGGCTTCC-3'), which were designed based on the published rat CDNA sequence of the P2X-R gene (28). Total RNA isolated from the J774 A.1 mouse monocyte/macrophage cell line was used as the template RNA. This polymerase chain reaction product was 401 base pairs long and was cloned and sequenced to verify that it corresponded to the mouse P2X-R gene. The probe was used to screen a gene. The probe was used to screen a gene. A targeting vector containing the neomycin resistance gene inserted into the 5' coding sequence of the P2X-R gene product (38). 129Ola-derived E14Tg2a ES cells (39) were grown, transfected, and screened using standard methods (40). Targeted ES cells and mice carrying the mutant allele were identified using a probe specific to a genomic region upstream of the targeted locus. Chimeric mice derived from targeted ES cells were mated with B6D2 (C57 BL/6 × DBA/2 F1) or C57BL/6 mice.

**Peritoneal Macrophage Isolation**—Mouse peritoneal macrophages were harvested by injecting 5 ml of RPMI medium containing 5% FBS into each peritoneal cavity; immediately prior to injection, the animals were euthanized. The injected medium was dispersed throughout the peritoneal cavity, after which a hole in the skin covering the peritoneum was introduced and the injected fluid was recovered with the aid of a transfer pipette. Lavage fluids from multiple animals were pooled and the cells were collected by centrifugation (300 x g). These cell pellets were washed twice by centrifugation in RPMI containing 5% fetal calf serum. A cell count was performed before the final wash.

**Western Analysis**—Peritoneal macrophage cell pellets were washed once in cation buffer (25 mM Hepes, pH 7.0, 30 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 gm/l leupeptin, and 1 gm/l pepstatin) by centrifugation. Cell pellets then were suspended in 2.5 ml of cation buffer, and the cells were disrupted by nitrogen cavitation (15 min on ice at 750 psi). The resulting cell lysates were adjusted to 0.1% saponin, incubated on ice for 30 min, and cell membranes subsequently were recovered by centrifugation (50,000 rpm for 30 min at 4 °C in a Beckman Ti70 rotor). The membrane pellet was suspended in 2 ml of cation buffer with the aid of a glass tube-tenflo pestle homogenizer, and an aliquot of the suspension was set aside for analysis of total protein (Pierce, Rockford, IL). The membranes again were collected by centrifugation, and the pellets were suspended in 100 ml of 2% Laemmli sample buffer (41).

40 gm of protein were loaded into wells of a 4–20% Tris-glycine gel (Novex, San Diego, CA), and after separation the proteins were transferred to nitrocellulose. These blots were blocked overnight at 4 °C in 1× Western blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN) in TBS-T (10 mM Tris, pH 8.5, 150 mM NaCl, 0.1% Tween 20). Blots then were incubated for 2 h at room temperature in a TBS-T solution containing a 1:2000 dilution of anti-P2X-R serum (Alomone, Jerusalem, Israel) and 1× Western blocking reagent. Blots were washed in TBS-T (three rinses, 5 min each) and then incubated for 1 h with TBS-T containing a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (New England BioLabs, Beverly, MA) and 1× Western blocking reagent. Blots were washed in TBS-T (three rinses, 5 min each), and then developed with Super Signal (Pierce) and imaged with a Lum-i-mager (Roche Molecular Biochemicals).

For Cox-2 Western analysis, peritoneal macrophages (in RPMI, 5% FBS) were seeded into 6-well plates (1 × 10⁶ cells/well) and incubated overnight at 37 °C in a 5% CO₂ environment. Cells were washed twice with RPMI, 5% FBS and then 1 ml of medium containing 100 mg/ml LPS (type 055:B5, Sigma) was added to each well and the cells were incubated overnight at 37 °C for h. Medium and supernatants then were discarded, the adherent cells were washed twice with PBS, and then the cells were solubilized by addition of 200 µl of 2× Laemmli sample buffer; the resulting samples were boiled for 3 min. 20 µl of each sample were fractionated on a 4–20% Tris-glycine gel, after which the proteins were transferred to nitrocellulose. These blots were processed as described above except that the primary antibody employed was anti-anti-prostaglandin synthase-2 (Oxford, Oxford, MI; 1:2000 dilution) and the secondary antibody was horseradish peroxidase-conjugated rabbit anti-IgG (New England BioLabs, 1:2000 dilution).

**YoPro Yellow Uptake**—Peritoneal macrophages were washed with isometric medium (15 mM Hepes, pH 7.2, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂) by centrifugation, and the resulting cell pellet was suspended in isometric medium to achieve a final cell concentration of 1 × 10⁶ cells/ml. 50 µl of this cell suspension then was placed into wells of a Microfluor “B” U-bottom plate (Dynatech, Chantilly, VA), and 50 µl of 2 µM YoPro Yellow (Molecular Probes, Eugene, OR) was introduced; the fluorescent dye was dissolved in isometric medium. Each well then was adjusted to 5 mM ATP or 0.0075% saponin by addition of concentrated stock solutions of these agents. Fluorescence was monitored as a function of time at 37 °C; excitation, 450 nm; emission, 530 nm.

**Stimulus-induced IL-1β Post-translational Processing in Vitro**—Macrophages from wild-type and P2X-R\(-/-\) animals (2 × 10⁶ cells seeded per well of 6-well cluster plate) were stimulated with 1 µg/ml LPS for 75 min and then rinsed with 2 ml of methionine-free RPMI medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 1% FBS, 25 mM Hepes (pH 7.4), and 1 µg/ml IL-4 (Peprotech, London, UK). One ml of pulse medium containing 83 µCi/ml of [³⁵S]methionine (Amershams Pharmacia Biotech) then was added to each well, and the cells were labeled at 37 °C for 1 h. These labeled cells subsequently were rinsed twice with RPMI 1640 medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 1% FBS, 2 mM glutamine, 1 µg/ml LPS, and 25 mM Hepes, pH 7.3 (chase medium). One ml of chase medium containing no effector, 5 mM ATP or 20 µM nigericin then was added to each well, and the cells were chased at 37 °C for 30 min. Media were harvested and clarified by centrifugation (6000 × g for 5 min) to remove cells and/or cell debris. Cell monolayers were suspended in 1 ml of a lysis buffer composed of 1% Triton X-100, 150 mM NaCl, 25 mM Hepes, pH 7.0, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml ovalbumin, 1 mM iodoacetate, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. Clarified medium samples were adjusted to the same final Triton X-100 and protease inhibitor concentrations by addition of concentrated stocks of these reagents. After a 30-min incubation on ice, all samples were clarified by centrifugation at 45,000 rpm for 30 min in a TLA-45 rotor (Beckman). The resulting supernatants were recovered and IL-1β was immunoprecipitated from these samples using a goat anti-murine IL-1β (Oxford, Oxford, MI; 1:2000 dilution) and 1 µg/ml protein A-Sepharose (Pharmacia, Piscataway, CT). Immunoprecipitates were fractionated by SDS-gel electrophoresis; the quantity of radioactivity associated with individual IL-1β polypeptide species was determined by scanning dried gels with a phosphorimager.

**ATP-induced IL-1β Post-translational Processing in Vivo**—Groups of mice were injected ip with 1 µg of LPS. Two hours after this LPS injection, mice were injected ip with 1 µg of ATP and the mice were killed 1 h after the ATP injection. The liver was harvested and clarified by centrifugation at 45,000 rpm for 30 min in a TLA-45 rotor (Beckman). The resulting supernatants were recovered and IL-1β was immunoprecipitated from these samples using a goat anti-murine IL-1β (Oxford, Oxford, MI; 1:2000 dilution) and 1 µg/ml protein A-Sepharose (Pharmacia, Piscataway, CT). Immunoprecipitates were fractionated by SDS-gel electrophoresis; the quantity of radioactivity associated with individual IL-1β polypeptide species was determined by scanning dried gels with a phosphorimager.
**FIG. 1. Disruption of the P2X7R gene in ES cells and mice.**

A, schematic representation of the P2X-R locus segment, the P2X-R targeting vector, and the targeted P2X-R allele. Filled gray boxes indicate exons and labeled boxes indicate the PGK-TK and PGK-Neo selection cassettes. Homologous recombination of the targeting vector with the endogenous P2X7R gene disrupts the carboxyl-terminal coding region of the P2X7R gene. Relevant restriction sites are abbreviated as follows: B, BamHI; RV, EcoRV; K, KpnI; S, SalI. B, detection of targeted and endogenous P2X7R alleles by Southern blot analysis of DNA from pups derived from a heterozygous mating. DNA was digested with EcoRV and hybridized with the probe indicated by a dotted line in A. This probe detects a 16-kb band from the endogenous locus and a 10-kb band from the targeted locus. C, total RNA was prepared from cultured bone marrow-derived mast cells from wild-type (+/+) and P2X7R−/− animals and was assessed by Northern analysis using a cDNA probe specific for the region that is amino-terminal to the disrupted portion of the P2X7R gene (corresponding to amino acids Gly313–Leu492). Equal loading of the lanes and the integrity of the mRNA obtained from the P2X7R−/− cells were confirmed by analysis of the Northern blot with an actin specific probe.

Injection, mice were injected ip with either 0.5 ml of 30 mM ATP (adjusted to pH 7) or PBS. Mice were euthanized 30 min or 120 min after the ATP or PBS injection, and each peritoneal cavity was lavaged with 3 ml of media. Individual lavages were centrifuged, supernatants were collected and tested by ELISA for the presence of IL-1β, IL-6 and IL-10. After the ATP or PBS injection, and each peritoneal cavity was lavaged (adjusted to pH 7) or PBS. Mice were euthanized 30 min or 120 min

**FIG. 2. Peritoneal macrophages isolated from knockout animals lack the P2X7R.** Membranes isolated from peritoneal macrophages (Macs) were fractionated by SDS-polyacrylamide gel electrophoresis, and the polypeptides were transferred to nitrocellulose. The blot then was probed for the presence of the P2X7R. Control lanes contained membrane proteins isolated from human MRC-5 fibroblasts (Fb), which do not express P2X7R and membranes recovered from HEK293 cells transfected with the human P2X7R.

**RESULTS**

**Generation and Characterization of P2X7R−/− Mice—Mouse ES cells in which the P2X7R gene was disrupted by homologous recombination were generated using the scheme shown in Fig. 1. Integration of the targeting vector into the mouse genome by homologous recombination resulted in replacement of the region that is amino-terminal to the disrupted portion of the P2X7R gene.**

A hallmark of the P2X-R is its ability to facilitate translocation of large organic molecules such as the fluorescent dye YoPro Yellow in response to ATP activation (32, 33, 42, 43). When mouse peritoneal macrophages isolated from wild-type animals were activated with 5 mM ATP in the presence of extracellular YoPro Yellow, a time-dependent increase in fluorescence intensity was observed (Fig. 3). This increase in fluorescence results from internalization of the dye molecules followed by their binding to DNA; when bound to DNA, their fluorescence intensity increases (43). In the absence of ATP, no
significant increase in fluorescence intensity was observed, indicating that YoPro Yellow is impermeable to the plasma membrane in the absence of the nucleotide triphosphate. In contrast, addition of ATP to macrophages isolated from P2X7R-/- animals did not result in a time-dependent increase in fluorescence intensity (Fig. 3). Macrophages isolated from the P2X7R+/- animals demonstrated the same low fluorescence in the absence and presence of extracellular ATP. In the presence of saponin, a detergent that permeabilizes the plasma membrane, YoPro Yellow accumulates to the same extent in the absence and presence of extracellular ATP. In the presence of saponin, a detergent that permeabilizes the plasma membrane, YoPro Yellow accumulates to the same extent in the absence and presence of extracellular ATP. In the absence of a secretory stimulus was 43,900 PSL/LDH equivalent and 44,100 PSL/LDH equivalent, respectively, from the wild-type and knockout macrophages. This similarity suggests that the two cell-types generated comparable amounts of pro-IL-1β in response to LPS activation. Neither the wild-type nor the P2X7R-/- macrophages released radiolabeled IL-1β to the medium in the absence of a secretory stimulus (Fig. 4).

Stimulus-coupled IL-1β Post-translational Processing

Peritoneal macrophages isolated from wild-type and P2X7R-/- animals were stimulated with LPS and labeled with [35S]methionine. These radiolabeled cells then were chased in the absence or presence of a secretory stimulus, after which cells and media were harvested separately, cells were solubilized by detergent extraction, and IL-1β was recovered from these samples by immunoprecipitation. The immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis, and autoradiograms of the dried gels are shown for the cell-associated (top) and media (bottom) samples. Arrows indicate the migration positions of the 35-kDa pro-IL-1β, 17-kDa mature IL-1β, and a 28-kDa alternate caspase-1 cleavage fragment. Each condition was performed on duplicate cultures.

In response to the potassium ionophore nigericin, on the other hand, both wild-type and P2X7R-/- macrophages produced and released a 17-kDa IL-1β (Fig. 4). The extracellular mature cytokine represented 66 and 74%, respectively, of the total [35S]methionine-labeled IL-1β recovered from the wild-type and P2X7R-/- cultures (Fig. 5A). Moreover, cytokine recovered from extracts of the ATP-treated P2X7R-/- macrophages persisted as the 35-kDa procytokine (Fig. 4). ATP-treated wild-type but not P2X7R-deficient macrophages demonstrated enhanced release of LDH relative to non-ATP treated cultures (Fig. 5B). In response to the potassium ionophore nigericin, on the other hand, both wild-type and P2X7R-/- macrophages produced and released a 17-kDa IL-1β (Fig. 4). The extracellular mature cytokine represented 66 and 74%, respectively, of the total [35S]methionine-labeled IL-1β recovered from the wild-type and P2X7R-/- cultures (Fig. 5A). Moreover, both macrophage populations released comparable quantities of LDH in response to nigericin (Fig. 5B). Thus, P2X7R-/- macrophages produce pro-IL-1β in response to LPS challenge, and they posttranslationally process this procytokine in response to nigericin stimulation. However, as a result of the absence of the P2X7R, these cells do not produce or release mature IL-1β in response to ATP challenge.

The P2X7R recently was implicated as a component of the pathway by which LPS induces Cox-2 expression (45). To determine whether peritoneal macrophages isolated from...
P2X<sub>7</sub> Receptor Deficiency

Peritoneal macrophages from wild-type (P2X<sub>7</sub>R<sup>+/+</sup>) and knockout (P2X<sub>7</sub>R<sup>−/−</sup>) animals were incubated for 4 h in the absence and presence of LPS, after which cellular polypeptides were separated by SDS-polyacrylamide gel electrophoresis. The presence of Cox-2 subsequently was probed by Western analysis. Each condition was performed on duplicate cultures. The left lane of the blot contains standards of the indicated molecular masses.

**Fig. 6.** Lack of the P2X<sub>7</sub>-R does not impair Cox-2 expression in response to LPS stimulation. Peritoneal macrophages from wild-type (P2X<sub>7</sub>R<sup>+/+</sup>) and knockout (P2X<sub>7</sub>R<sup>−/−</sup>) animals were incubated for 4 h in the absence and presence of LPS, after which cellular polypeptides were separated by SDS-polyacrylamide gel electrophoresis. The presence of Cox-2 subsequently was probed by Western analysis. Each condition was performed on duplicate cultures. The left lane of the blot contains standards of the indicated molecular masses.

**Fig. 7.** P2X<sub>7</sub>-R-deficient peritoneal macrophages fail to generate IL-1β in response to ATP stimulation in vivo. Wild-type (+/+), heterozygous (+/−), and homozygous P2X<sub>7</sub>-R-deficient (−/−) mice were subjected to three different regimens: 1) untreated, 2) ip injection of LPS followed by a second ip injection of PBS (+LPS/PBS), 3) ip injection of LPS followed by a second injection of ATP (+LPS+ATP). Following these treatments, peritoneal lavage fluids were analyzed for IL-1β content by ELISA. The amount of IL-1β recovered (ng/ml) is indicated as a function of treatment. Each bar is the mean ± S.D. for five mice.

**Characterization of In Vivo Cytokine Production Capabilities**—Peritoneal macrophages exposed to LPS in vivo also require a secretion stimulus to elicit efficient externalization of mature IL-1β (25). To determine whether absence of the P2X<sub>7</sub>-R affects IL-1 release in vivo, animals were primed with LPS, and 2 h later they received an ip injection of PBS with or without ATP. Peritoneal lavage fluids from these animals then were assessed for IL-1β content by ELISA. Wild-type and heterozygous LPS-primed animals yielded no significant IL-1β in response to PBS (Fig. 7), but abundant quantities of IL-1β were detected following ATP challenge (Fig. 7). In contrast, LPS-primed P2X<sub>7</sub>R<sup>−/−</sup> animals failed to generate significant levels of IL-1β in response to ATP challenge (Fig. 7).

As noted earlier, IL-1 signaling often leads to the production of other cytokines such as IL-6 (4), and cytokine networks can be initiated in vivo in response to IL-1 generation. For example, mice primed with an ip injection of LPS and subsequently challenged with ATP to promote IL-1 post-translational processing generate elevated levels of IL-6 compared with animals challenged with phosphate-buffered saline (25). To determine whether absence of the P2X<sub>7</sub>-R altered the efficiency of IL-6 production in this type of network response, wild-type and P2X<sub>7</sub>R<sup>−/−</sup> animals were compared in a two-stage production assay format. Animals were administered a priming ip injection of LPS followed 2 h later by a PBS or ATP challenge. Peritoneal lavage fluids subsequently were collected at 30 and 120 min, and these were assessed for cytokine content by ELISA. Wild-type animals primed with LPS and challenged with PBS or ATP for 30 min yielded 0.05 ng/ml and 0.4 ng/ml of IL-1β, respectively (Table I). When the time of the challenge reaction was extended to 2 h, IL-1β levels declined slightly to 0.033 ng/ml in the ATP-treated animals, but levels of IL-1β within lavage fluids recovered from PBS-challenged animals remained at the lower limit of detection (Table I). Minimal levels of IL-1β were recovered from P2X<sub>7</sub>R<sup>−/−</sup> animals challenged with PBS or ATP at both the 30 and 120 min time points (Table I). Quantities of IL-6 generated by wild-type and P2X<sub>7</sub>-R-deficient animals in response to PBS challenge were comparable at the 30 min harvest, representing 6 and 5 ng/ml, respectively (Table I). After 120 min of PBS challenge, IL-6 levels declined in both sets of animals to baseline values (Table I). After 30 min of ATP challenge, both wild-type and P2X<sub>7</sub>R<sup>−/−</sup>
animals yielded 8–9 ng/ml of IL-6; these values are slightly elevated over the quantities of IL-6 recovered from the PBS-challenged animals at 30 min (Table I). After 120 min of ATP challenge, on the other hand, wild-type animals yielded higher levels of IL-6 (17 ng/ml) than the P2X7R−/− animals (6 ng/ml) (Table I). Although levels of IL-6 generated by the ATP-challenged mutant animals at this time were lower than those generated by their wild-type counterparts, they were elevated above those generated in response to PBS challenge (6 ng/ml when challenged with ATP versus 0 ng/ml when challenged with PBS; Table I). This suggests that ATP affects IL-6 production via both a P2X7R-dependent and -independent mechanism. No IL-1β or IL-6 could be detected in lavage fluids obtained from animals that were primed with saline (data not shown).

**DISCUSSION**

Pharmacological evidence suggests that the P2X7R has a restricted cellular distribution and is expressed primarily on cells of hematopoietic origin including monocytes, macrophages, mast cells, lymphocyte populations, and dendritic cells (35, 47–51). Several other reports suggest that expression is not exclusive to this lineage as there is evidence of P2X7R expression on sperm (52) and some cancer cells of non-hematopoietic lineage (53). Despite extensive studies, the physiological function of this receptor remains unclear. It has been reported to participate in a diverse list of cellular activities including lymphocyte proliferation (54), fertilization (52), giant cell formation (55), cell death (36, 37), killing of invading mycobacteria (56), and IL-1 post-translational processing (20, 22). In the absence of specific receptor agonists and antagonists, however, P2X7R participation has generally been inferred based on ATP concentration requirements and the use of non-selective antagonists. Following its cloning, overexpression studies established that the P2X7R is responsible for the ability of ATP to promote pore formation within the plasma membrane of cells expressing this receptor (28, 29). These pores allow passage of large organic molecules such as YoPro Yellow and ethidium bromide, a feature not shared with other members of the P2X family of ligand-gated ion channels. Ligation of the P2X7R also has been associated with activation of phospholipase D and activation of some transcription factors (57–59). To what extent these reported activities are manifested in vivo remains to be determined. In this study we report the generation of a mouse line that fails to express the P2X7R polypeptide. These P2X7R−/− mice are healthy and fertile. The absence of striking phenotypic traits associated with mature knockout mice suggests that the P2X7R is dispensable for normal development. Moreover, homozygous male animals are fertile, suggesting that absence of the P2X7R does not compromise sperm development and function (52). However, although their macrophages can respond to LPS and promote pro-IL-1β and Cox-2 expression, the receptor-deficient cells fail to generate mature IL-1β in response to subsequent ATP challenge. As a consequence, P2X7R-deficient animals demonstrate an attenuated response in a cytokine signaling network in vivo.

Several previous studies demonstrated that overexpression of the P2X7R led to the ability of ATP to promote pore formation and fluorescent dye accumulation (28, 29). Macrophages isolated from wild-type, but not from P2X7R−/− mice, responded to extracellular ATP and accumulated YoPro Yellow. Thus, the knockout provides the corollary to the overexpression experiments; depletion of the receptor leads to an inability to form pores that facilitate passage of YoPro yellow. These data, however, do not prove that the P2X7R is the actual pore-forming polypeptide. BW5147 cells express the murine P2X7R, yet these cells do not accumulate ethidium bromide in response to ATP challenge (60). Moreover, the marine toxin maitotoxin activates P2X7R-like pores in cells that do not express the P2X7R (61). These observations led to the proposal that the P2X7R itself may not constitute the pore but, rather, may link to and/or activate the pore-forming unit (61).

One of the most intriguing activities attributed to the P2X7R is its ability to induce post-translational processing of pro-IL-1β (18, 20, 22). Data previously have been presented establishing that efficient release of mature IL-1β from LPS-activated monocytes and macrophages requires the cytokine-producing cells to encounter a secretion stimulus. Agents or treatments that have been shown to function in this capacity in vitro include ATP, nigericin, hypotonic stress, cytolytic T-cells, and bacterial toxins (19–24). All of these effectors appear to initiate IL-1 post-translational processing reactions by inducing changes to the intracellular ionic environment. For example, treatment of LPS-activated monocytes with ATP or nigericin promotes loss of intracellular K+ (22). When this loss is prevented by exposing the cells to the secretion stimuli in the presence of elevated extracellular K+ concentrations, IL-1β post-translational processing is completely inhibited (18, 22, 62). Therefore, depletion of intracellular K+ appears to be a necessary element of the stimulus-induced process. Likewise, inhibitors of anion transport, such as tenidap, ethacrynic acid, and glyburide, disrupt ATP-induced IL-1β post-translational processing suggesting that anion movements are a necessary feature of the cellular process (17, 26, 63).

Peritoneal macrophages isolated from the P2X7R−/− mice responded to LPS activation and produced quantities of pro-IL-1β and Cox-2 comparable with those produced by wild-type macrophages. Although we cannot rule out the possibility of the involvement of a compensatory mechanism, these data suggest that the P2X7R is not required for induction of LPS-inducible gene products. In contrast to our findings, a previous study concluded that ATP released from macrophages as a result of LPS activation served as an autocrine-type stimulus.
to promote expression of Cox-2 via ligation of the P2X7R (45). Conclusions reached in this previous study, however, were based on the use of non-selective inhibitors of the P2X7-R, and these compounds may elicit their effects by disrupting other cellular processes. Although expression of pro-IL-1β was normal in macrophages that lacked the P2X7-R, post-translational processing of the procytokine in response to ATP challenge was totally ablated. Therefore, the P2X7-R is a necessary element of the post-translational processing mechanism. Interestingly, LPS-activated P2X7-R-deficient macrophages continued to produce mature IL-1β in response to nigericin challenge. Nigericin, therefore, must promote cytokine post-translational processing independently of the P2X7-R. The mechanisms by which nigericin and ATP initiate pro-IL-1β post-translational processing appear to share common elements. For example, both mechanisms require extracellular Na+ (62), both are inhibited by high extracellular K+ (22), and both result in similar morphological transformations (22). Perhaps, these two diverse stimuli link to a common pore-forming subunit to initiate the cytokine production pathway.

IL-1 is known to elicit complex cytokine signaling networks when administered to animals, and injection of the recombiant cytokine locally within animal joints can lead to an inflammatory response and attendant structural changes that mimic the pathophysiological process taking place in joints of patients suffering from rheumatoid arthritis (Ref. 64). Many gene products are known to be up-regulated in response to IL-1 signaling including IL-6 (4). Ip injection of LPS into wild-type mice produced no extracellular IL-1β and only small quantities of IL-6. However, following challenge with extracellular ATP, these LPS-primed mice generated cell-dissociated IL-1β and, in turn, much greater levels of IL-6. In contrast, LPS-primed P2X7 R−/− animals failed to generate IL-1 in response to ATP challenge and failed to match the large increase in IL-6 production demonstrated by their wild-type counterparts. These observations indicate that IL-1 released as a result of the P2X-R can serve as a functional element of a cytokine network. Although the ATP-dependent IL-6 response of the knockout animals was greatly attenuated relative to the wild-type, the knockout animals did appear to respond to ATP. LPS-primed P2X7 R−/− animals that were subsequently challenged with PBS yielded no significant IL-6 at 4-h post-LPS priming. On the other hand, comparable animals challenged with ATP yielded 6 ng/ml of IL-6 at the 4-b time point. This suggests that ATP may work through other purinoreceptors to affect IL-6 production. Perhaps ATP activation of G-protein-coupled P2Y-type receptors leads to changes in intracellular cAMP and/or Ca2+ that enhance the LPS-induced IL-6 production response (48). Additional work will be required to understand this P2X7-R-independent response, and studies are ongoing to further characterize in vivo inflammatory processes within the receptor-deficient mice to gain a more complete understanding of how absence of this receptor affects immune system function.

IL-1 is a potent mediator of inflammatory responses (1). Not surprisingly perhaps, animals appear to have developed a number of safeguards to ensure that IL-1 activity is tightly regulated. For example, a natural receptor antagonist, IL-1ra, exists and this protein competes with IL-1 for binding to the type-1 IL-1 receptor (65). Unlike IL-1α and IL-1β, IL-1ra binding does not lead to receptor activation. Rather, the receptor antagonist appears to be produced via an endogenous process to suppress IL-1 signaling events. Another safeguard exists in the type-2 IL-1 receptor. Binding of IL-1 to this receptor does not initiate signaling cascades within target cells (66). Rather, the type-2 receptor appears to act as a decoy and provides a mechanism to buffer and/or blunt an IL-1 biologic response. Finally, the unique post-translational requirements of IL-1 appear to offer yet another additional safeguard. Monocytes and macrophages may become activated to produce the procytokine polypeptides, but these remain latent unless the producing cells encounter a secondary stimulus that promotes their post-translational processing and release. The P2X7 R−/− mice establish that ATP acting through the P2X7-R represents one potential mechanism by which IL-1 post-translational processing is achieved in vivo. Understanding how P2X7-R activity is regulated, therefore, may provide important insights for designing strategies to control inflammatory response mechanisms.

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