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Altered Cytokine Production in Mice Lacking $P2X_7$ Receptors*

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The $P2X_7$ receptor ($P2X_7R$) 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 $P2X_7R$-deficient mouse line. $P2X_7R^{-/-}$ 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 $P2X_7R^{-/-}$ cells is not externalized or activated by caspase-1. Nigericin, an alternate secretion stimulus, promotes release of 17-kDa IL-1β from $P2X_7R^{-/-}$ macrophages. In response to *in vivo* lipopolysaccharide injection, both wild-type and $P2X_7R^{-/-}$ animals display increases in peritoneal lavage IL-6 levels but no detectable IL-1. Subsequent ATP injection to wild-type animals promotes an increase in IL-1, which in turn leads to additional IL-6 production; similar increases did not occur in ATP-treated, LPS-primed $P2X_7R^{-/-}$ animals. Absence of the $P2X_7R$ thus leads to an inability of peritoneal macrophages to release IL-1 in response to ATP. As a result of the IL-1 deficiency, *in vivo* cytokine signaling cascades are impaired in $P2X_7R$-deficient animals. Together these results demonstrate that $P2X_7R$ activation can provide a signal that leads to maturation and release of IL-1β and initiation of a cytokine cascade.

Interleukin (IL)[1]-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 <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 $P2X_7$ purinergic receptor (17, 18, 26, 27). Like other members of the P2X receptor family, the $P2X_7$ receptor ($P2X_7R$) is an ATP-gated ion channel (28–30). The $P2X_7R$, however, demonstrates attributes that clearly distinguish it from other members of the family. For example, the $P2X_7R$ requires levels of ATP in excess of 1 mM to achieve activation, whereas other P2X receptors activate at ATP concentrations of $\leq$100 μM (31, 32); the higher concentration requirement reflects, in part, the preference of the $P2X_7R$ for ATP^{4-} as its ligand and the relatively low abundance of this species in media containing physiological

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[1] The abbreviations used are: IL, interleukin; LPS, lipopolysaccharide; $P2X_7R$, $P2X_7$ receptor; ES, embryonic stem; ip, intraperitoneal; Cox-2, cyclooxygenase-2, LDH, lactate dehydrogenase; PSL, phosphospecific luminescence; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HEK, human embryonic kidney cells; ELISA, enzyme-linked immunoadsorbent assay.

concentrations of divalent cations (*e.g.* Ca^{2+} and Mg^{2+}). An additional unique feature of the $P2X_7R$ is found in its conductance properties. All P2X receptors demonstrate non-selective channel-like properties following ligation, but the channels formed by the $P2X_7R$ 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 $P2X_7R$ participates in pore complex formation (28, 29); the carboxyl-terminal domain of the $P2X_7R$ 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 $P2X_7$ receptor for times of >15 min can lead to cell death (35–37).

In this study we employ a genetic approach to inactivate the $P2X_7R$. Mice lacking the $P2X_7R$ are healthy and fertile and demonstrate no overt phenotype. However, in contrast to their wild-type counterparts, LPS-activated peritoneal macrophages from $P2X_7R^{-/-}$ animals fail to generate mature IL-1β when challenged with ATP. This defect is not because of an inability of the macrophages to produce pro-IL-1β 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-1β post-ATP challenge, $P2X_7R^{-/-}$ animals generate reduced quantities of IL-6 relative to their wild-type controls. Therefore, the knockout animals establish that the $P2X_7R$ 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 $P2X_7R$ Targeting Vector and Generation of the Knockout Mice—A cDNA probe specific to the mouse $P2X_7R$ gene was synthesized by reverse transcription-polymerase chain reaction using primers $P2X_7$-F1 (5′-CGGCGTGCGTTTTGACATCCT-3′) and $P2X_7$-R2 (5′-AGGGCCCTGCGGTTCTC-3′), which were designed based on the published rat cDNA sequence of the $P2X_7R$ 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 $P2X_7R$ gene. The probe was used to screen a 129/Sv mouse genomic library and to isolate a single positive genomic clone. Sequence analysis of *Bam*HI subcloned fragments confirmed that this clone corresponded to the mouse $P2X_7R$ gene. A targeting vector was constructed that inserted the neomycin resistance gene from the pJNS2 plasmid directly after the Arg^{505} codon, deleting from Cys^{506} to Pro^{532}, which is in the carboxyl-terminal domain of the $P2X_7R$ gene product (38).

129/Ola-derived E14Tg2a ES cells (39) were grown, transformed, 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 × *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 cavitation buffer (25 mM Hepes, pH 7, 30 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) by centrifugation. Cell pellets then were suspended in 2.5 ml of cavitation 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 cavitation buffer with the aid of a glass tube-teflon 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 after which the pellets were suspended in 100 μl of 2× Laemmli sample buffer (41).

40 μg 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, 150 mM NaCl, 0.1% Tween 20). Blots then were incubated for 2 h at room temperature in a TBS-T solution containing a 1:200 dilution of anti-$P2X_7R$ 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 Lumi-imager (Roche Molecular Biochemicals).

For Cox-2 Western analysis, peritoneal macrophages (in RPMI, 5% FBS) were seeded into 6-well plates (1×10^6 cells/well) and incubated overnight at 37 °C in a 5% CO_2 environment. Cells were washed twice with RPMI, 5% FBS and then 1 ml of medium containing 100 ng/ml LPS (type 055:B5, Sigma) was added to each well and the cells were incubated at 37 °C for 4 h. Medium supernatants then were discarded, the adherent cells were washed twice with PBS, and then they 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-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 isotonic medium (15 mM Hepes, pH 7.2, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$) by centrifugation, and the resulting cell pellet was suspended in isotonic medium to achieve a final cell concentration of 1×10^6 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 isotonic 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 $P2X_7R^{-/-}$ animals (2×10^6 cells seeded per well of 6-well cluster plates) 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% dialyzed FBS, 1 μg/ml LPS, and 25 mM Hepes, pH 7.3 (pulse medium). One ml of pulse medium containing 83 μCi/ml of [^{35}S]methionine (Amersham 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, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml ovalbumin, 1 mM iodoacetic acid, 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 serum obtained from Dr. Ivan Otterness (Pfizer Central Reseach, Groton, 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

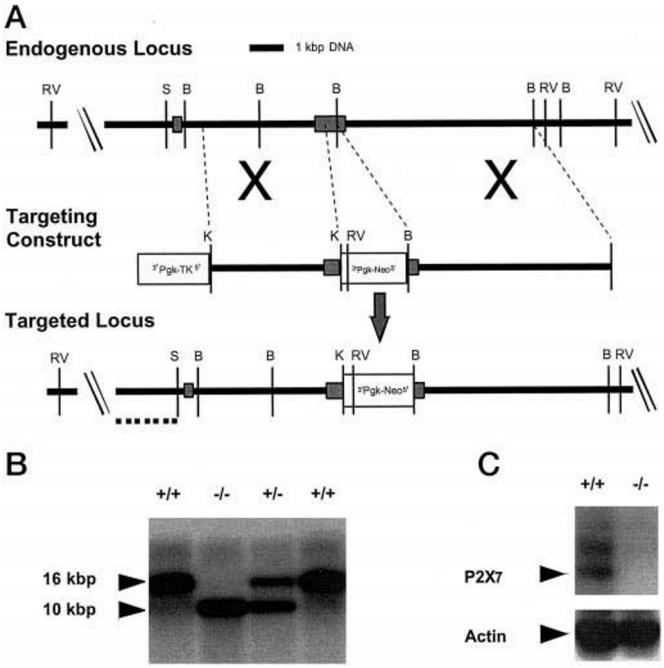

FIG. 1. **Disruption of the $P2X_7R$ gene in ES cells and mice.** *A,* schematic representation of the $P2X_7R$ locus segment, the $P2X_7R$ targeting vector, and the targeted $P2X_7R$ allele. *Filled gray boxes* indicate exons and *labeled boxes* indicate the PGK-TK and PGK-Neo selection cassettes. Homologous recombination of the targeting vector with the endogenous *$P2X_7R$* gene disrupts the carboxyl-terminal coding region of the *$P2X_7R$* gene. Relevant restriction sites are abbreviated as follows: *B*, *Bam*HI; *RV*, *Eco*RV; *K*, *Kpn*I; *S*, *Sal*I. *B,* detection of targeted and endogenous *$P2X_7R$* alleles by Southern blot analysis of DNA from pups derived from a heterozygous mating. DNA was digested with *Eco*RV 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 *$P2X_7R^{-/-}$* 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 *$P2X_7R$* gene (corresponding to amino acids Gly^{313}–Leu^{492}). Equal loading of the lanes and the integrity of the mRNA obtained from the *$P2X_7R^{-/-}$* cells were confirmed by analysis of the Northern blot with an actin specific probe.

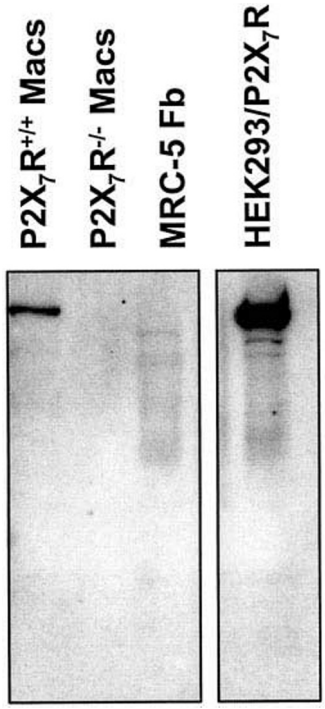

FIG. 2. **Peritoneal macrophages isolated from knockout animals lack the $P2X_7R$.** Membranes isolated from peritoneal macrophages (Macs) were fractionated by SDS-polyacrylamide gel electrophoresis, and the polypeptides were transferred to nitrocellulose. The blot then was probed for the presence of the $P2X_7R$. Control lanes contained membrane proteins isolated from human MRC-5 fibroblasts (*Fb*), which do not express $P2X_7R$ and membranes recovered from HEK293 cells transfected with the human *$P2X_7R$*.

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 (Amersham Pharmacia Biotech) and IL-6 (Endogen, Inc. Woburn, MA). All procedures involving mice were approved by the Institutional Animal Care and Use Committee at Pfizer Inc.

RESULTS

Generation and Characterization of $P2X_7R^{-/-}$ Mice—Mouse ES cells in which the *$P2X_7R$* 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 results in replacement of the region of the gene encoding Cys^{506} to Pro^{532} with the neomycin resistance gene. ES cells containing the mutant *$P2X_7R$* allele were identified by Southern blot analysis and used to generate the *$P2X_7R^{\Delta 506-532}$* mouse line. Homozygous null animals were recovered in the F2 generation with the expected Mendelian frequencies. *$P2X_7R^{\Delta 506-532}$* (*$P2X_7R^{-/-}$*) animals were viable and fertile and could not be identified among littermates by observation alone.

Northern analysis of RNA isolated from cultured bone marrow mast cells signified that the transcript for the $P2X_7R$ was present in wild-type (+/+) but not knockout (−/−) animals (Fig. 1*C*). To further demonstrate that the mutation introduced into the $P2X_7R$ locus resulted in loss of expression of this gene, peritoneal macrophages obtained from wild-type or $P2X_7R$-deficient mice were compared by Western analysis for the presence of the receptor polypeptide. An equivalent number of macrophages were recovered from wild-type and $P2X_7R$-deficient animals, suggesting that absence of the receptor did not alter macrophage development. Membranes isolated from wild-type peritoneal macrophages contained a 76-kDa polypeptide that cross-reacted with the $P2X_7R$ antiserum. The size of the murine polypeptide is comparable with that displayed by the human $P2X_7R$ when overexpressed in HEK293 cells (Fig. 2). In contrast, macrophage membranes prepared from cells isolated from the $P2X_7R$-deficient animals did not contain a similarly sized polypeptide (Fig. 2). In addition, no smaller cross-reacting polypeptides were observed in the Western blot of the *$P2X_7R^{-/-}$* cells, suggesting that the mutation introduced into the *$P2X_7R$* gene did not lead to expression of a truncated version of the receptor (Fig. 2). However, because the antibody employed was prepared against a peptide epitope that resides at the extreme carboxyl terminus of the receptor polypeptide, we cannot exclude the possibility that a truncated form of the receptor lacking the entire carboxyl terminus is present and not detected by the analysis.

A hallmark of the $P2X_7R$ 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

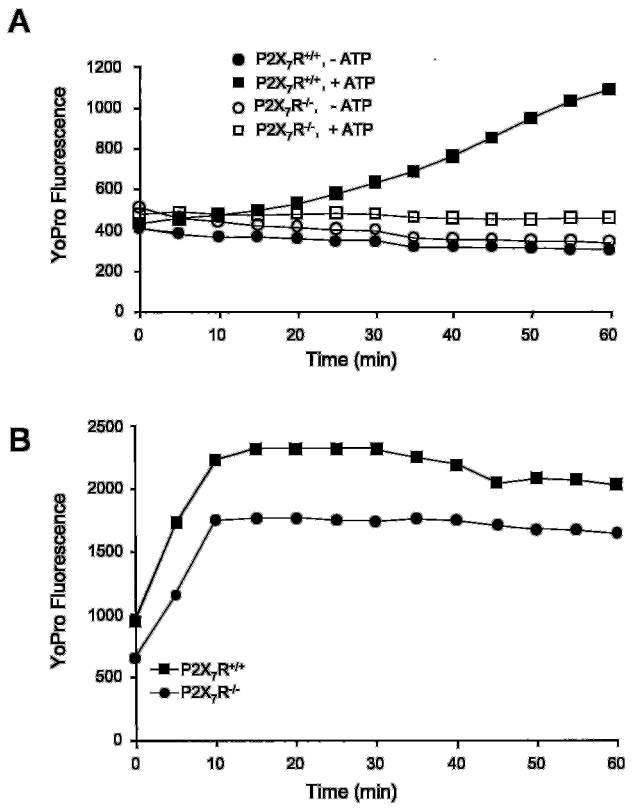

FIG. 3. **Wild-type but not knockout macrophages accumulate YoPro Yellow in response to ATP activation.** *A,* peritoneal macrophages were incubated with 1 μM YoPro Yellow in the presence (*+ATP*) or absence (*−ATP*) of 5 mM ATP, and resulting fluorescence intensity changes were recorded as a function of time at 37 °C. *B,* wild-type and knockout peritoneal macrophages were incubated with 1 μM YoPro Yellow in the presence of 0.0075% saponin, and resulting fluorescence intensity changes were recorded as a function of time.

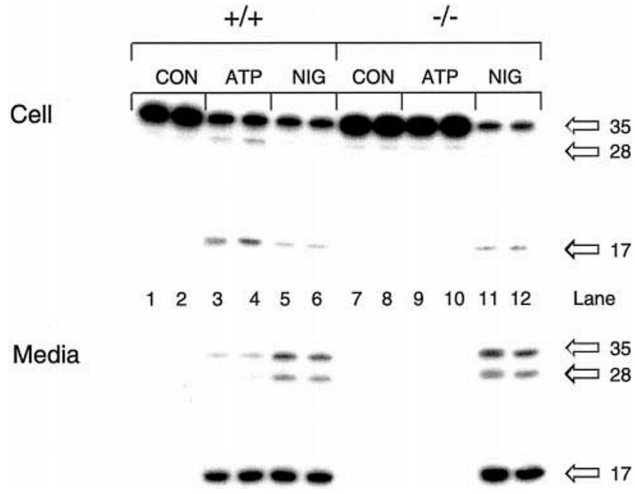

FIG. 4. **$P2X_7R$ deficiency abolishes ATP-induced IL-1β post-translational processing but does not affect nigericin-induced processes.** Peritoneal macrophages were stimulated with LPS for 90 min after which the cells were pulse-labeled with [^{35}S]methionine for 60 min. The radiolabeled cells then were incubated in the absence of any effector (*CON*) or presence of 5 mM ATP or 20 μM nigericin (*NIG*) for 30 min. Cell and media fractions were collected separately, 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.

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 $P2X_7R^{-/-}$ animals did not result in a time-dependent increase in fluorescence intensity (Fig. 3). Macrophages isolated from the $P2X_7R^{-/-}$ 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 both wild-type and $P2X_7R^{-/-}$ macrophages (Fig. 3). This demonstrates that the $P2X_7R$ mediates ATP-dependent YoPro Yellow accumulation.

Stimulus-coupled IL-1β Post-translational Processing—Peritoneal macrophages isolated from wild-type and $P2X_7R^{-/-}$ animals were stimulated with LPS and labeled with [^{35}S]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 the medium and cell extracts by immunoprecipitation. Radiolabeled 35-kDa pro-IL-1β was recovered from cell extracts derived from both wild-type and $P2X_7R^{-/-}$ macrophages (Fig. 4). The amount of [^{35}S]methionine recovered as the cell-associated 35-kDa polypeptide (assessed by phosphorimager analysis) after the chase 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 $P2X_7R^{-/-}$ macrophages released radiolabeled IL-1β to the medium in the absence of a secretory stimulus (Fig. 4).

Treatment of LPS-activated [^{35}S]methionine-labeled wild-type macrophages with extracellular ATP promoted formation and release of a 17-kDa IL-1β (Fig. 4). Cytokine that remained cell-associated was for the most part recovered as the 35-kDa procytokine (Fig. 4). After correcting for the 2-fold loss of radioactivity that occurs when the 35-kDa procytokine is proteolytically processed by caspase-1 (44), the extracellular 17-kDa polypeptide represents 64% of the total (sum of cell-associated and medium species) radiolabeled IL-1β recovered from these cultures (Fig. 5). In sharp contrast, LPS-activated [^{35}S]methionine-labeled $P2X_7R^{-/-}$ macrophages did not release any radiolabeled IL-1β to the medium in response to ATP (Fig. 4). Moreover, cytokine recovered from extracts of the ATP-treated $P2X_7R^{-/-}$ macrophages persisted as the 35-kDa procytokine species (Fig. 4). ATP-treated wild-type but not $P2X_7R$-deficient macrophages demonstrated enhanced release of LDH relative to non-ATP treated cultures (Fig. 5*B*).

In response to the potassium ionophore nigericin, on the other hand, both wild-type and $P2X_7R^{-/-}$ macrophages produced and released a 17-kDa IL-1β (Fig. 4). The extracellular mature cytokine represented 66 and 74%, respectively, of the total [^{35}S]methionine-labeled IL-1β recovered from the wild-type and $P2X_7R^{-/-}$ cultures (Fig. 5*A*). Moreover, both macrophage populations released comparable quantities of LDH in response to nigericin (Fig. 5*B*). Thus, $P2X_7R^{-/-}$ macrophages produce pro-IL-1β in response to LPS challenge, and they post-translationally process this procytokine in response to nigericin stimulation. However, as a result of the absence of the $P2X_7R$, these cells do not produce or release mature IL-1β in response to ATP challenge.

The $P2X_7R$ recently was implicated as a component of the pathway by which LPS induces Cox-2 expression (45). To determine whether peritoneal macrophages isolated from

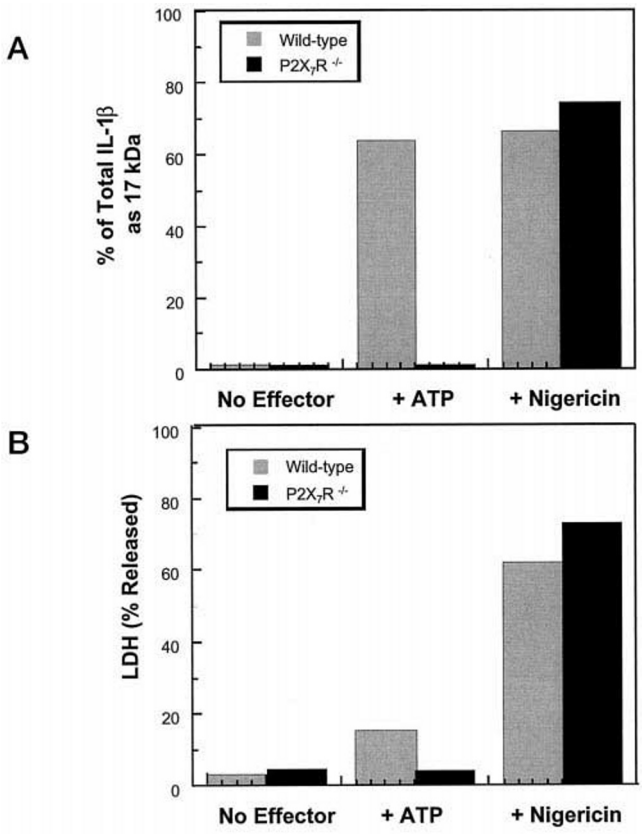

FIG. 5. **$P2X_7R$ deficiency selectively affects release of mature IL-1β.** *A,* regions of the gel shown in Fig. 4 containing radiolabeled IL-1β were scanned by phosphorimager analysis, and the sum of all species (corrected for the 2-fold loss of radioactivity that occurs when pro-IL-1 is converted to the 17-kDa species) was determined. The % of this total that was represented by the 17-kDa species is indicated as a function of effector treatment. Each *bar* is an average of duplicate determinations. *B,* the amount of LDH released into the medium (expressed as a % of the total culture-associated activity) is indicated as a function of effector treatment. Each *bar* is an average of duplicate determinations.

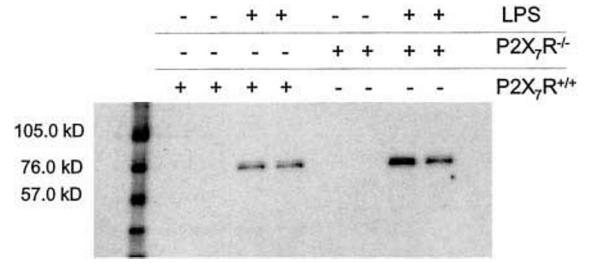

FIG. 6. **Lack of the $P2X_7R$ does not impair Cox-2 expression in response to LPS stimulation.** Peritoneal macrophages from wild-type ($P2X_7R^{+/+}$) and knockout ($P2X_7R^{-/-}$) animals were incubated for 4 h in the absence and presence of LPS, after which cellular polypeptides were separated by 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.

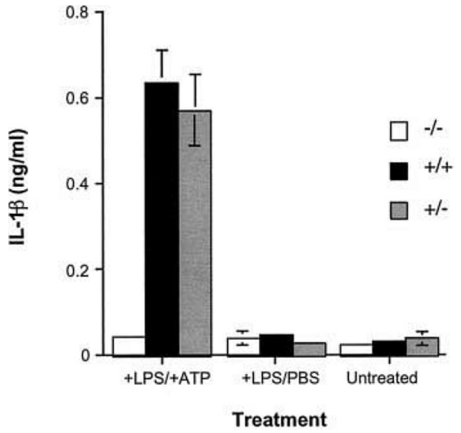

FIG. 7. **$P2X_7R$-deficient peritoneal macrophages fail to generate IL-1β in response to ATP stimulation *in vivo*.** Wild-type (+/+), heterozygous (+/−), and homozygous $P2X_7R$-deficient (−/−) mice were subjected to three different regimens: 1) untreated, 2) ip injection of LPS followed by a second 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.

$P2X_7R^{-/-}$ animals were impaired in their ability to generate Cox-2 following LPS activation relative to their wild-type counterparts, cultures of the two cell types were stimulated with LPS and Cox-2 expression was examined by Western analysis. In the absence of LPS, neither wild-type nor $P2X_7R^{-/-}$ macrophages demonstrated the presence of Cox-2 cross-reacting polypeptides (Fig. 6). However, after LPS activation, both macrophage populations possessed a 70-kDa immunogenic polypeptide (Fig. 6); this is the expected molecular mass of murine Cox-2 (46).

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 $P2X_7R$ 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 $P2X_7R^{-/-}$ 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 $P2X_7R$ altered the efficiency of IL-6 production in this type of network response, wild-type and $P2X_7R^{-/-}$ 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 $P2X_7R^{-/-}$ 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 $P2X_7R$-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 $P2X_7R^{-/-}$

TABLE I

P2X$_7$ receptor-deficient mice generate less IL-6 in response to LPS/ATP challenge

Wild-type (WT) and P2X$_7$R$^{-/-}$ mice were primed with an ip injection of LPS, and 2 hr later these animals were challenged with an additional ip injection of PBS or ATP. At 30- and 120-min post-challenge, separate animals were sacrificed, and peritoneal lavages were collected; cytokine content within the clarified lavage fluids subsequently was assessed by ELISA. Each indicated value is the mean and where indicated S.D. of six separate animals. The limit of detection of the ELISAs, based on comparison of the assay response to recombinant cytokine standards, was 0.05 ng/ml for the IL-1 kit and 0.05 ng/ml for the IL-6 kit.

Time post-challenge	PBS Challenge				ATP Challenge			
	IL-1		IL-6		IL-1		IL-6	
	WT	*P2X$_7$R$^{-/-}$*	WT	*P2X$_7$R$^{-/-}$*	WT	*P2X$_7$R$^{-/-}$*	WT	*P2X$_7$R$^{-/-}$*
min	*ng/ml*		*ng/ml*		*ng/ml*		*ng/ml*	
30	0.05	0.04	5.6 ± 0.7	4.7 ± 0.6	0.4 ± 0.12	0.04	8.2 ± 1.7	9 ± 1
120	0.02	0.02	1 ± 0.6	0	0.33 ± 0.08	0.02	16.8 ± 2.3	5.7 ± 1.2

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 *P2X$_7$R$^{-/-}$* 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 P2X$_7$R-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 P2X$_7$R 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 P2X$_7$R 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, P2X$_7$R 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 P2X$_7$R 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 P2X$_7$R 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 P2X$_7$R polypeptide. These P2X$_7$R-deficient mice are healthy and fertile. The absence of striking phenotypic traits associated with mature knockout mice suggests that the P2X$_7$R is dispensable for normal development. Moreover, homozygous male animals are fertile, suggesting that absence of the P2X$_7$R 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, P2X$_7$R-deficient animals demonstrate an attenuated response in a cytokine signaling network *in vivo*.

Several previous studies demonstrated that overexpression of the P2X$_7$R led to the ability of ATP to promote pore formation and fluorescent dye accumulation (28, 29). Macrophages isolated from wild-type, but not from *P2X$_7$R$^{-/-}$* 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 P2X$_7$R is the actual pore-forming polypeptide. BW5147 cells express the murine P2X$_7$R, yet these cells do not accumulate ethidium bromide in response to ATP challenge (60). Moreover, the marine toxin maitotoxin activates P2X$_7$R-like pores in cells that do not express the P2X$_7$R (61). These observations led to the proposal that the P2X$_7$R 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 P2X$_7$R is its ability to induce post-translational processing of pro-IL-1 (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 *P2X$_7$R$^{-/-}$* 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 P2X$_7$R 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 P2X$_7$R (45). Conclusions reached in this previous study, however, were based on the use of non-selective inhibitors of the P2X$_7$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 P2X$_7$R, post-translational processing of the procytokine in response to ATP challenge was totally ablated. Therefore, the P2X$_7$R is a necessary element of the post-translational processing mechanism. Interestingly, LPS-activated P2X$_7$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 P2X$_7$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 recombinant 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 *P2X$_7$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$_7$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 *P2X$_7$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-h time point. This suggests that ATP may work through other purinoceptors to affect IL-6 production. Perhaps ATP activation of G-protein-coupled P2Y-type receptors leads to changes in intracellular cAMP and/or Ca^{2+} that enhance the LPS-induced IL-6 production response (48). Additional work will be required to understand this P2X$_7$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 an 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 *P2X$_7$R$^{-/-}$* mice establish that ATP acting through the P2X$_7$R represents one potential mechanism by which IL-1 post-translational processing is achieved *in vivo*. Understanding how P2X$_7$R activity is regulated, therefore, may provide important insights for designing strategies to control inflammatory response mechanisms.

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