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Effect of the Purinergic Receptor $P2X_7$ on *Chlamydia* Infection in Cervical Epithelial Cells and Vaginally Infected Mice[1]

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Ligation of the purinergic receptor, $P2X_7R$, with its agonist ATP has been previously shown to inhibit intracellular infection by chlamydiae and mycobacteria in macrophages. The effect of $P2X_7R$ on chlamydial infection had never been investigated in the preferred target cells of chlamydiae, cervical epithelial cells, nor in vaginally infected mice. In this study, we show that treatment of epithelial cells with $P2X_7R$ agonists inhibits partially *Chlamydia* infection in epithelial cells. Chelation of ATP with magnesium or pretreatment with a $P2X_7R$ antagonist blocks the inhibitory effects of ATP. Similarly to previous results obtained with macrophages, ATP-mediated inhibition of infection in epithelial cells requires activation of host-cell phospholipase D. Vaginal infection was also more efficient in $P2X_7R$-deficient mice, which also displayed a higher level of acute inflammation in the endocervix, oviduct, and mesosalpingeal tissues than in infected wild-type mice. However, secretion of IL-1β, which requires $P2X_7R$ ligation during infection by other pathogens, was decreased mildly and only at short times of infection. Taken together, these results suggest that $P2X_7R$ affects *Chlamydia* infection by directly inhibiting infection in epithelial cells, rather than through the ability of $P2X_7R$ to modulate IL-1β secretion. ***The Journal of Immunology,*** **2007, 179: 3707–3714.**

The pathologic consequences of female genital infection by *Chlamydia trachomatis* include pelvic inflammatory disease, ectopic pregnancy, and infertility (1–4). An inflammatory response is required for the resolution of primary *C. trachomatis* infection, but inflammation is also responsible for the scarring process observed in trachoma and chlamydial genital disease. A number of proinflammatory mediators are present during infection, including multiple chemokines, IL-1β, and TNF-α (4–6). Inflammation is initiated in large part due to ligation of receptors from the innate immune system such as TLR that recognize pathogen-associated molecular patterns (PAMP)[3] expressed by microbial pathogens, including LPS and peptidoglycan (7–9). However, recent studies have uncovered a prominent role for danger signals released from dying or stressed cells in promoting an inflammatory response. Thus, molecules that are normally found within the host cell, such as uric acid, ATP, and the chromatin-associated high mobility group box 1 protein, can initiate secretion of inflammatory cytokines after binding to specific receptors on immune effector cells that recognize the extracellular form of these molecules (10–12).

The danger signal, ATP, is released by infected or stressed cells at sites of inflammation, and helps to control infection through its ability to stimulate secretion of proinflammatory cytokines. ATP binds to the purinergic receptor, $P2X_7R$, which is expressed primarily in hemopoietic cells and a limited number of other cell types, including fibroblasts and some epithelial cells (13, 14). Engagement of the $P2X_7R$ by extracellular ATP (ATPe) leads to maturation and secretion of the proinflammatory cytokines IL-1β and IL-18 (11, 13, 15–19). Macrophages from $P2X_7R$-deficient mice do not release IL-1β in response to ATPe, and the mice are resistant to the development of collagen-induced arthritis (20, 21).

Although ATPe is viewed largely as a proinflammatory mediator, recent in vitro studies have demonstrated that ATPe ligation of the $P2X_7R$ also stimulates killing of intracellular chlamydiae and mycobacteria in infected macrophages (22–24). ATPe stimulation of $P2X_7R$ in macrophages results in a marked increase in the activity of phospholipase D (PLD) (23, 25, 26), an enzyme that has been previously linked to antimicrobial mechanisms, including phagocytosis and generation of reactive oxygen species. PLD activation appears to be directly responsible for killing of intracellular chlamydiae and mycobacteria, because PLD inhibitors decrease the level of killing of the intracellular pathogens (22–24, 26).

Although there is agreement that ATPe ligation of $P2X_7R$ can inhibit infection by intracellular pathogens in vitro, a study with $P2X_7R$-deficient mice suggested that $P2X_7R$ may not be important for control of pulmonary *Mycobacterium tuberculosis* infection in vivo (27). However, recent work showed that various polymorphisms in the human $P2X_7R$ gene abrogate IFN-γ/ATPe-induced killing of mycobacteria by macrophages, and hence may contribute to variability in susceptibility to mycobacterial infections in humans (28). Furthermore, a significant protective association against tuberculosis was found for one single nucleotide polymorphism in the $P2X_7$ promoter gene in a Gambian population (29). This association supports a role for ATPe/$P2X_7R$-mediated regulation of *M. tuberculosis* infection in humans. These different conclusions may simply indicate that $P2X_7R$-mediated mycobacterial

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[3] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; ATPe, extracellular ATP; BzATP, 3-*O*-(4-benzoylbenzoyl) ATP; IFU, inclusion-forming unit; oATP, oxidized ATP; PLD, phospholipase D.

killing is more effective in humans than in rodents, but they point out that further investigations of the role of $P2X_7R$ in host defense against mycobacteria are warranted.

Previous studies on effects of $P2X_7R$ on infection were done with macrophages (22, 24). However, epithelial cells lining mucosal surfaces are the first and main host cells for all *Chlamydia* strains. They secrete chemoattractant and proinflammatory cytokines in response to infection with *Chlamydia*, suggesting that these cells contribute directly to early host defense during infection (30). Given the expression of $P2X_7R$ on human cervical epithelial cells (31, 32) and vaginal epithelium in rats (33), we therefore also investigated whether ATPe could induce killing of chlamydiae in epithelial cells, and, if so, whether activation of PLD is required for the bactericidal activity.

The effect of $P2X_7R$ deficiency has not been described in an in vivo model of *Chlamydia* infection. Genital tract infection of mice with *Chlamydia muridarum* closely mimics genital infection in women (34). We therefore determined the role of $P2X_7R$ in the in vivo response to chlamydial genital tract infection by examining the course of infection, the local inflammatory response, and chronic histopathology in infected mice deficient for $P2X_7R$.

Materials and Methods

Animals

The $P2X_7R$-deficient mice were previously described (20) and were used as a source of macrophages and thymocytes as in our previous studies (22, 35). These animals were maintained on a mixed genetic background (129/Ola × C57BL/6 × DBA/2) backcrossed five times onto C57BL/6. To maintain a colony of receptor-deficient mice, $P2X_7R^{-/-}$ males were bred with $P2X_7R^{-/-}$ females. Genetically comparable wild-type mice were used as controls in experiments with $P2X_7R$-deficient mice. The mice were given food and water ad libitum in an environmentally controlled room with a cycle of 12 h of light and 12 h of darkness. All animal experiments were preapproved by the University Institutional Animal Care and Use Committee.

Bacteria, cells, and reagents

C. muridarum, also known as the mouse pneumonitis biovar of *C. trachomatis* (Nigg) (36, 37), was grown in *Mycoplasma*-free McCoy cells, and elementary bodies were harvested from infected cells by centrifugation, as previously described (38). The human endocervical adenocarcinoma cell lines, HeLa 229 and CaSki, were from American Type Culture Collection. The cells were cultured in a humidified incubator at 37°C with 5% CO_2 in DMEM with Glutamax-1 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS and 25 μg/ml gentamicin. ATP, 3-*O*-(4-benzoylbenzoyl) ATP (BzATP), oxidized ATP (oATP), and UTP were purchased from Sigma-Aldrich.

RT-PCR for $P2X_7R$ mRNA from HeLa and CaSki cells

RNA from HeLa or CaSki cells was isolated using an RNeasy kit (Qiagen), following the manufacturer's instructions. Total RNA was converted into cDNA by standard reverse transcription with Superscript II RNase H^- reverse transcriptase in the manufacturer's buffer (Invitrogen Life Technologies). cDNAs were amplified using the iCycler (Bio-Rad) in a 50-μl reaction mixture containing 1/15 of the cDNA generated from reverse-transcription reaction, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM (each) dNTPs, 0.5 μM forward and reverse primers, and 1 U of HotGoldstar DNA polymerase (Eurogentec). For GAPDH, the sequences of the primers used were as follows: forward primer, 5′-AACGGATTTGGTCGTATTGGGC-3′; reverse primer, 5′-CTTGACGGTGCCATGGAATTTG-3′. For $P2X_7R$, the sequences of the primers used were as follows: forward primer, 5′-TGATAAAAGTCTTCGGGATCCGT-3′; reverse primer, 5′-TGGAC AAATCTGTGAAGTCCATC-3′. To detect specifically the truncated $P2X_7R$ variant ($P2X_{7\text{-}j}$), the following sequences, previously described (32), were used: forward primer, 5′-TTTCAGATGTGGCAATTCA GATA-3′; reverse primer, 5′-AAGTAGGAGAGGGTTGAGCC-3′. The following sequences were used for the full-length $P2X_7$: forward primer, 5′-ATACAGTTTCCGTCGCCTTG-3′; reverse primer, 5′-AACGGATC CCGAAGACTTTT-3′, as described (32). The PCR cycling protocol for all primers was 94°C at 45 s, 60°C at 45 s, and 72°C at 45 s. The protocol was repeated for 40 cycles and included an initial 10-min enzyme activation step at 95°C and a final 10-min extension step at 72°C. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. PBLs were used as a positive control for the expression of $P2X_7R$.

Real-time PCR for $P2X_7R$ mRNA from HeLa cells

RNA from HeLa cells was isolated using an RNeasy kit (Qiagen) following the manufacturer's instructions. Total RNA was converted into cDNA by standard reverse transcription with Superscript II Rnase H^- reverse transcriptase in the manufacturer's buffer (Invitrogen Life Technologies). Quantitative PCR was performed with 1/50 of the cDNA preparation in the Mx3000P (Stratagene) in a 25-μl final volume with Brilliant QPCR Master Mix (Stratagene). cDNA was amplified using 300 nM $P2X_7R$ forward primer and 100 nM $P2X_7R$ reverse primer, or 400 nM GAPDH forward and reverse primers. We also used 300 nM fluorogenic oligonucleotides specific for the gene segments in which a reporter fluorescent dye on 5′ (FAM) and a quencher dye on 3′ (TAMRA) were attached. For GAPDH, the primers used were as follows: forward primer, 5′-GAGAAGGCT GGGGCTCAT-3′; reverse primer, 5′-TGCTGATGATCTTGAGGCTG-3′; probe, 5′-CTCTGCTGATGCCCCCATGTTCGT-3′. For $P2X_7R$, the primers used were as follows: forward primer, 5′-CTTTCTCAAAACA GAAGGCCAAGA-3′; reverse primer, 5′-CAACCTCGGTCAGAGGAA CAGA-3′; probe, 5′-TGTGTCCCGAGTATCCCACCCGC-3′. Real-time PCR was conducted at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s and one cycle at 60°C for 1 min. The sp. act. of cDNA from $P2X_7R$ were compared with GAPDH, and normalized with respect to untreated control HeLa cells by the comparative cycle threshold method, as described by the manufacturer (Stratagene).

Measurement of infectious activity of chlamydiae

To measure effects of extracellular nucleotides on survival of intracellular chlamydiae, HeLa or CaSki cells that had been infected with *C. muridarum* at a multiplicity of infection of 0.75 for 24 h were incubated with ATP, ATP analogues, or nucleotides with inhibitors in serum-free DMEM for 2 h at 37°C in 5% CO_2, and the medium was then replaced by fresh cell culture medium. The infection was allowed to proceed for an additional 4 h at 37°C in a 5% CO_2 humidified incubator. Alternatively, infected HeLa cells were incubated with the $P2X_7R$ antagonist, oATP, for 2 h, and oATP was then removed from the medium before ATP was added for an additional 2 h. The medium was replaced, and infection was then allowed to proceed for an additional 4 h, as above. Except where noted otherwise, PLD-dependent generation of phosphatidic acid was inhibited by incubating infected HeLa cells with butan-1-ol (0.03–0.3%) for 15 min at 37°C before the addition of ATP for an additional 2 h (26). Fresh culture medium was then added, and the infection was allowed to proceed for an additional 4 h. In all cases, the cells and supernatant were combined and centrifuged for 60 min at 13,000 rpm in a Jouan MR1822 rotor. The pellet was resuspended in ice-cold culture medium with a 21-gauge 2-ml syringe to dissociate aggregates, giving the final suspension of *Chlamydia* used to reinfect HeLa cells. Serial dilutions of the chlamydial preparation from each well were used to reinfect HeLa cells on coverslips for 24 h, as described (22). The chlamydial vacuoles were detected using an anti-*Chlamydia* genus mAb (1/400 dilution; Argene), followed by FITC-conjugated anti-mouse IgG plus IgM (1/200 dilution; Argene). Samples were examined with a Leica fluorescence microscope attached to a camera ORCA-ER (Nikon). Images were acquired with Lucia v4.5 software and analyzed with Adobe Photoshop software. *Chlamydia* inclusions were identified by fluorescence staining. At least 10 separate fields containing an average of 200–300 HeLa cells were counted per sample, and the experiment was repeated on two or three separate occasions.

Infection of wild-type and $P2X_7R$-deficient mice

Mice received 2.5 mg of medroxyprogesterone acetate (Depo-Provera; Pfizer) in 0.1 ml of saline s.c. 7 days before vaginal infection. Mice anesthetized with sodium pentobarbital were infected by placing 30 μl of 250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid containing 1.0×10^7 inclusion-forming units (IFU) of McCoy cell-grown *C. muridarum* (1500 ID_{50}) into the vaginal vault. Infection was monitored by enumeration of IFUs from cervicovaginal swabs (Spectrum Medical Industries) obtained at various times after infection (39). Mice were infected in groups of five, and each experiment was repeated at least once.

Measurement of cytokine secretion in infected wild-type and $P2X_7R$-deficient mice

Genital tract secretions collected from mice on multiple days throughout the course of infection were analyzed by ELISA for cytokines of interest.

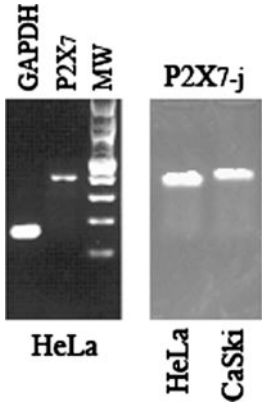

FIGURE 1. $P2X_7R$ and truncated $P2X_7R$ mRNA are expressed in HeLa cells. *Left panel*, Gel analysis of RT-PCR products from HeLa cells. PCR amplification of a constitutively expressed control mRNA encoding GAPDH was used as a control for the presence of cDNA in the reactions. Primers detecting full-length and truncated $P2X_7R$ detected a band of the expected size. *Right panel*, mRNA for truncated P2X7 ($P2X_{7\text{-}j}$) was detected in both HeLa and CaSki cells. All PCR amplifications were performed at least three times.

At intervals before and after infection, an aseptic surgical sponge (ear wicks, 2 × 5 mm) (DeRoyal) was inserted into the vagina of an anesthetized mouse and retrieved 30 min later. Samples were eluted from the sponges, as described (39), and eluates were analyzed individually for IL-1β using a commercial cytokine ELISA kit (R&D Systems).

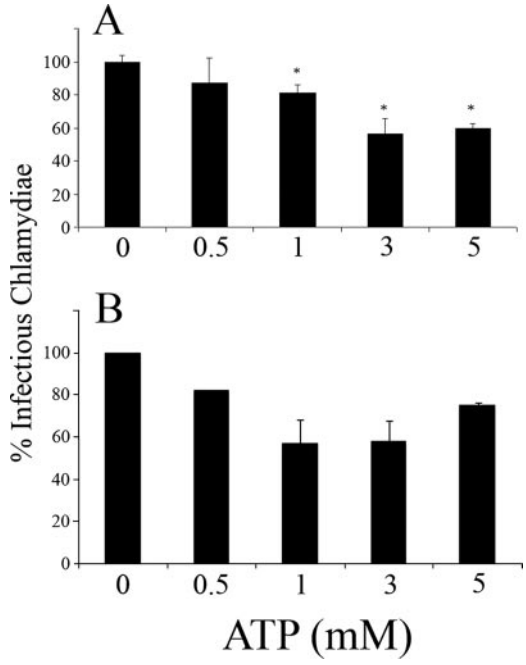

FIGURE 2. ATPe inhibits survival of intracellular chlamydiae. HeLa cells (*A*) or CaSki cells (*B*) were infected with *C. muridarum* for 24 h, and then incubated for 2 h with the indicated concentration of ATPe. The medium was replaced with cell culture medium, and the infection was allowed to proceed for an additional 4 h. The chlamydiae in cells and supernatant were collected, and the relative infectious activity was titrated on HeLa cells, as described in *Materials and Methods*. ∗, $p < 0.01$ for 1, 3, and 5 mM ATP, compared with 0 mM ATP in HeLa cells. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments.

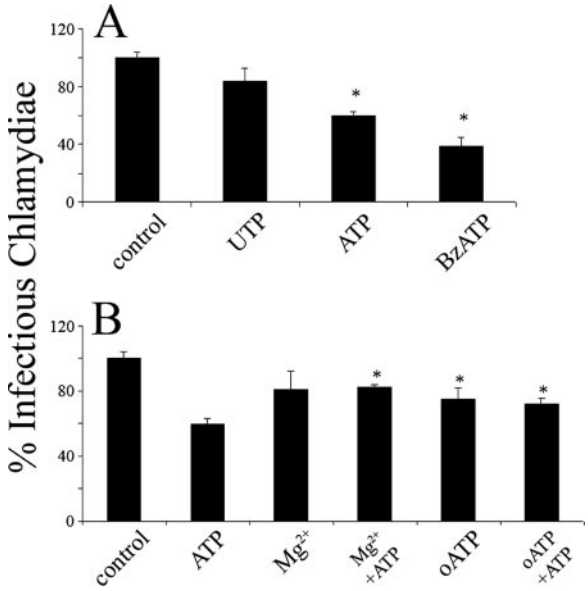

FIGURE 3. $P2X_7R$ agonists inhibit intracellular chlamydiae, and these effects are blocked by $P2X_7R$ antagonists. HeLa cells were infected with *C. muridarum* for 24 h and then incubated for 2 h with the indicated nucleotides. After an additional incubation for 4 h at 37°C in the absence of nucleotides, the chlamydiae in cells and supernatant were collected, and the relative infectious activity was titrated on HeLa cells, as described in *Materials and Methods*. *A*, The $P2X_7R$ agonist BzATP inhibits survival of chlamydiae. HeLa cells were incubated with ATP (5 mM), UTP (5 mM), or BzATP (0.5 mM), as described in *Materials and Methods*. ∗, $p < 0.01$ for ATP, BzATP, compared with control. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments. *B*, The reversible $P2X_7R$ antagonist, Mg^{2+}, and an irreversible antagonist, oATP, block the chlamydial inhibitory effects of ATP in HeLa cells. HeLa cells were incubated with 5 mM ATP alone, 10 mM $MgCl_2$ alone, 5 mM ATP plus 10 mM $MgCl_2$, 0.3 mM oATP alone, or 0.3 mM oATP for 2 h, followed by 5 mM ATP for 2 h. ∗, $p < 0.01$ for Mg^{2+} plus ATP, oATP, oATP plus ATP, compared with ATP alone. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments.

Histopathology

Mice were sacrificed on days 10, 17, and 42 after infection, and the entire genital tract was removed en bloc, fixed in 10% buffered formalin, and embedded in paraffin. Longitudinal 4-μm sections were cut, stained with H&E, and evaluated by a pathologist blinded to the experimental design. Each anatomic site (exocervix, endocervix, uterine horn, oviduct, and mesosalpinx) was assessed independently for the presence of acute inflammation (neutrophils), chronic inflammation (lymphocytes), plasma cells, and fibrosis. Luminal distention of the uterine horns and dilatation of the oviducts were graded from 1 to 4, with grade 4 representing severe hydrosalpinx. Right and left uterine horns and right and left oviducts were evaluated individually. A four-tiered semiquantitative scoring system was used to quantitate the inflammation and fibrosis: 0 = normal; 1^+ = rare foci (minimal presence) of parameter; 2^+ = scattered (1–4) aggregates or mild diffuse increase in parameter; 3^+ = numerous aggregates (>4) or moderate diffuse or confluent areas of parameter; 4^+ = severe diffuse infiltration or confluence of parameter.

Statistics

The effects of ATPe, the $P2X_7R$ agonists and antagonists, and PLD inhibitor on the survival of intracellular chlamydiae were evaluated statistically using Student's *t* test. Statistical comparisons between the wild-type and $P2X_7R$-deficient mice for level of infection and cytokine production over the course of infection were made by two-way ANOVA with post hoc Tukey test as a multiple comparison procedure. The Wilcoxon rank sum test was used to compare the duration of infection in the respective strains over time. The Kruskal, Wallis one-way ANOVA on ranks was used to determine significant differences in the pathological data between groups.

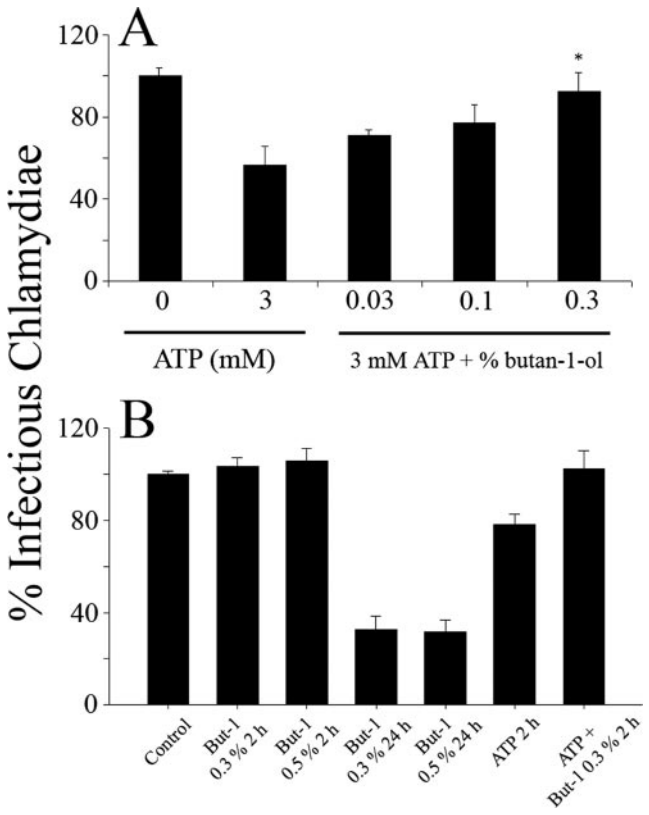

FIGURE 4. ATPe-induced chlamydial inactivation requires host-cell PLD activation. *A*, HeLa cells were infected for 24 h and then incubated in the presence or absence of the indicated concentration of butan-1-ol for 15 min. ATP (3 mM) was then added for 2 h, the medium was replaced with cell culture medium, and the infection was allowed to proceed for an additional 4 h. The chlamydiae in cells and supernatant were collected and titrated on HeLa cell monolayers. *, $p < 0.01$ for 3 mM ATP with 0.3% butan-1-ol, compared with 3 mM ATP alone. Values represent the mean and SD of two experiments. *B*, HeLa cells were infected for 24 h and then incubated in the presence or absence of 0.3 or 0.5% butan-1-ol (But-1) for 2 or 24 h. Infected cells were harvested at 48 h postinfection, and infectious chlamydiae were titrated. Alternatively, 3 mM ATP was added to 24-h infected cells for 2 h in the presence or absence of 0.3% butan-1-ol, the medium was replaced with cell culture medium, and the infection was allowed to proceed for an additional 22 h. The chlamydiae in cells and supernatant were collected, and the relative infectious activity was titrated. *, $p < 0.05$ for 3 mM ATP with 0.3% butan-1-ol, compared with 3 mM ATP alone. Values represent the mean and SD of two experiments.

The *z* test for determination of significant differences in sample proportions was used to compare frequencies of pathological findings between specific groups. SigmaStat software was used (SPSS).

Results

$P2X_7R$ mRNA is detected in HeLa cells, and is not affected by infection with C. muridarum

Prior studies revealed that primary human cervical epithelial cells express normal $P2X_7R$ protein, but the endocervical epithelial cell line, HeLa 229, was found to express high levels of a truncated version of $P2X_7R$ protein, referred to as $P2X_{7\text{-j}}$ (32). Because $P2X_7R$ gene expression in HeLa cells was not investigated, we therefore analyzed the levels of $P2X_7R$ mRNA in HeLa cells by RT-PCR. PBLs were used as a positive control for $P2X_7R$ expression (13). A positive cDNA band for $P2X_7R$ was found in HeLa cells (Fig. 1, *left panel*). The truncated $P2X_7R$ protein was previously described in HeLa cells (32), although the $P2X_{7\text{-j}}$ mRNA was isolated from another cervical cell line, CaSki. To confirm that

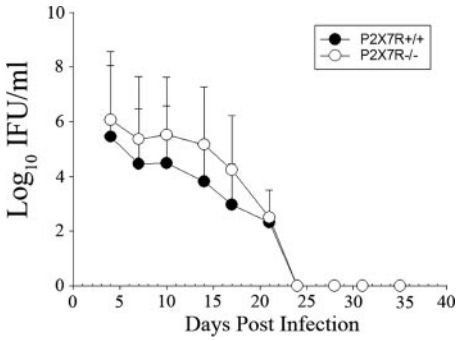

FIGURE 5. The intensity, but not the duration of in vivo lower genital tract infection, is increased by $P2X_7R$ deficiency. Quantitative isolations from cervicovaginal swabs obtained after infection with *C. muridarum* in wild-type and $P2X_7R$-deficient mice. Data points represent means ± SEM of duplicate determinations of $\log_{10}$ IFU/ml for infected animals on each day. ●, Wild-type mice; ○, $P2X_7R$-deficient mice. $p < 0.001$ by two-factor ANOVA for intensity of infection during the first 22 days postinoculation in wild-type vs $P2X_7R$-deficient mice. A representative experiment of two is shown with $n = 5$ mice per strain per experiment.

HeLa cell-truncated $P2X_7R$ protein may in fact be due to translation of $P2X_{7\text{-j}}$ mRNA, we verified the presence of $P2X_{7\text{-j}}$ in HeLa cells using primers that detect only the truncated form, as previously described (32). As expected, CaSki cells express $P2X_{7\text{-j}}$, which was also found in HeLa cells (Fig. 1, *right panel*). Using primers that detect only the full-length $P2X_7R$ mRNA (32), amplicons were detected in both HeLa and CaSki cells (data not shown). To determine whether the relative level of gene expression for $P2X_7R$ may change during chlamydial infection, we quantified mRNA for $P2X_7R$ by real-time PCR. HeLa cells infected with *C. muridarum* for 2, 4, 8, 16, or 24 h expressed the same amount of $P2X_7R$ mRNA as uninfected cells (data not shown), indicating that infection had no effect on the level of $P2X_7R$ gene expression.

ATPe decreases the viability of intracellular chlamydiae in HeLa cells

We have previously shown that ATPe stimulates killing of intracellular chlamydiae within infected macrophages (22). To determine whether ATPe might have a similar effect against intracellular chlamydiae in epithelial cells, we infected HeLa cells with *C. muridarum* for 24 h. We then incubated the infected cells with the indicated concentration (0.5–5 mM) of ATPe for 2 h and, after incubating the infected cells for an additional 4 h at 37°C in the absence of ATPe, harvested the bacteria from the supernatant and adherent infected cells. Infectious activity was then titrated on a new monolayer of HeLa cells, as described in *Materials and Methods*. Incubation of infected HeLa cells with 5 mM ATP led to a 40% (range 37–43%) inhibition of chlamydial infectious activity (Fig. 2*A*).

A previous report showed that ATPe, via stimulation of the $P2X_7R$, can trigger apoptosis of human cervical epithelial cells (31), although the HeLa cell line expresses high levels of a truncated $P2X_7R$ protein that makes it resistant to ATPe-induced apoptosis (32). The chlamydial inactivation induced by ATPe was observed within 6 h postincubation, at which time there was no measurable HeLa cell death (data not shown). As we observed previously for macrophages (22), killing of chlamydiae in HeLa cells was therefore dissociable from ATPe-induced host-cell death.

Although both truncated and full-length $P2X_7R$ protein were previously found in both HeLa and CaSki cells, higher levels of the the truncated protein were observed in HeLa cells (32). Because

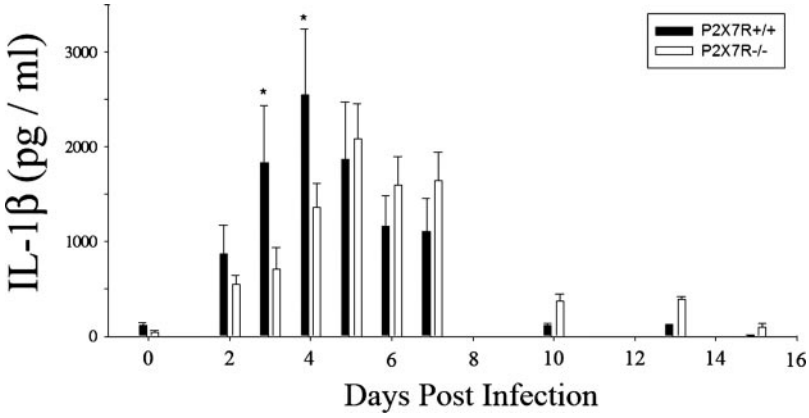

FIGURE 6. Secretion of IL-1β is decreased early in infection, but the overall IL-1β response is not affected by $P2X_7R$ deficiency. Wild-type and $P2X_7R$-deficient mice were vaginally infected with *C. muridarum*, and IL-1β levels in genital tract secretions were measured by ELISA, as described in *Materials and Methods*. The overall response was not different between the groups ($p = 0.36$ by two-way ANOVA). ■, Wild-type mice; □, $P2X_7R$-deficient mice. *, $p < 0.05$ for wild-type vs $P2X_7R$-deficient mice on days 3 and 4 by posthoc analysis (Tukey). A representative experiment of three is shown.

CaSki cells express mostly full-length $P2X_7R$ protein, we also investigated whether ATPe may affect chlamydial infection in these cells. Chlamydiae were able to infect CaSki cells at comparable levels to HeLa cells (data not shown), but 1 mM ATP had a higher inhibitory effect on chlamydial infection in CaSki cells than in HeLa cells (Fig. 2*B*). For both HeLa cells and CaSki cells, the ability to stimulate $P2X_7R$ decreased at ATP concentrations greater than 5 mM, as previously observed for ATP-induced apoptosis of macrophages, which decreases at high ATP concentrations (40).

$P2X_7R$ agonists inhibit chlamydiae, and inhibition is blocked by $P2X_7R$ antagonists

ATPe-induced chlamydial killing has been attributed to $P2X_7R$ in macrophages (22). To evaluate whether $P2X_7R$ is responsible for killing of chlamydiae in epithelial cells, the effect of various agonists and antagonists of the $P2X_7R$ on the viability of intracellular chlamydiae was investigated. The nucleotide UTP is able to engage other members of the P2R family, but not $P2X_7R$ (41). As shown in Fig. 3*A*, UTP did not have an appreciable effect on the survival of chlamydiae. BzATP is a selective agonist of the $P2X_7R$ (41, 42), and Fig. 3*A* shows that it induces inactivation of chlamydiae in HeLa cells. In fact, 0.5 mM BzATP, which is a more selective agonist for $P2X_7R$ than ATP and in some cases is more potent than ATP (43), can induce chlamydial killing better than 5 mM ATP. Conversely, the $P2X_7R$ antagonist, oATP, which can block ATPe-dependent stimulation of $P2X_7R$, inhibits irreversibly ATPe-dependent chlamydial killing (Fig. 3*B*). Because the active form of ATP that binds the $P2X_7R$ is the tetrabasic ATP^{4-}, the receptor is also antagonized by coincubation with Mg^{2+}, which chelates ATP^{4-}. Consistent with these findings, ATPe-induced chlamydial inactivation is inhibited by Mg^{2+} (Fig. 3*B*).

ATP results in inhibition of chlamydial infection due to host-cell PLD activation

$P2X_7R$-dependent PLD activation has been shown to promote killing of intracellular chlamydiae and mycobacteria in macrophages (22, 23, 26). To determine whether ATPe-induced PLD activation is responsible for killing of intracellular chlamydiae in HeLa cells, the effect of a PLD inhibitor on the survival of chlamydiae was evaluated.

PLD is an enzyme that mainly hydrolyzes phosphatidylcholine into phosphatidic acid and choline. If cells are pretreated with butan-1-ol, PLD preferentially uses the primary alcohol as a substrate, producing the nonsignaling phosphatidylbutanol instead of phosphatidic acid. Incubation of infected HeLa cells with butan-1-ol for 15 min at 37°C, before addition of 3 mM ATP for 2 h,

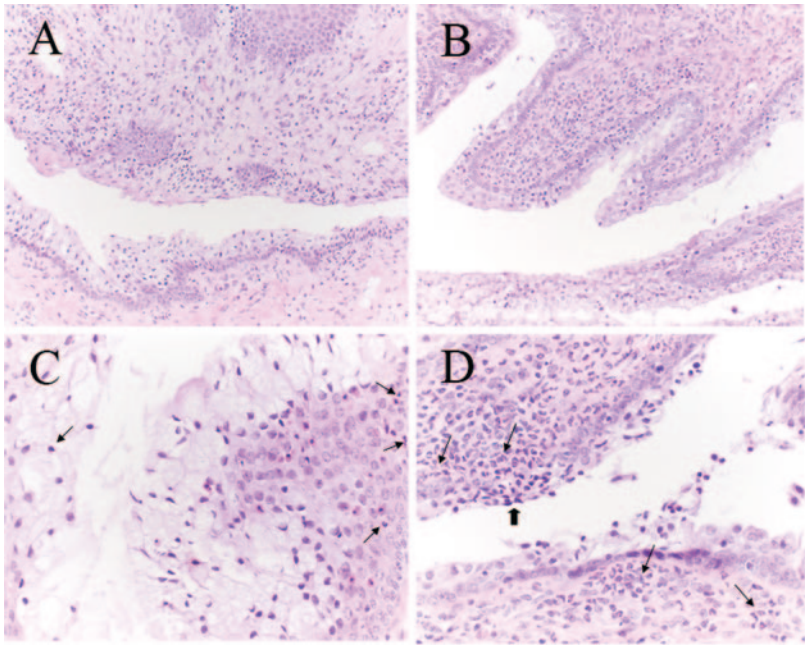

FIGURE 7. A higher degree of neutrophil infiltrates is observed in endocervical tissues of infected $P2X_7R$-deficient mice compared with infected wild-type mice 10 days postinfection. Low power (×10) magnification of representative endocervical tissue of an infected wild-type mouse (*A*) and infected $P2X_7R$-deficient mouse (*B*) on day 10 of infection. The overall density of acute inflammatory cells within and beneath the endocervical epithelium is increased in the infected $P2X_7R$-deficient mouse tissue. High power (×40) magnification of representative endocervical tissue of an infected wild-type mouse (*C*) and infected $P2X_7R$-deficient mouse (*D*) on day 10 of infection. Arrows in *C* point to scattered neutrophils present in the wild-type mouse tissue, and to groups of neutrophils in the $P2X_7R$-deficient mouse tissue (*D*). The thick arrow in *D* indicates an area of eroded epithelium in the $P2X_7R$-deficient mouse tissue.

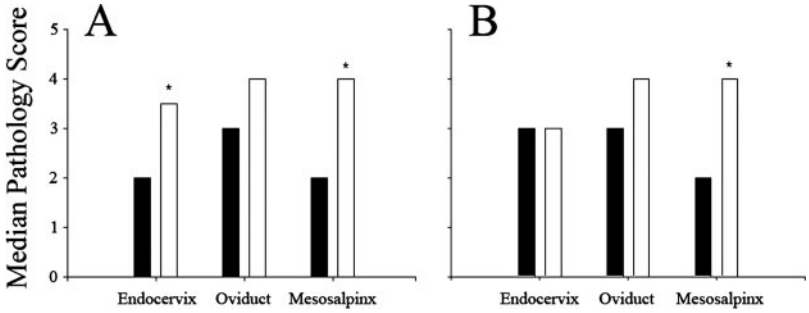

FIGURE 8. Inflammation is increased, but chronic oviduct pathology is not enhanced in $P2X_7R$-deficient mice. Wild-type and $P2X_7R$-deficient mice were vaginally infected with *C. muridarum*. The mice were sacrificed after 10 days of infection, and median pathology scores in the genital tract were measured semiquantitatively, as described in *Materials and Methods*. Levels of acute (*A*) and chronic (*B*) inflammatory cells were scored in tissues from wild-type (■) and $P2X_7R$-deficient (□) mice. *, $p < 0.05$ for wild-type vs $P2X_7R$-deficient mice by one-way ANOVA.

resulted in concentration-dependent inhibition of chlamydial killing (Fig. 4*A*), suggesting that ATPe-induced PLD activity is responsible for inhibition of infection.

Several species of *Chlamydia* express a family of plasticity zone PLD enzymes (44). Incubation of infected cells with 0.5% butan-1-ol during the last 24 h of infection with *C. trachomatis* inhibited infection, suggesting a role for the plasticity zone PLD in late stages of development (44). Because the primary alcohol could affect either host-cell PLD or chlamydial plasticity zone PLD, we incubated infected cells for 24 h, treated with 0.3 or 0.5% butan-1-ol for 2 or 24 h, and then measured viable chlamydiae at 48 h postinfection by titrating the bacteria on a fresh monolayer of HeLa cells. Although a 24-h incubation with the primary alcohol inhibited infection dramatically, in agreement with previous results (44), the effect of 2-h incubation with butan-1-ol was negligible (Fig. 4*B*). However, when 24-h infected cells were incubated with 3 mM ATP for 2 h, there was a significant reduction in the number of viable chlamydiae recovered. Moreover, there was no longer an inhibitory effect of ATP on chlamydial growth when 24-h infected cells were coincubated with 3 mM ATP and 0.3% butan-1-ol for 2 h (Fig. 4*B*). These results suggest that the 2-h incubation of infected cells with ATP results in inhibition of chlamydial infection due to host-cell PLD activation.

$P2X_7R$ deficiency results in a higher infectious burden in vivo

Given the ability of $P2X_7R$ ligands to inhibit chlamydial infection in HeLa cells (Fig. 5), we investigated whether $P2X_7R$ could influence chlamydial mouse genital tract infection. Wild-type and $P2X_7R$-deficient mice were infected vaginally with *C. muridarum*, and the efficiency of lower genital infection was followed as a function of time. The infectious burden was 10-fold higher in $P2X_7R$-deficient mice than in wild-type mice, but the duration of infection was not affected by $P2X_7R$ deficiency (Fig. 5). Although the effect of $P2X_7R$ deficiency was partial, it was statistically significant ($p < 0.001$ by two-way ANOVA) and is consistent with the partial effect of ATPe on infection in HeLa cells.

$P2X_7R$ deficiency results in inhibition of early IL-1β secretion in infected mice

ATPe has also been identified as a second signal that induces secretion of IL-1β and IL-18 from cells that had been stimulated with a PAMP. Stimulation of cells with PAMP alone leads to synthesis and intracellular accumulation of pro-IL-1β, but subsequent stimulation with ATPe results in caspase-1 activation and secretion of mature IL-1β (11, 16–18). IL-1β secretion was therefore measured in genital secretions from wild-type and $P2X_7R$-deficient mice that had been infected with *C. muridarum*. Two-way ANOVA revealed no difference in the IL-1β response between the two groups over time; however, on days 3 and 4 of infection, a significant increase was detected in wild-type mice by multiple comparison analysis (Tukey). On later days, IL-1β levels in $P2X_7R$-deficient mice slightly exceeded those of wild-type mice (Fig. 6). These results suggest that although $P2X_7R$ stimulation may contribute to IL-1β secretion in vivo, much of the IL-1β produced occurs independently of $P2X_7R$ activation and relates directly to the degree of local infection.

$P2X_7R$ deficiency leads to enhanced inflammation early during infection, but has no effect on chronic oviduct pathology

Histological examination of genital tract tissues taken from mice sacrificed on day 10 of infection revealed a higher level of acute inflammation in the endocervix, oviduct, and mesosalpingeal tissues of the $P2X_7R$-deficient mice vs wild-type mice (Figs. 7 and 8), and increased numbers of chronic inflammatory cells in the oviduct and mesosalpingeal tissues. This increase in inflammation agrees with the increased level of infection detected in the $P2X_7R$-deficient mice (Fig. 5). Despite the 10-fold increase in infectious burden seen through day 17 in $P2X_7R$-deficient mice, the frequency and severity of chronic oviduct pathology were not enhanced (12 of 20 wild-type oviducts and 13 of 20 $P2X_7R$-deficient oviducts exhibited moderate to severe oviduct dilation) (Fig. 8).

Discussion

In this study, we show that $P2X_7R$ ligation leads to inhibition of chlamydial infection in a cervical epithelial cell line. $P2X_7R$ also affects the efficiency of infection in vaginally infected mice. The effects observed were partial, but may underestimate the role played by $P2X_7R$ in controlling chlamydial infection in humans. Thus, $P2X_7R$ deficiency has been reported to have no effect on *M. tuberculosis* infection in mice (27), but polymorphisms in the $P2X_7R$ gene and its expression have been associated with resistance against tuberculosis or progression of disease in humans (29, 45, 46). Part of the discrepancy may be due to the finding that the strain of mice used in this study and to study *M. tuberculosis* infection (27) are on the C57BL/6 genetic background. These mice, along with the DBA/2 strain, carry a P451L mutation in a cytosolic domain of $P2X_7R$ that is absent in wild mice, rats, or humans (47). The P451L mutation results in a large decrease in the activity of $P2X_7R$ (47). Similarly, the preferred cell line used in this study and in most *Chlamydia* studies, the cervical epithelial carcinoma, HeLa, does not undergo apoptosis following ATP incubation, unlike primary cervical epithelial cells from humans, which are sensitive to ATP-induced apoptosis (31, 32). The difference between HeLa and primary epithelial cells has been ascribed to a truncated variant of $P2X_7R$ expressed in HeLa, which shows diminished ATP-binding and $P2X_7R$-dependent signaling activity, compared with the full-length $P2X_7R$ expressed exclusively in most humans (32). Because the activity of $P2X_7R$ depends on oligomerization of the receptor (42), it has been proposed that hetero-oligomerization between full-length and truncated variants of $P2X_7R$ results in lower activity of $P2X_7R$ in HeLa cells

(32). We thus expect that $P2X_7R$ should play a larger role in controlling infection by *C. trachomatis* in primary cells and humans than observed by us in this study. Furthermore, given the small effect of extracellular UTP on chlamydial infection in HeLa cells, we cannot exclude the possibility that several nucleotides released simultaneously at sites of infection may also stimulate other purinergic receptors expressed on infected cells.

Previous studies with macrophages have shown that ATPe ligation of $P2X_7R$ leads to a large increase in the activity of PLD (25), and that ATPe inhibits macrophage infection by mycobacteria and chlamydiae by activating host-cell PLD, which leads to fusion between lysosomes and the vacuoles harboring the pathogens (22, 23, 26, 48). Chlamydiae also express PLD orthologs during infection of HeLa cells, and infection can be inhibited by incubating infected cells with PLD inhibitors (primary alcohols) for 24 h or more (44). To determine whether the PLD inhibitors affect host-cell PLD or chlamydial PLD in our experiments, we confirm in this work that treatment of infected cells with primary alcohols for 24 h blocks chlamydial infection, but also show that a 2-h incubation with the PLD inhibitor, the same condition as used in our previous study (22), does not affect the efficiency of chlamydial infection in untreated HeLa cells. Interestingly, coincubation with alcohol and ATP for 2 h reverses significantly the inhibition of chlamydial infection due to ATPe treatment, suggesting that short incubation with the PLD inhibitor affects mainly host-cell PLD. Taken together, these results suggest that blocking the activity of either chlamydial or host-cell PLD inhibits infection by chlamydiae.

Besides inhibiting intracellular infection directly, $P2X_7R$ is also involved in activation of caspase-1 and subsequent processing and secretion of IL-1β (16–19). IL-1β is a proinflammatory cytokine released upon invasion of the host by multiple bacterial pathogens. Thus, IL-1β is produced by ovine alveolar macrophages infected with *Chlamydia caviae* (49) and human monocytes or dendritic cells infected with *C. trachomatis* or *C. caviae* (50–52). In turn, IL-1β inhibits chlamydial growth through induction of IDO activity and enhancement of IFN-mediated inhibition of chlamydial growth (53).

Ligation of TLR is sufficient for transcription, synthesis, and secretion of cytokines such as IL-8, IFN-γ, and IL-12 (7, 8). But the production and secretion of IL-1β typically require two separate signals (11, 16–18, 54). The first signal, from a PAMP such as LPS, promotes production and intracellular accumulation of the immature cytokines. The second signal, provided by extracellular danger signals such as ATP or uric acid, results in activation of an inflammasome containing Nalp3 (from the nucleotide-binding oligomerization domain family of proteins), which in turn activates caspase-1 and leads to processing and secretion of the mature cytokines (11, 16, 18). Unlike most pathogens studied to date, it was thought that *Chlamydia* may activate caspase-1 directly, in the absence of an exogenous danger signal, during infection of monocytes, dendritic cells, or epithelial cells in vitro (51, 52, 55, 56). *Salmonella* and *Shigella* were also believed to activate caspase-1 directly, via secretion of the type III secretion-dependent substrates, SipB and IpaB; and it had been proposed that *Chlamydia* may express homologues of SipB and Ipa B (56). However, more recent data have demonstrated that activation of caspase-1 by *Shigella* and *Salmonella* infection involves an inflammasome containing the nucleotide-binding oligomerization domain family member, Ipaf (57). Thus, chlamydiae could also activate caspase-1 through induction of an inflammasome, despite type III secretion and independently of $P2X_7R$ ligation.

We have addressed this issue by measuring IL-1β secretion from $P2X_7R$-deficient and wild-type mice infected vaginally with *C. muridarum*. Our results show that the absence of $P2X_7R$ leads to lower levels of IL-1β secretion mainly at early times of infection, but may have the opposite effect at longer times of infection. Although ATP can induce caspase-1 activation and IL-1β secretion, we cannot rule out the possibility that other danger signals, such as uric acid (10), may also influence IL-1β secretion during chlamydial infection. Nonetheless, the observation that chlamydial infection, in the absence of exogenous ATP, can induce caspase-1 activation in vitro (51, 52, 55, 56) suggests that the higher levels of infection in $P2X_7R$-deficient mice could account for the more efficient IL-1β secretion in vivo at longer times. In aggregate, these results suggest that $P2X_7R$ may exert most of its effect in vivo by directly inhibiting chlamydial infection in infected cells, rather than by modulating infection via IL-1β secretion.

Disclosures

The authors have no financial conflict of interest.

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