Immunity, Vol. 19, 403–412, September, 2003, Copyright ©2003 by Cell Press

Inhibition of Chlamydial Infectious Activity due to $P2X_7R$-Dependent Phospholipase D Activation

Robson Coutinho-Silva,[1,2,3] Lynn Stahl,[1] Marie-Noëlle Raymond,[4] Thomas Jungas,[1] Philippe Verbeke,[1] Geoffrey Burnstock,[2] Toni Darville,[5] and David M. Ojcius[1,*]

[1]Université Paris 7
Institut Jacques Monod
CNRS UMR 7592
2 place Jussieu
75251 Paris cedex 5
France

[2]Autonomic Neuroscience Institute
Department of Anatomy and Developmental Biology
Royal Free and University College Medical School
London NW3 2PF
United Kingdom

[3]Instituto de Biofisica Carlos Chagas Filho
Universidade Federal do Rio de Janeiro
21949-900 Rio de Janeiro
Brazil

[4]Université Paris-Sud
Laboratoire de l'activation cellulaire et transduction des signaux
UMR8619
91405 Orsay
France

[5]Department of Microbiology and Immunology
University of Arkansas for Medical Sciences
Little Rock, Arkansas 72205

Summary

Chlamydia trachomatis **survives within host cells by inhibiting fusion between *Chlamydia* vacuoles and lysosomes. We show here that treatment of infected macrophages with ATP leads to killing of chlamydiae through ligation of the purinergic receptor, $P2X_7R$. Chlamydial killing required phospholipase D (PLD) activation, as PLD inhibition led to rescue of chlamydiae in ATP-treated macrophages. However, there was no PLD activation nor chlamydial killing in ATP-treated $P2X_7R$-deficient macrophages. $P2X_7R$ ligation exerts its effects by promoting fusion between *Chlamydia* vacuoles and lysosomes. $P2X_7R$ stimulation also resulted in macrophage death, but fusion with lysosomes preceded macrophage death and PLD inhibition did not prevent macrophage death. These results suggest that $P2X_7R$ ligation leads to PLD activation, which is directly responsible for inhibition of infection.**

Introduction

Chlamydia trachomatis are obligate intracellular bacteria that cause ocular and genital tract infection. Consequences of chronic disease include blindness and female infertility. Occasionally, the organism disseminates from the genital tract to bony joints and causes reactive arthritis (Schachter, 1988). The sexually transmitted biovars of *C. trachomatis* infect superficial columnar epithelial cells of the genital tract, but some strains can also infect other cells, including macrophages (La Verda and Byrne, 1994; Moulder, 1991).

All *Chlamydia* species have a characteristic developmental cycle involving two morphologically and functionally distinct forms (Bavoil et al., 2000; Hackstadt, 1999; Moulder, 1991; Wyrick, 2000). The elementary bodies (EB) are metabolically inert and infectious. Following uptake by epithelial cells or macrophages into small entry vacuoles, the EB differentiate into metabolically active reticulate bodies (RB), which proliferate within the same membrane-bound vacuole, called an inclusion. The chlamydiae survive within the host cell by inhibiting fusion between entry vacuoles and lysosomes, through as-yet-uncharacterized mechanisms. After several rounds of division, the RB redifferentiate into EB. The EB exit from the host cell and begin a new cycle of infection in neighboring cells.

There are no unifying rules for predicting how well *Chlamydia* may survive and replicate in macrophages. Nonetheless, in general, the oculogenital biovars of *C. trachomatis* do not appear to grow within any type of macrophage, while the lymphogranuloma venereum (LGV) biovar grows in unactivated but not activated macrophages. The 6BC avian strains of *C. psittaci* proliferate in unactivated but not activated macrophages, while the guinea pig inclusion conjunctivitis (GPIC) strain is not believed to proliferate in any type of macrophage (La Verda and Byrne, 1994). Interferon-γ produced at sites of infection inhibits *Chlamydia* infection via host-cell indoleamine 2,3-dioxygenase activity, which depletes host-cell concentrations of tryptophan (Beatty et al., 1994), but the molecular basis for control of chlamydial growth by macrophages remains to be fully characterized.

Chlamydiae and their nucleic acids have been detected in synovial tissues of patients with reactive arthritis, and the macrophage is the main host cell where persistent forms are found (Gerard et al., 1998a, 2002; Nanagara et al., 1995; Taylor-Robinson et al., 1992). One potential method of transport of chlamydiae from the genital epithelium to extragenital sites is via peripheral blood monocytes. *C. trachomatis* serovar K survives in a persistent, transcriptionally active, but nonreplicative form in human monocytes in vitro (Gerard et al., 1998b; Koehler et al., 1997). Intraperitoneal innoculation of mice with the mouse pneumonitis (MoPn) biovar of *C. trachomatis* leads to spreading of the infection to liver, lung, and spleen. At the same time, the infection induces NOS in peritoneal macrophages, and an inhibitor of NOS causes the intensity and duration of the infection to increase significantly (Khatsenko et al., 1998). Likewise, in vaginally infected mice, dissemination of the bacteria to spleen and lungs is greater in iNOS-deficient mice than in wild-type mice (Igietseme et al., 1998), and immunosuppressive treatment after apparent resolution of genital tract infection leads to recrudescence of infection in iNOS-deficient mice but not in wild-type mice

*Correspondence: ojcius@ijm.jussieu.fr

(Ramsey et al., 2001). These results suggest that macrophages are involved in dissemination of infection from the genital tract to remote sites and in establishment of persistent infection.

Extracellular ATP (ATP_e) is thought to be produced at sites of inflammation (Di Virgilio, 1995). In vitro, ATP_e has been shown to modify survival of another intracellular pathogen that inhabits macrophages, *Mycobacterium tuberculosis*. ATP_e interacts with purinergic $P2X_7$ receptors ($P2X_7R$), which are expressed on the surface of macrophages and dendritic cells (DC) (Coutinho-Silva et al., 1999; Di Virgilio, 1995, 2001; Mutini et al., 1999). Engagement of the $P2X_7R$ leads to macrophage and DC death, and concomitantly maturation and secretion of IL-1β (MacKenzie et al., 2001; Solle et al., 2001). ATP_e-induced death of *M. tuberculosis*-infected macrophages is associated with killing of the intracellular mycobacteria (Fairbairn et al., 2001; Kusner and Adams, 2000; Kusner and Barton, 2001; Lammas et al., 1997; Molloy et al., 1994; Stober et al., 2001). In studies with monocytes infected with bacillus Calmette-Guerin, both H_2O_2 and ATP_e killed the monocytes, but only ATP_e treatment killed the mycobacteria (Molloy et al., 1994). In a comparison with other ligands that can trigger death of macrophages, including complement-mediated cytolysis, Fas ligation, and CD69 activation, only ATP_e treatment led to death of both host cells and intracellular mycobacteria (Lammas et al., 1997).

ATP_e stimulation of the $P2X_7R$ is associated with a large increase in the activity of phospholipase D (PLD) (Fairbairn et al., 2001; Humphreys and Dubyak, 1996; Kusner and Adams, 2000; Kusner and Barton, 2001), an enzyme that has been previously linked to leukocyte antimicrobial mechanisms, including phagocytosis and generation of reactive oxidants. PLD activation appears to be directly responsible for killing of intracellular mycobacteria, since a PLD inhibitor decreased significantly the level of $P2X_7R$-mediated killing of virulent strains of *M. tuberculosis* (Fairbairn et al., 2001; Kusner and Adams, 2000).

At the same time, as macrophage death would be detrimental to the intracellular pathogens, mycobacteria secrete ATP-scavenging enzymes such as ATPase to minimize the cytolytic effect of ATP_e (Zaborina et al., 1999a). Conversely, ATP_e does not affect the growth of extracellular pathogens such as *Pseudomonas aeruginosa* (Zaborina et al., 1999b), which secretes virulence factors that increase the sensitivity of macrophages to ATP_e-induced death, presumably via ligation of the $P2X_7R$ on the macrophage surface. *C. psittaci*, like the intracellular mycobacteria, protects J774 macrophages partially from ATP_e-induced cell death (Coutinho-Silva et al., 2001). Thus, intracellular mycobacteria and chlamydiae protect the macrophage, which may be used for microbial dissemination, while *P. aeruginosa*, which does not need macrophages for its propagation, secretes factors that enhance cytolysis of macrophages.

The objectives of this study were to determine if ATP_e has an effect on the infectious activity of the MoPn serovar of *C. trachomatis* in murine peritoneal macrophages, and if the effect requires PLD activity. We show conclusively a role for the $P2X_7R$ by characterizing the infection in peritoneal macrophages from wild-type and $P2X_7R$-deficient mice.

Results

Effect of Extracellular Nucleotides and the $P2X_7R$ on PLD Activation

ATP_e is known to modulate a myriad of cellular responses in macrophages and other cell types, including cytokine secretion, generation of reactive oxygen and nitrogen species, and activation of PLD (Di Virgilio et al., 2001; Gargett et al., 1996; Humphreys and Dubyak, 1996; Kusner and Adams, 2000). The effects of antagonists or agonists of the $P2X_7R$ suggested that ATP_e-mediated PLD activation relied on stimulation of the $P2X_7R$ (Gargett et al., 1996; Humphreys and Dubyak, 1996; Kusner and Adams, 2000), although PLD activity was not tested in $P2X_7R^{-/-}$ macrophages.

After incubation with 1 mM ATP_e, but not 1 mM extracellular UTP, there was a significant increase in the PLD activity of peritoneal macrophages (Figure 1A), as previously observed for human macrophages and lymphocytes (Gargett et al., 1996; Humphreys and Dubyak, 1996; Kusner and Adams, 2000). Similarly, the $P2X_7R$ agonist BzATP (200 μM) also induced PLD activation (Figure 1A), suggesting that ATP_e activated PLD via the $P2X_7R$.

In the presence of water, PLD produces PA from phosphatidylcholine. In cells pretreated with butan-1-ol, PLD uses preferentially the alcohol as a substrate, producing the nonsignaling phosphatidylbutanol (PBut) instead of PA, thus inhibiting PLD-dependent responses (Yang et al., 1967). As expected, coincubation with 0.3% butan-1-ol inhibited ATP_e-induced PLD activation, as assayed by PA production (Figure 1B).

Effect of Extracellular Nucleotides on PLD Activation in Macrophages from $P2X_7R$-Deficient Mice

To demonstrate directly a role for the $P2X_7R$ in ATP_e-dependent PLD activation, peritoneal macrophages were isolated from wild-type and $P2X_7R^{-/-}$ mice. Incubation of wild-type macrophages with ATP_e resulted in a large increase in the activity of PLD, assayed by measuring PBut production in the presence of butan-1-ol, while the same treatment with ATP_e had no effect on PLD activity of $P2X_7R^{-/-}$ macrophages (Figure 1C). Thus, ATP_e-mediated PLD activation in peritoneal macrophages requires stimulation of the $P2X_7R$.

Effect of ATP_e and PLD on Macrophage Death

Previous reports have shown that J774 cells or human or murine macrophages can be induced to die after treatment with ATP_e but not other nucleotides, via stimulation of the $P2X_7R$ (Chow et al., 1997; Di Virgilio et al., 1998). We confirm that murine peritoneal macrophages are also susceptible to ATP_e-induced cell death. When macrophages were treated with ATP_e for 2 hr and then incubated in the absence of ATP_e for an additional 24 hr, many macrophages became annexin positive (Figure 2A) and permeable to trypan blue (data not shown). In agreement with previous studies with the J774 macrophage cell line (Coutinho-Silva et al., 2001), death of peritoneal macrophages became significant after treatment with $\geq$2 mM ATP (Figure 2B). There was no macrophage death after a 40 min or 3 hr incubation with ATP_e, as measured by trypan blue exclusion (data not shown).

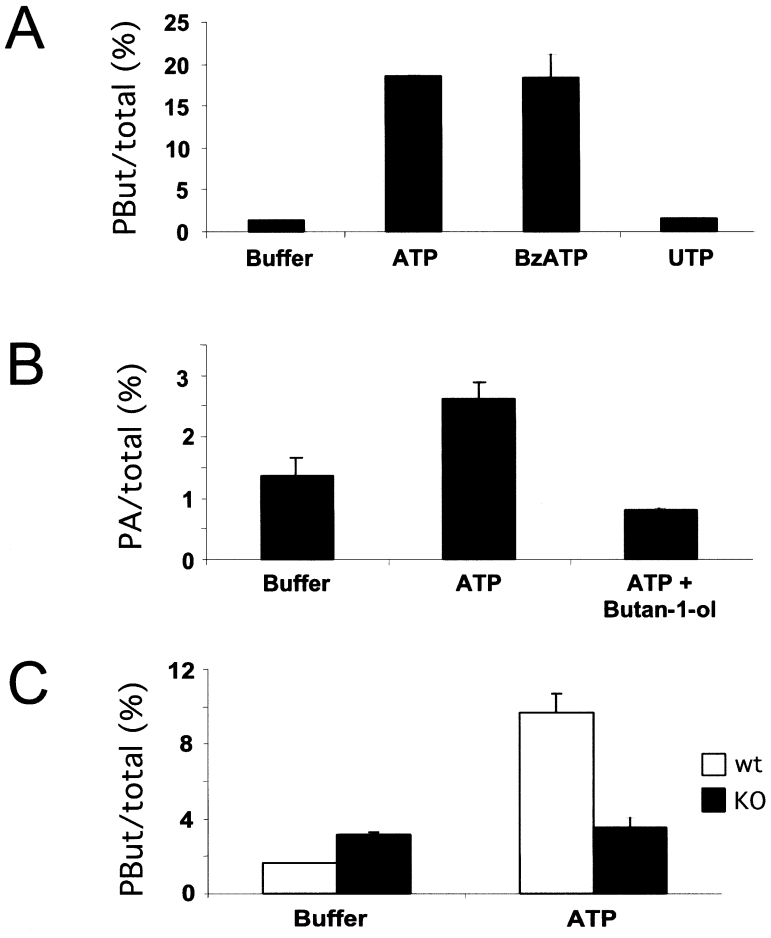

Figure 1. $P2X_7R$ Ligation with ATP_e Leads to PLD Activation

(A) Peritoneal macrophages from wild-type mice were stimulated for 45 min with ATP, BzATP, or UTP in the presence of 0.3% butan-1-ol. The lipids were extracted, and the amount of PBut was measured as described in Experimental Procedures. PBut is expressed as the % of total radiolabeled lipids.

(B) Peritoneal macrophages from wild-type mice were stimulated with ATP_e in the presence or absence of 0.3% butan-1-ol. The lipids were extracted, and the amount of PA was measured as described in Experimental Procedures. PA is expressed as the % of total radiolabeled lipids.

(C) Peritoneal macrophages from wild-type mice (white bar) or $P2X_7R^{-/-}$ mice (black bar) were stimulated with ATP_e in the presence of 0.3% butan-1-ol. PBut is expressed as the % of total radiolabeled lipids. The experiments were performed on at least 2 separate days, and the values represent the mean and SD of a representative experiment.

Annexin labeling was not used for macrophages incubated with ATP_e for up to 3 hr, since early PS surface-exposure following brief $P2X_7R$ stimulation is reversible and unrelated to cell death (MacKenzie et al., 2001). As expected, ATP_e had no effect on cell death of macrophages isolated from $P2X_7R^{-/-}$ mice (Figure 2A).

As ATP_e activates PLD via the $P2X_7R$, and the activation could be inhibited with butan-1-ol, we investigated whether PLD activation is required for $P2X_7R$-dependent macrophage death. Incubation of peritoneal macrophages with 5 mM ATP in the presence of 0.3% butan-1-ol had no effect on macrophage death (data not shown), implying that PLD is not involved in $P2X_7R$-dependent macrophage killing.

Effect of *C. trachomatis* Infection on PLD Activation

Although no putative genes encoding PLD have been identified in the *Chlamydia* genome (http://chlamydia-www.berkeley.edu:4231), extracts of *M. tuberculosis* contain PLD activity, and genes homologous to a phospholipase C of *P. aeruginosa* have been cloned in virulent mycobacterial species (Johansen et al., 1996). In addition, PLD is activated during macrophage phagocytosis of either *M. tuberculosis* or opsonized zymosan (Kusner et al., 1996).

As we aimed to explore the effects of ATP_e-induced PLD activation on the infection by *C. trachomatis*, we verified whether chlamydial internalization or infection by itself could modify PLD activity. Infected and uninfected wild-type macrophages were therefore incubated with ATP, UTP, or BzATP, and PLD activity was measured. As before, both ATP and BzATP activated the macrophage PLD, whereas UTP had no effect. A previous 24 hr infection with *C. trachomatis* did not affect PLD activity of untreated cells, nor did it modify PLD activation due to ATP or BzATP treatment (Figure 3). Thus, any effects of PLD on intracellular chlamydiae could be attributable only to ATP_e stimulation of the $P2X_7R$.

Effect of $P2X_7R$ Stimulation and PLD Activation on Chlamydial Infectious Activity

ATP_e-mediated PLD activation has been shown to promote killing of intracellular mycobacteria in macrophages (Fairbairn et al., 2001; Kusner and Adams, 2000). In order to determine whether ATP_e-mediated apoptosis affects survival or growth of chlamydiae in infected cells, macrophages were infected with MoPn for 24 hr, the macrophages were treated with the indicated concentration of ATP for 2 hr, and the incubation was allowed to continue in the absence of ATP for an additional 24 hr (Figure 4A) or 4 hr (Figure 4B). The bacteria from supernatant and remaining macrophages were collected and titrated on HeLa epithelial cells. The number of infectious chlamydiae was then measured on HeLa cells by immunofluorescence, using anti-*Chlamydia* an-

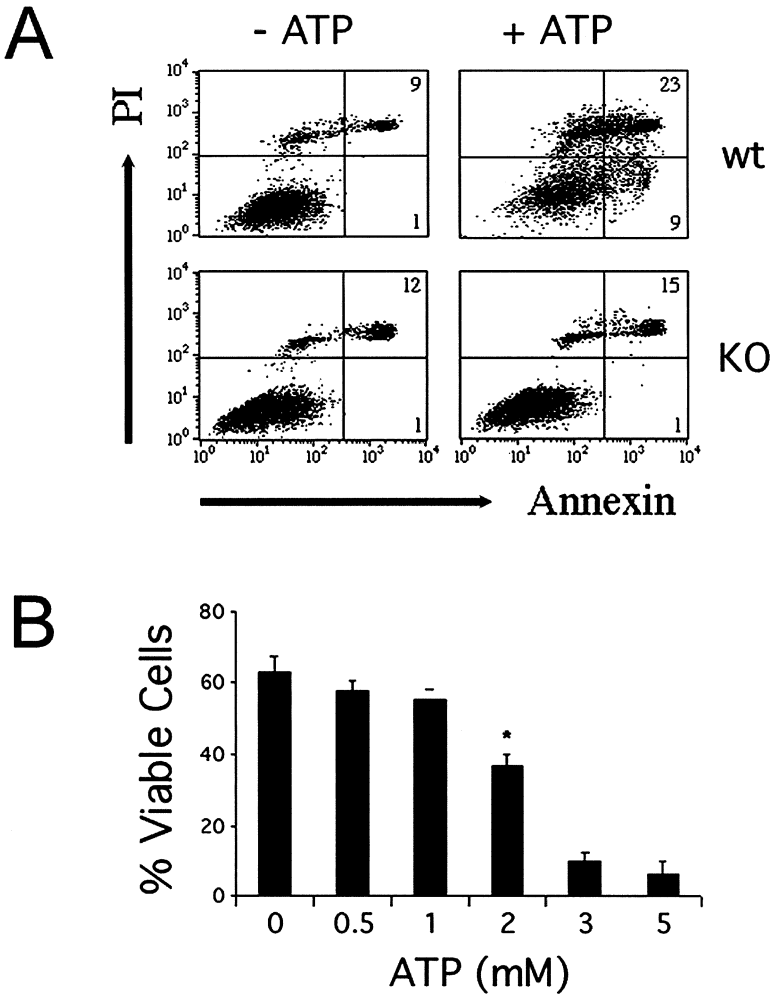

Figure 2. ATP_e-Induced Macrophage Death Requires $P2X_7R$

(A) Peritoneal macrophages from wild-type or $P2X_7R^{-/-}$ (KO) mice were incubated with 5 mM ATP as described in Experimental Procedures, and cell death was measured after 24 hr. Cell death was measured by cytofluorimetry with PI-annexin V-FITC double staining, as described in Experimental Procedures. Trypan blue exclusion measurements confirmed that annexin-labeling corresponded to macrophage death at 24 hr.

(B) Peritoneal macrophages form wild-type mice were incubated with the indicated concentration of ATP. Cell death was measured as above, and cells that did not label with annexin V or PI were plotted as a function of the ATP concentration. *, $p < 0.05$ for 2 mM ATP compared with 0 mM ATP; $p < 0.01$ for 3 or 5 mM ATP compared with 0 mM ATP. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments.

tibodies. Incubation of infected macrophages with 5 mM ATP_e led to almost complete inhibition of chlamydial infectious activity, although half of the activity was inhibited by concentrations as low as 0.5 mM ATP_e (Figure 4A). Most of the bacteria were also killed by 3 mM ATP_e within 6 hr postincubation (Figure 4B), at which time there is no measurable macrophage death, indicating that inactivation of chlamydiae precedes macrophage death.

The inhibition was due in large part to PLD activation, as co-treatment with butan-1-ol, which had no effect on macrophage death, or 2,3-diphosphoglycerate (2,3-DPG) reversed much of the ATP_e-induced inhibition of infectious activity (Figure 4B). As a negative control, butan-2-ol (0.03–0.3%), which is not a substrate for transphosphatidylation, had no effect on ATP_e-mediated inhibition of infection (data not shown). In the absence of ATP_e, butan-1-ol and 2,3-DPG did not alter the viability of intracellular chlamydiae (data not shown).

To rule out conclusively a role for $P2X_7R$-independent pathways in PLD-mediated effects on chlamydial survival, the experiments were repeated by infecting perito-

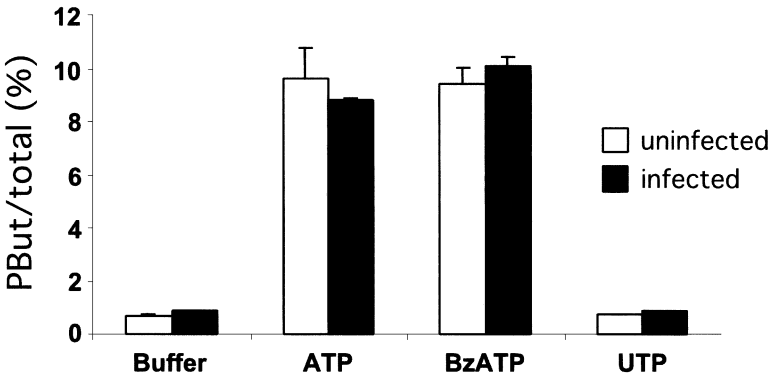

Figure 3. Infection of Macrophages with *C. trachomatis* Does Not Affect PLD Activation

Peritoneal macrophages from wild-type mice were infected for 1 day with *C. trachomatis* at an moi of 0.25 (black bar) or left uninfected (white bar), and were then incubated for 45 min with extracellular ATP, UTP, BzATP, or control buffer in the presence of butan-1-ol. The lipids were extracted, and the amount of PBut was measured as described in Experimental Procedures. PBut is expressed as the % of total radiolabeled lipids. The experiments were performed on 2 separate days, and the values represent the mean and SD of a representative experiment.

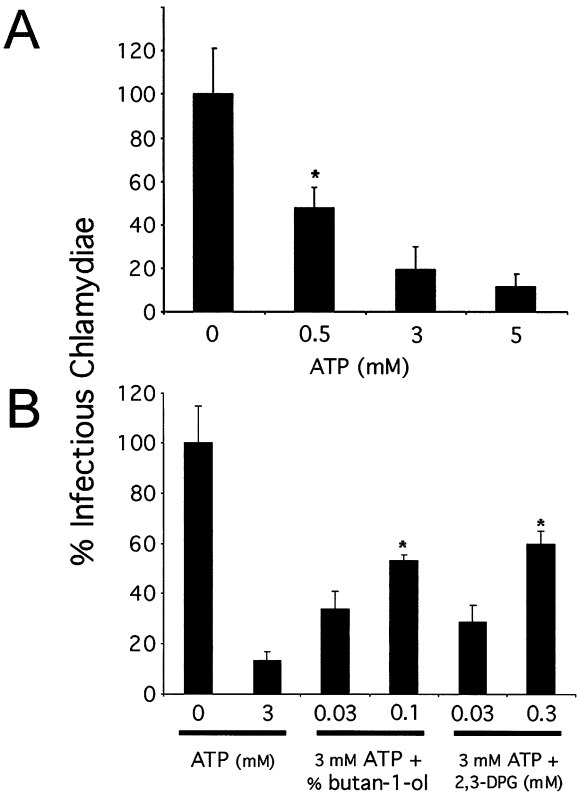

Figure 4. ATP_e-Induced Chlamydial Inactivation Requires PLD Activation

(A) J774 macrophages were infected with *C. trachomatis* for 1 day, and then incubated for 2 hr with the indicated concentration of extracellular ATP. The medium was replaced with cell culture medium and the infection was allowed to proceed for an additional 24 hr. The chlamydiae in cells and supernatant were collected, and the relative infectious activity was measured by titrating the bacteria on HeLa cells, as described in Experimental Procedures. *, $p < 0.01$ for 0.5 mM ATP compared with 0 mM ATP. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments.

(B) J774 macrophages were infected with *C. trachomatis* for 1 day, and then incubated for 2 hr with extracellular ATP in the presence or absence of butan-1-ol or 2,3-DPG. The medium was replaced with cell culture medium and the infection was allowed to proceed for an additional 4 hr. The chlamydiae in cells and supernatant were collected, and the relative infectious activity was measured as above. *, $p < 0.02$ for 3 mM ATP with 0.1% butan-1-ol or 0.3 mM 2,3-DPG, compared to 3 mM ATP alone. The experiments were performed on 3 separate days, and the values represent the mean and SD of the three experiments.

neal macrophages from wild-type and $P2X_7R^{-/-}$ mice with *C. trachomatis*. While most of the infectious activity was inhibited by treatment of wild-type macrophages with 5 mM ATP, incubation of infected $P2X_7R^{-/-}$ macrophages with the same concentration of ATP_e had no effect on chlamydial infectious activity (data not shown), implying that $P2X_7R$-dependent PLD-activation was responsible for the inhibition.

Fusion of Lysosomes and *Chlamydia* Inclusions due to $P2X_7R$ Stimulation

Extracellular ATP kills mycobacteria (Fairbairn et al., 2001; Kusner and Adams, 2000) and induces phagosome-lysosome fusion and acidification of the mycobacteria-containing phagosomes (Fairbairn et al., 2001; Kusner and Barton, 2001; Stober et al., 2001). To investigate the mechanism whereby the $P2X_7R$ inhibits survival of intracellular chlamydiae, wild-type macrophages were infected for 24 hr with *C. trachomatis* and then treated with ATP_e. Infected macrophages were then fixed and double-stained with anti-*Chlamydia* antibodies and antibodies against a lysosomal marker, LAMP-1. In general, *Chlamydia* entry vacuoles in epithelial cells maintain a neutral pH by avoiding fusion with host-cell lysosomes (Hackstadt, 1999), and specifically, many vacuoles harboring the MoPn serovar avoid fusion with lysosomes in macrophages (Figure 5A). These results are consistent with previous observations that MoPn can infect alveolar macrophages and macrophages following intraperitoneal infection of mice (Gogolak, 1953; Khatsenko et al., 1998). While some of the chlamydiae that had entered macrophages remained in small vacuoles, most of these also avoided fusion with lysosomes (Figure 5C). Occasionally, small vacuoles that had fused with lysosomes were observed in macrophages that contained a large inclusion (Figure 5A) or only small vacuoles. However, most *Chlamydia* inclusions contained the LAMP-1 antigen after the infected macrophages were treated with ATP_e for 40 min (Figures 5D and 6), indicating that $P2X_7R$ ligation leads to lysosome fusion with mature *Chlamydia* inclusions. Similarly, most chlamydiae were in acidic compartments following a 3 hr incubation with ATP_e (Figure 5E), suggesting fusion with acidic lysosomes.

Interestingly, most *Chlamydia* vacuoles expressed the LAMP-1 antigen after a 40 min treatment of infected macrophages with ATP_e (Figure 6), before macrophage death is observed. These results suggest that $P2X_7R$-dependent fusion of *Chlamydia* vacuoles with host-cell lysosomes is responsible for inhibition of chlamydial growth, independently of macrophage death.

Discussion

C. trachomatis is a major cause of sexually transmitted disease, afflicting 4 million people and costing $2 billion annually in the United States (Pearlman and McNeely, 1994). In women, the manifestations of *C. trachomatis* infection range from asymptomatic cervicitis to pelvic inflammatory disease, infertility, and ectopic pregnancy (Gerbase et al., 1998). More recently, there has been increasing interest in characterizing infections by *C. pneumoniae*, which infects endothelial cells and circulating macrophages and is responsible for approximately a tenth of pneumonia cases in industrialized countries, to determine if there is a link between previous infections with *C. pneumoniae* and increased risk of developing atherosclerosis (Rosenfeld et al., 2000).

Despite extensive work to better characterize the biology of the infection, an effective vaccine against *Chlamydia* does not exist. Determination of mechanisms that cause genital tract or lung pathology may enable us to develop alternative treatment strategies that prevent the morbidity of chlamydial disease. Any improvement of either pharmacologic or vaccine-based therapies would therefore benefit from a better understanding of the mechanisms that promote natural immunity to *Chlamydia*. A salient feature of dissemination of invasive

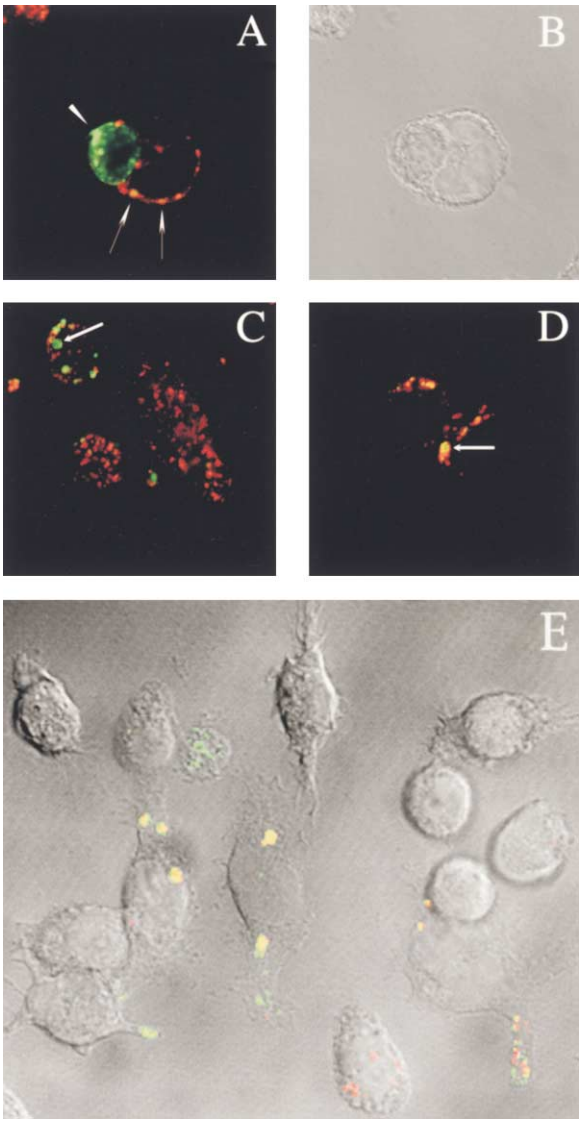

Figure 5. Treatment with Extracellular ATP Leads to Fusion between *Chlamydia* Inclusions and Host-Cell Lysosomes

J774 macrophages were infected with *C. trachomatis* for 24 hr and then incubated with control medium or ATP for 40 min or 3 hr, as described in Experimental Procedures. For LAMP-1 localization (A–C), cells were immediately fixed, and lysosomes (LAMP-1) and *Chlamydia* inclusions were revealed by immunofluorescence, as described in Experimental Procedures. For localization of acidic compartments (E), cells were incubated with LysoTracker during the last 30 min of the experiment.

(A) Image of an infected macrophage revealed by confocal microscopy, in the absence of ATP treatment. Arrowhead points to a productive *Chlamydia* inclusion that has not fused with lysosomes (LAMP-1 antigen); thin arrows point to small vacuoles that have fused with lysosomes.

(B) Contours of the macrophage in (A) shown in a contrast image.

(C) Image of infected macrophages with small multiple *Chlamydia* vacuoles that have not fused with lysosomes (LAMP-1 antigen) (arrow), in the absence of ATP treatment.

(D) Image of infected macrophages with *Chlamydia* vacuoles that have fused with lysosomes (arrow), after 40 min of treatment with ATP. Red, lysosomes (LAMP-1 antigen); green, *Chlamydia*; yellow, colocalization.

(E) Low magnification image of infected macrophages with *Chlamydia* vacuoles that have fused with acidic compartments, after 3 hr of treatment with ATP. Red, acidic compartment (LysoTracker); green, *Chlamydia*; yellow, colocalization.

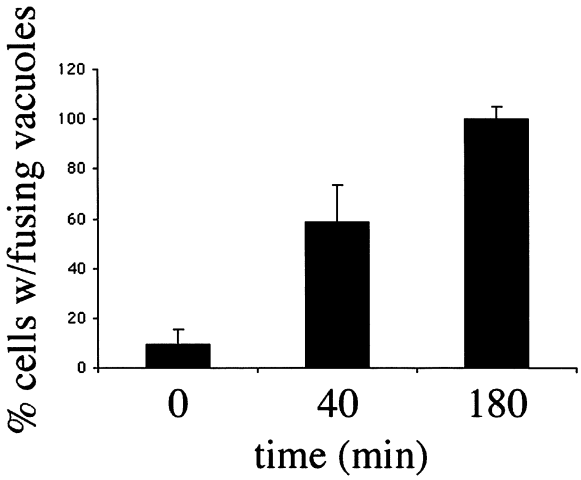

Figure 6. $P2X_7R$ Stimulation Results in Fusion of *Chlamydia* Inclusions with Host-Cell Lysosomes

Macrophages were infected with *C. trachomatis* for 24 hr and then incubated for 0 min, 40 min, or 180 min with ATP. Cells were fixed, and lysosomes (LAMP-1) and *Chlamydia* inclusions were revealed by immunofluorescence, as described in Experimental Procedures. Samples were examined with a fluorescence microscope, and the number of cells with inclusions that colocalized with LAMP-1 were counted. Values are expressed as the % of cells with inclusions that had fused with lysosomes. The average and SD were calculated from the values obtained from at least fifty microscope fields. The experiment was performed on 2 separate days, and results from a representative experiment are shown.

strains of *Chlamydia* is the ability of the bacteria to survive within host macrophages (Igietseme et al., 1998; Khatsenko et al., 1998; La Verda and Byrne, 1994; Rosenfeld et al., 2000). However, the specific mechanisms that regulate chlamydial growth within macrophages remain poorly characterized.

Based on reports that ATP_e stimulates killing of intracellular mycobacteria within macrophages (Fairbairn et al., 2001; Kusner and Adams, 2000; Kusner and Barton, 2001; Lammas et al., 1997; Molloy et al., 1994; Stober et al., 2001), we tested the hypothesis that ATP_e could have a similar effect against *C. trachomatis* in macrophages. We found that ATP_e exerted a profound inhibitory effect on the infectious activity of the MoPn serovar of *C. trachomatis* through a mechanism requiring PLD activation. Killing of chlamydiae was also dissociable from ATP_e-induced macrophage death, since an inhibitor of PLD rescued chlamydiae but did not prevent macrophage death, and the infectious activity of *Chlamydia* was inhibited at time points when macrophages were still viable.

Characterization of ATP_e-mediated killing of *C. trachomatis* revealed both similarities and differences compared with killing of intracellular bacillus Calmette-Guérin (BCG) or virulent *M. tuberculosis* (Fairbairn et al., 2001; Kusner and Adams, 2000; Kusner and Barton, 2001; Sikora et al., 1999; Stober et al., 2001). The most

significant similarity was the dependence on $P2X_7R$ for microbial killing. The effects of ATP_e on killing of both the BCG bacillus and virulent *M. tuberculosis* strains were ascribed to $P2X_7R$ stimulation based on the effects of different agonists and antagonists of $P2X_7R$. The effects of ATP_e were also absent or diminished in $P2X_7R^{-/-}$ macrophages (Fairbairn et al., 2001; Sikora et al., 1999; Stober et al., 2001). Although some residual activity against mycobacteria remained, $P2X_7R$ is thus the primary receptor for the pathway leading to mycobacterial killing following ATP_e treatment of infected macrophages. The residual activity suggests that additional purinergic receptors may contribute to ATP_e-mediated mycobacterial killing.

In contrast, $P2X_7R$ appears to be indispensable for ATP_e-mediated inhibition of *C. trachomatis* infectious activity in murine peritoneal macrophages, as no inhibition was observed in macrophages isolated from $P2X_7R^{-/-}$ mice. Part of the difference observed between previous reports on mycobacteria and our studies on *Chlamydia* may be due to macrophage host types, since ATP_e-induced mycobacterial killing is more effective in human macrophages than in murine macrophages, the latter being more dependent on $P2X_7R$ for ATP_e-mediated killing (Stober et al., 2001). Hence, we can not exclude the possibility that additional, $P2X_7R$-independent mechanisms of ATP_e-mediated chlamydial killing may be operative in human macrophages.

ATP_e treatment led to PLD activation and killing of mycobacteria in both human and murine macrophages (Fairbairn et al., 2001; Kusner and Adams, 2000; Stober et al., 2001). We find that PLD activation is required for inhibition of chlamydial infectious activity, since the chlamydiae were rescued to a large extent when infected macrophages were treated with ATP_e in the presence of PLD inhibitors.

PLD activation has been previously associated with lysosomal trafficking and fusion of intracellular organelles (Brown et al., 1998), and ATP_e promotes $P2X_7R$-dependent acidification of phagosomes containing mycobacteria and fusion of phagosomes and lysosomes (Fairbairn et al., 2001; Kusner and Barton, 2001; Molloy et al., 1994). In macrophages infected with mycobacteria, PLD participates in maturation of phagosomes to microbicidal phagolysosomes (Kusner and Adams, 2000), and we propose that a similar mechanism may be operative in *Chlamydia*-infected macrophages. Chlamydiae normally survive in epithelial cells and macrophages by inhibiting fusion between *Chlamydia* vacuoles and host-cell lysosomes (Hackstadt, 1999; Schramm et al., 1996; Wyrick, 2000). We observed numerous small *Chlamydia* vacuoles that are acidic and express the lysosomal marker, LAMP-1, in macrophages that were treated with ATP_e, suggesting that $P2X_7R$-dependent PLD activation stimulates fusion between the mature *Chlamydia* inclusions and lysosomal markers, leading to fragmentation of the inclusion. The fusion was also a rapid event, since inclusions that had avoided interactions with lysosomes for 24 hr would express lysosomal markers within 40 min after ATP_e treatment.

For both mycobacteria (Fairbairn et al., 2001) and chlamydiae (this work), inhibition of PLD activity blocked microbial killing, without affecting macrophage death. The time-course of ATP_e-mediated killing of mycobacteria and chlamydiae was also more rapid than the induction of macrophage death (Fairbairn et al., 2001). Previous studies have shown that $P2X_7R$ agonists do not lead to significant cell death for at least 4 hr after $P2X_7R$ activation (Grahames et al., 1999; MacKenzie et al., 2001). These results imply that $P2X_7R$ stimulation is required for both microbial inactivation and macrophage death, but the signaling pathways diverge downstream of $P2X_7R$ ligation. Thus, for both mycobacteria and chlamydiae, microbial killing is not a direct consequence of host-cell death.

What could be the source of ATP_e in vivo? The cytosolic concentration of ATP in most cells is 5 to 10 mM, and increases during *Chlamydia* infection of epithelial cells (Ojcius et al., 1998b). Although the plasma membrane is impermeable to ATP, release of ATP has been observed from a variety of cells and virtually all tissues under conditions of stress, inflammation, and necrosis (Bodin and Burnstock, 1995, 1998; Pedersen et al., 1999). Sufficient concentrations of ATP_e to stimulate $P2X_7R$ are known to be produced in vivo, as shown in a mouse model of arthritis induced by anti-collagen antibody (Labasi et al., 2002). In addition, ATP could be secreted by cytotoxic T cells and platelets (Di Virgilio, 1995, 1998). It has been reported that a cytosolic enzyme, lactate dehydrogenase, is released from cells infected with *Chlamydia* (Moulder, 1991), making it more than likely that ATP is also released. Consistent with this view, macrophages infected with *M. tuberculosis* release ATP in a dose-dependent manner (Sikora et al., 1999). At the same time, proinflammatory cytokines secreted during *Chlamydia* infection would enhance the microbicidal activity of the $P2X_7R$, since IFNγ increases the expression of the $P2X_7R$ (Humphreys and Dubyak, 1996, 1998). Inflammatory responses during *Chlamydia* infection should therefore allow the $P2X_7R$ to function optimally in limiting dissemination of chlamydiae via infected macrophages. Finally, in view of the fact that $P2X_7R$ stimulation leads to inhibition of infection by two different pathogens—*M. tuberculosis* and *C. trachomatis*—we propose that $P2X_7R$, by activating PLD, could play a general role in regulating infection by bacteria and protozoan parasites that survive in macrophages within membrane-bound vacuoles that avoid fusion with lysosomes.

Experimental Procedures

Mice, Cells, and Materials

The $P2X_7R^{-/-}$ mice were described previously (Solle et al., 2001). These animals were maintained on a mixed genetic background (129/Ola x C57BL/6 x DBA/2) backcrossed five times onto C57BL/6. Breeding $P2X_7R^{-/-}$ males with $P2X_7R^{-/-}$ females was used to maintain the colony of receptor-deficient animals, and genetically comparable wild-type animals were maintained by crossing homozygous animals.

The human cervical adenocarcinoma cell line, HeLa 229, and the mouse macrophage cell lines, J774, were from the American Type Culture Collection (Manassas, VA). The cells were cultured in a humidified incubator at 37°C with 5% CO_2 in Dulbecco's modified minimal essential medium (DMEM) with Glutamax-1 (Life Technologies, Inc.; Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 25 μg/ml gentamicin (cell culture medium for HeLa cells), or RPMI 1640 medium containing 10% FCS, 1 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol,

and 25 μg/ml gentamicin (for J774 cells). The mouse pneumonitis (MoPn) strain of *C. trachomatis*, obtained originally from American Type Culture Collection, was grown in McCoy cells and purified as previously described (Ramsey et al., 1989). Butan-1-ol, butan-2-ol, ATP, 3-*O*-(4-benzoylbenzoyl)ATP (BzATP), 2,3-diphosphoglycerate, and UTP were from Sigma (St. Louis, MO). [^{3}H]Myristic acid was purchased from Perkin Elmer Life Science. Other reagents were previously described (Coutinho-Silva et al., 2001).

Isolation of Peritoneal Macrophages

Peritoneal macrophages were recovered from $P2X_7R^{+/+}$ and $P2X_7R^{-/-}$ mice as described (Albuquerque et al., 1993). In brief, the peritoneal macrophages were obtained by lavage on the intraperitoneal cavity with cold balanced salt solution. Isolated cells were washed with RPMI 1640 medium containing 10% heat-inactivated FCS, 1 mM L-glutamine, and then seeded into 12-well plates (Costar; Corning, NY) at a density of 5–10 $\times$ 10^5 cells/well. After incubation for 1 hour or overnight at 37°C in a 10% CO_2 humidified incubator, nonadherent cells were removed by vigorous washing and the adherent cells were kept under the same conditions for 24–48 hr, until ready for use. After the 24–48 hr incubation in the incubator, the media were removed, and macrophages were used for infection or treatment with nucleotides.

Measurement of Cell Death

Macrophages from wild-type or $P2X_7R^{-/-}$ mice were treated with the indicated concentration of ATP in the presence or absence of 0.3% butan-1-ol in RPMI medium for 2 hr, after which the medium was replaced with RPMI 1640 medium containing 1 mM L-glutamine, 10% heat-inactivated FCS, 25 μg/ml gentamicin, in the absence of nucleotides or butan-1-ol. After an additional 24 hr in the incubator at 37°C, both adherent cells and cells in suspension were collected, washed twice in PBS, and analyzed by cytofluorimetry. Phosphatidylserine exposure on dying cells was measured by labeling with Annexin V-FITC (R&D Systems; Minneapolis, MN), and loss of plasma membrane integrity was measured by PI staining, as described (Perfettini et al., 2002). In parallel, macrophages were incubated with 5 mM ATP for 40 min, 3 hr, 6 hr, or 24 hr, and cell viability was measured by trypan blue exclusion.

Infection of Macrophages

Peritoneal macrophages were seeded on 12-well plates (Costar) at a density of 5–10 $\times$ 10^5 cells per well. After 24 hr in a humidifed incubator at 37°C with 5% CO_2, the cells were washed and infected with MoPn at a multiplicity of infection (moi) of 0.25. After 1 hour, the medium was changed and cells were incubated for an additional 24 hr, and prepared for immunofluorescence microscopy, quantification of infectious activity, or measurement of PLD activity.

Measurement of PLD Activity

PLD activity was measured by following phosphatidylbutanol (PBut), the nonmetabolizable product of the transphosphatidylation reaction (Denmat-Ouisse et al., 2001; Yang et al., 1967). Uninfected peritoneal macrophages (0.3 million macrophages per measurement), or macrophages that had been incubated for 1 hr with *C. trachomatis* at an moi of 0.25, were washed in serum-free RPMI, and the infection was allowed to proceed for an additional 24 hr, while the macrophages were labeled for 24 hr with [^{3}H]myristic acid (47 Ci/mmol, 3 μCi/ml medium) in RPMI containing 1 mg/ml lipid-free bovine serum albumin. The macrophages were then washed with lipid-free BSA in PBS and preincubated at 37°C for 15 min in 1 ml of RPMI containing 1 mg/ml lipid-free bovine serum albumin (BSA) and 0.92 mM $CaCl_2$, supplemented with 0.3% butan-1-ol; 1 mM ATP or other extracellular nucleotides were then added for a further 45 min incubation. At the end of the experiment, the macrophages were collected and the lipids were extracted as previously described (Auger et al., 1999). The amounts of PBut formed were expressed as percentages of the amount of total radiolabeled lipids. Alternatively, phosphatidic acid (PA) production was measured in the presence or absence of butan-1-ol. The amount of PA or PBut was expressed as the percentage of the total amount of radiolabeled lipids.

Immunofluorescence Microscopy and Measurement of Infectious Activity

Following infection of peritoneal macrophages or J774 macrophages for 24 hr, control serum-free RPMI medium or the indicated concentration of ATP was added for 40 min or 3 hr at 37°C. The supernatant was then removed and cells were fixed with paraformaldehyde, as described (Ojcius et al., 1998a). Chlamydial inclusions were detected using an anti-*Chlamydia* genus mAb (1:250 dilution; Argene, France), followed by a FITC-conjugated anti-mouse IgG + IgM (1:200 dilution; Argene). Lysosomes were detected by staining with rat antibody against murine LAMP-1 (lysosome associated membrane protein-1) (1:100 dilution; from PharMingen BD Bioscience; Le Pont de Chaix, France), followed by biotinylated $F(ab')_2$ goat anti-rat Igs (1:200 dilution, from Pharmingen) and revealed by streptavidin-Texas Red (1:100 dilution, from Pharmingen). Compartments with acidic pH were identified with LysoTracker Red DND-99 (Molecular Probles; Eugene, Oregon), by preincubating infected macrophages with 1 μM LysoTracker at 37°C during the last 30 min of the experiment, before washing cells once with PBS and fixing with paraformaldehyde. Samples were examined with either a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss; Jena, Germany) attached to a cooled charge-coupled device camera, or a LSM 510 Zeiss confocal microscope. Images were analyzed with Adobe Photoshop software, and *Chlamydia* inclusions were identified by fluorescence staining. The number of cells with inclusions that colocalized with LAMP-1 was counted in each microscope field, and the average and standard deviation per condition was calculated for the % of cells with colocalizing inclusions in at least fifteen fields, containing an average of ~100 cells per field.

In order to measure effects of extracellular nucleotides on survival of intracellular chlamydiae, J774 macrophages and wild-type and $P2X_7R^{-/-}$ macrophages that had been infected with *C. trachomatis* at an moi of 0.25 for 24 hr were incubated with the indicated concentration of ATP in serum-free RPMI medium for 2 hr at 37°C, in the presence or absence of the indicated concentrations of butan-1-ol, butan-2-ol, or 2,3-DPG (Kusner and Adams, 2000), and the medium was then replaced by fresh cell culture medium. The infection was allowed to proceed for an additional 4 hr or 24 hr at 37°C in the 5% CO_2 humidified incubator. The cells and supernatant were combined and centrifuged for 60 min at 12,000 rpm in a Sorvall type GSA rotor. Macrophages in the pellet were lysed, and the infectious activity of the chlamydiae was measured by resuspending the pellet in ice-cold culture medium, and using serial dilutions of the chlamydial preparation from each well to infect HeLa cells on coverslips for 24 hr, as described (Perfettini et al., 2003). The chlamydial vacuoles were revealed by fixing the cells with methanol and incubating with anti-*Chlamydia* antibodies, as above.

Acknowledgments

We are grateful to Dr. Christopher Gabel (PGRD, Pfizer Inc, Groton, CT) for generously providing the $P2X_7R^{-/-}$ mice, Dr. Jean Kanellopoulos (Université Paris-Sud) for helpful discussions, and Emmanuelle Perret and Pascal Roux of the Centre d'Imagerie Dynamique for assistance with microscopy. This work was financed by National Institutes of Health grant R01 AI054624, Fondation pour la Recherche Médicale, Université Paris 7, Université Paris-Sud, PTR 94 (Institut Pasteur), and CNRS. R.C.-S. was supported by a fellowship from the Wellcome Trust.

Received: February 5, 2003
Revised: May 20, 2003
Accepted: July 3, 2003
Published: September 16, 2003

References

Albuquerque, C., Oliveira, S.M., Coutinho-Silva, R., Oliveira-Castro, G.M., and Persechini, P.M. (1993). ATP- and UTP-induced currents in macrophages and macrophage polykaryons. Am. J. Physiol. *265*, C1663–C1673.

Auger, R., Robin, P., Camier, B., Vial, G., Rossignol, B., Tenu, J., and Raymond, M.N. (1999). Relationship between phosphatidic acid

level and regulation of protein transit in colonic epithelial cell line HT29-cl19A. J. Biol. Chem. *274*, 28652–28659.

Bavoil, P.M., Hsia, R.-c., and Ojcius, D.M. (2000). Closing in on *Chlamydia* and its intracellular bag of tricks. Microbiol. *146*, 2723–2731.

Beatty, W.L., Morrison, R.P., and Byrne, G.I. (1994). Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. Microbiol. Rev. *58*, 686–699.

Bodin, P., and Burnstock, G. (1995). Synergistic effect of acute hypoxia on flow-induced release of ATP from cultured endothelial cells. Experientia *51*, 256–259.

Bodin, P., and Burnstock, G. (1998). Increased release of ATP from endothelial cells during acute inflammation. Inflamm. Res. *47*, 351–354.

Brown, F.D., Thompson, N., Saqib, K.M., Clark, J.M., Powner, D., Thompson, N.T., Solari, R., and Wakelam, M.J.O. (1998). Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. Curr. Biol. *8*, 835–838.

Chow, S.C., Kass, G.E., and Orrenius, S. (1997). Purines and their roles in apoptosis. Neuropharmacology *36*, 1149–1156.

Coutinho-Silva, R., Persechini, P.M., Bisaggio, R.C., Perfettini, J.L., Sa Neto, A.C.T., Kanellopoulos, J.M., Motta, I., Dautry-Varsat, A., and Ojcius, D.M. (1999). P2Z–P2X$_7$ receptor-mediated apoptosis of dendritic cells. Am. J. Physiol. *276*, C1139–C1147.

Coutinho-Silva, R., Perfettini, J.L., Persechini, P.M., Dautry-Varsat, A., and Ojcius, D.M. (2001). Modulation of P2Z/P2X$_7$ receptor activity in macrophages infected with *Chlamydia psittaci*. Am. J. Physiol. *280*, C81–C89.

Denmat-Ouisse, L.A., Phebidias, C., Honkavaara, P., Robin, P., Geny, B., Min, D.S., Bourgoin, S., Frohman, M.A., and Raymond, M.N. (2001). Regulation of constitutive protein transit by phospholipase D in HT29-cl19A cells. J. Biol. Chem. *276*, 48840–48846.

Di Virgilio, F. (1995). The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. Immunol. Today *16*, 524–528.

Di Virgilio, F., Chiozzi, P., Falzoni, S., Ferrari, D., Sanz, J.M., Venketaraman, V., and Baricordi, O.R. (1998). Cytolytic P2X purinoceptors. Cell Death Differ. *5*, 191–199.

Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J.M., Morelli, A., Torboli, M., Bolognesi, G., and Baricordi, O.R. (2001). Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood *97*, 587–600.

Fairbairn, I.P., Stober, C.B., Kumararatne, D.S., and Lammas, D.A. (2001). ATP-mediated killing of intracellular mycobacteria by macrophages is a P2X$_7$-dependent process inducing bacterial death by phagosome-lysosome fusion. J. Immunol. *167*, 3300–3307.

Gargett, C.E., Cornish, E.J., and Wiley, J.S. (1996). Phospholipase D activation by P2Z-purinoceptor agonists in human lymphocytes is dependent on bivalent cation influx. Biochem. J. *313*, 529–535.

Gerard, H.C., Branigan, P.J., Schumacher, H.R., and Hudson, A.P. (1998a). Synovial *Chlamydia trachomatis* in patients with reactive arthritis/Reiter's syndrome are viable but show aberrant gene expression. J. Rheumatol. *25*, 734–742.

Gerard, H.C., Kohler, L., Branigan, P.J., Zeidler, H., Schumacher, H.R., and Hudson, A.P. (1998b). Viability and gene expression in *Chlamydia trachomatis* during persistent infection of cultured human monocytes. Med. Microbiol. Immunol. *187*, 115–120.

Gerard, H.C., Wang, Z., Whittum-Hudson, J.A., El-Gabalawy, H., Goldbach-Mansky, R., Bardin, T., Schumacher, H.R., and Hudson, A.P. (2002). Cytokine and chemokine mRNA produced in synovial tissue chronically infected with *Chlamydia trachomatis* and *C. pneumoniae*. J. Rheumatol. *29*, 1827–1835.

Gerbase, A.C., Rowley, J.T., and Mertens, T.E. (1998). Global epidemiology of sexually transmitted diseases. Lancet *351*, 2–4.

Gogolak, F.M. (1953). The histopathology of murine pneumonitis infection and the growth of the virus in the mouse lung. J. Infect. Dis. *92*, 254–272.

Grahames, C.B.A., Michel, A.D., Chessell, I.P., and Humphrey, P.P.A. (1999). Pharmacological characterization of ATP- and LPS-induced IL-1β release in human monocytes. Br. J. Pharmacol. *127*, 1915–1921.

Hackstadt, T. (1999). Cell biology. In *Chlamydia*: Intracellular Biology, Pathogenesis, and Immunity, R.S. Stephens, ed. (Washington, D.C.: ASM Press), pp. 101–138.

Humphreys, B.D., and Dubyak, G.R. (1996). Induction of the P2Z/P2X7 nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-gamma in the human THP-1 monocytic cell line. J. Immunol. *157*, 5627–5637.

Humphreys, B.D., and Dubyak, G.R. (1998). Modulation of P2X7 nucleotide receptor expression by pro- and anti-inflammatory stimuli in THP-1 monocytes. J. Leukoc. Biol. *64*, 265–273.

Igietseme, J.U., Perry, L.L., Ananaba, G.A., Uriri, I.M., Ojior, O.O., Kumar, S.N., and Caldwell, H.D. (1998). Chlamydial infection in inducible nitric oxide synthase knockout mice. Infect. Immun. *66*, 1282–1286.

Johansen, K.A., Gill, R.E., and Vasin, M.L. (1996). Biochemical and molecular analysis of phospholipase C and phospholipase D activity in mycobacteria. Infect. Immun. *64*, 3259–3266.

Khatsenko, O.G., Barteneva, N.S., de la Maza, L.M., and Kikkawa, Y. (1998). Role of nitric oxide in the inhibition of cytochrome P450 in the liver of mice infected with *Chlamydia trachomatis*. Biochem. Pharmacol. *55*, 1835–1842.

Koehler, L., Nettelnbreker, E., Hudson, A.P., Ott, N., Gerard, H.C., Branigan, P.J., Schumacher, H.R., Drommer, W., and Zeidler, H. (1997). Ultrastructural and molecular analyses of the persistence of *Chlamydia trachomatis* (serovar K) in human monocytes. Microb. Pathog. *22*, 133–142.

Kusner, D.J., and Adams, J. (2000). ATP-induced killing of virulent *Mycobacterium tuberculosis* within human macrophages requires phospholipase D. J. Immunol. *164*, 379–388.

Kusner, D.J., and Barton, J.A. (2001). ATP stimulates human macrophages to kill intracellular virulent *Mycobacterium tuberculosis* via calcium-dependent phagosome-lysosome fusion. J. Immunol. *167*, 3308–3315.

Kusner, D.J., Hall, C.F., and Schlesinger, L.S. (1996). Activation of phospholipase D is tightly coupled to the phagocytosis of *Mycobacterium tuberculosis* or opsonized zymosan by human macrophages. J. Exp. Med. *184*, 585–595.

La Verda, D., and Byrne, G.I. (1994). Interactions between macrophages and chlamydiae. Immunol. Ser. *60*, 381–399.

Labasi, J.M., Petrushova, N., Donovan, C., McCurdy, S., Lira, P., Payette, M.M., Brissette, W., Wicks, J.R., Audoly, L., and Gabel, C.A. (2002). Absence of the P2X$_7$ receptor alters leukocyte function and attenuates an inflammatory response. J. Immunol. *168*, 6436–6445.

Lammas, D.A., Stober, C., Harvey, C.J., Kendrick, N., Panchalingam, S., and Kumararatne, D.S. (1997). ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X$_7$) receptors. Immunity *7*, 433–444.

MacKenzie, A., Wilson, H.L., Kiss-Toth, E., Dower, S.K., North, R.A., and Surprenant, A. (2001). Rapid secretion of interleukin-1β by microvesicle shedding. Immunity *15*, 825–834.

Molloy, A., Laochumroonvorapong, P., and Kaplan, G. (1994). Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. J. Exp. Med. *180*, 1499–1509.

Moulder, J.W. (1991). Interaction of chlamydiae and host cells in vitro. Microbiol. Rev. *55*, 143–190.

Mutini, C., Falzoni, S., Ferrari, D., Chiozzi, P., Morelli, A., Baricordi, O.R., Collo, G., Ricciardi-Castagnoli, P., and Di Virgilio, F. (1999). Mouse dendritic cells express the P2X$_7$ purinergic receptor: characterization and possible participation in antigen presentation. J. Immunol. *163*, 1958–1965.

Nanagara, R., Li, F., Beutler, A., Hudson, A.P., and Schumacher, H.R. (1995). Alteration of *Chlamydia trachomatis* biologic behavior in synovial membranes. Suppression of surface antigen production in reactive arthritis and Reiter's syndrome. Arthritis Rheum. *38*, 1410–1417.

Ojcius, D.M., Bravo de Alba, Y., Kanellopoulos, J.M., Hawkins, R.A., Kelly, K.A., Rank, R.G., and Dautry-Varsat, A. (1998a). Internalization of *Chlamydia* by dendritic cells and stimulation of *Chlamydia*-specific T cells. J. Immunol. *160*, 1297–1303.

Ojcius, D.M., Degani, H., Mispelter, J., and Dautry-Varsat, A. (1998b). Enhancement of ATP levels and glucose metabolism during an infection by *Chlamydia*. J. Biol. Chem. *273*, 7052–7058.

Pearlman, M.D., and McNeely, S.G. (1994). A review of the microbiology, immunology, and clinical implications of chlamydial infections. Obstet. Gynecol. Surv. *47*, 448–461.

Pedersen, S., Pederson, S.F., Nilius, B., Lambert, I.H., and Hoffmann, E.K. (1999). Mechanical stress induces release of ATP from Ehrlich ascites tumor cells. Biochim. Biophys. Acta. *1416*, 271–284.

Perfettini, J.-L., Gissot, M., Souque, P., and Ojcius, D.M. (2002). Modulation of apoptosis during infection by *Chlamydia*. Methods Enzymol. *358*, 334–344.

Perfettini, J.L., Ojcius, D.M., Andrews, C.W., Korsmeyer, S.J., Rank, R.G., and Darville, T. (2003). Role of proapoptotic BAX in propagation of *Chlamydia muridarum* (the mouse pneumonitis strain of *Chlamydia trachomatis*) and the host inflammatory response. J. Biol. Chem. *278*, 9496–9502.

Ramsey, K.H., Newhall, W.J., and Rank, R.G. (1989). Humoral immune response to chlamydial genital infection of mice with the agent of mouse pneumonitis. Infect. Immun. *57*, 2441–2446.

Ramsey, K.H., Miranpuri, G.S., Sigar, I.M., Ouellette, S., and Byrne, G.I. (2001). *Chlamydia trachomatis* persistence in the female mouse genital tract: inducible nitric oxide synthase and infection outcome. Infect. Immun. *69*, 5131–5137.

Rosenfeld, M.E., Blessing, E., Lin, T.M., Moazed, T.C., Campbell, L.A., and Kuo, C. (2000). *Chlamydia*, inflammation, and atherogenesis. J. Infect. Dis. *181* (*Suppl. 3*), S492–S497.

Schachter, J. (1988). Overview of human diseases. In Microbiology of *Chlamydia*, A.L. Barron, ed. (Boca Raton, FL: CRC Press), pp. 153–165.

Schramm, N., Bagnell, C.R., and Wyrick, P.B. (1996). Vesicles containing *Chlamydia trachomatis* serovar L2 remain above pH 6 within HEC-1B cells. Infect. Immun. *64*, 1208–1214.

Sikora, A., Liu, J., Brosnan, C., Buell, G., Chessel, I., and Bloom, B.R. (1999). Cutting edge: purinergic signaling regulates radical-mediated bacterial killing mechanisms in macrophages through a $P2X_7$-independent mechanism. J. Immunol. *163*, 558–561.

Solle, M., Labasi, J., Perregaux, D.G., Stam, E., Petrushova, N., Koller, B.H., Griffiths, R.J., and Gabel, C.A. (2001). Altered cytokine production in mice lacking $P2X_7$ receptor. J. Biol. Chem. *276*, 125–132.

Stober, C.B., Lammas, D.A., Li, C.M., Kumararatne, D.S., Lightman, S.L., and McArdle, C.A. (2001). ATP-mediated killing of *Mycobacterium bovis* bacille Calmette-Guerin within human macrophages is calcium dependent and associated with the acidification of mycobacteria-containing phagosomes. J. Immunol. *166*, 6276–6286.

Taylor-Robinson, D., Gibroy, C.B., Thomas, B.J., and Keat, A.C.S. (1992). Detection of *Chlamydia trachomatis* in the joints of reactive arthritis by polymerase chain reaction. Lancet *340*, 81–82.

Wyrick, P.B. (2000). Intracellular survival by *Chlamydia*. Cell. Microbiol. *2*, 275–282.

Yang, S.F., Freer, S., and Benson, A.A. (1967). Transphosphatidylation by phospholipase D. J. Biol. Chem. *242*, 477–484.

Zaborina, O., Li, X., Cheng, G., Kapatral, V., and Chakrabarty, A.M. (1999a). Secretion of ATP-utlizing enzymes, nucleoside diphosphate kinase and ATPase, by *Mycobacterium bovis* BCG: sequestration of ATP from macrophage P2Z receptors? Mol. Microbiol. *31*, 1333–1343.

Zaborina, O., Misra, N., Kostal, J., Kamath, S., Kapatral, V., El-Azami El-Idrissi, M., Prabhakar, B.S., and Chakrabarty, A.M. (1999b). P2Z-independent and P2Z receptor-mediated macrophage killing by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients. Infect. Immun. *67*, 5231–5242.