ATP-Mediated Killing of Intracellular Mycobacteria by Macrophages Is a P2X\textsubscript{7} -Dependent Process Inducing Bacterial Death by Phagosome-Lysosome Fusion

Ian P. Fairbairn, Carmel B. Stober, Dinakantha S. Kumararatne and David A. Lammas

*J Immunol* 2001; 167:3300-3307; doi: 10.4049/jimmunol.167.6.3300
http://www.jimmunol.org/content/167/6/3300

References

This article cites 46 articles, 23 of which you can access for free at: http://www.jimmunol.org/content/167/6/3300.full#ref-list-1

Why The *JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2001 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
ATP-Mediated Killing of Intracellular Mycobacteria by Macrophages Is a P2X<sub>7</sub>-Dependent Process Inducing Bacterial Death by Phagosome-Lysosome Fusion<sup>1</sup>

Ian P. Fairbairn, Carmel B. Stober, Dinakantha S. Kumararatne, and David A. Lammens

**Mycobacterium tuberculosis** survives within host macrophages by actively inhibiting phagosome fusion with lysosomes. Treatment of infected macrophages with ATP induces both cell apoptosis and rapid killing of intracellular mycobacteria. The following studies were undertaken to characterize the effector pathway(s) involved. Macrophages were obtained from p47<sup>phox</sup> and inducible NO synthase gene-disrupted mice (which are unable to produce reactive oxygen and nitrogen radicals, respectively) and P2X<sub>7</sub> gene-disrupted mice. RAW murine macrophages transfected with either the natural resistance-associated macrophage protein gene 1 (Nramp1)-resistant or Nramp1-susceptible gene were also used. The cells were infected with bacille Calmette-Guérin (BCG), and intracellular mycobacterial trafficking was analyzed using confocal and electron microscopy. P2X<sub>7</sub> receptor activation was essential for effective ATP-induced mycobacterial killing, as its bactericidal activity was radically diminished in P2X<sub>7</sub><sup>−/−</sup> macrophages. ATP-mediated killing of BCG within p47<sup>phox</sup>−/−, inducible NO synthase−/−, and Nramp<sup>−</sup> cells was unaffected, demonstrating that none of these mechanisms have a role in the ATP/P2X<sub>7</sub> effector pathway. Following ATP stimulation, BCG-containing phagosomes rapidly coalesce and fuse with lysosomes. Blocking of macrophage phospholipase D activity with butan-1-ol blocked BCG killing, but not macrophage death. ATP stimulates phagosome-lysosome fusion with concomitant mycobacterial death via P2X<sub>7</sub> receptor activation. Macrophage death and mycobacterial killing induced by the ATP/P2X<sub>7</sub> signaling pathway can be uncoupled, and diverge proximal to phospholipase D activation. The Journal of Immunology, 2001, 167: 3300–3307.

Approximately one-third of the world’s population is infected with **Mycobacterium tuberculosis**, yet 90% of infected individuals remain healthy (1). Of these, <10% will exhibit reactivation disease within their lifetime. Therefore, in the vast majority of individuals, their immune systems can effectively control the infection.

From HIV patients (2) and certain rare immunodeficient individuals (3, 4), we know that human immunity to mycobacterial infections constitutes an interplay between Ag-specific CD4<sup>+</sup> Th1 lymphocytes and host macrophages. The macrophages promote Th1 activity by production of IL-12 (3), IL-18 (5), and IL-23 (6), and the lymphocytes activate macrophages through the release of IFN-γ (4) and TNF-α (7). These two sets of effector cells are then thought to interact in a positive feedback manner to contain the infection within T cell-activated macrophages.

Mycobacteria, in turn, are thought to survive and replicate within nonactivated macrophages through a variety of strategies, including the avoidance of generation of reactive oxygen (8) and nitrogen intermediates (9) and the active inhibition of phagosome maturation (10, 11). The latter strategy preventing exposure to the antimicrobial peptides and enzymes presents within the acidic lysosomal granules. Infected cells are thought to become progressively unresponsive to further activation by Th1-derived cytokines as the pathogen undergoes intracellular replication (12).

Innate resistance to intracellular infections is also thought to be genetically determined, influencing the outcome of infection. The best characterized is the natural resistance-associated macrophage protein gene 1 (Nramp1)<sup>3</sup> (13). This gene codes for a membrane transporter protein that acts to inhibit the replication of various intracellular pathogens, including bacille Calmette-Guérin (BCG), by excluding Fe<sup>2+</sup> (14, 15) and other cations from the pathogen-containing phagosomes and promoting their acidification (16).

The macrophage acts as both the major reservoir of infection and the principal effector cell acting to regulate mycobacterial infections in vivo.

A major drawback in our understanding of the effector pathway(s) operating within human macrophages is the fact that, unlike in murine model cell systems (17), no mycobactericidal effect is observed in vitro following stimulation of macrophages with Th1-associated cytokines, including IFN-γ and TNF-α (18). This has been attributed to the inability of such mediators to induce NO generation in human cells. However, we know that in vivo macrophages are able to contain these organisms in the vast majority of infected individuals. This has initiated a search for alternative stimuli that may regulate mycobacteriastatic or mycobactericidal activity within infected human cells.

We have previously reported that a 15-min exposure to 1–3 mM ATP results in the apoptosis of BCG-infected monocyte-derived

---

<sup>1</sup> Abbreviations used in this paper: Nramp1, natural resistance-associated macrophage protein 1; ADC, albumin dextrose catalase; BCG, bacille Calmette-Guérin; BMDM, bone marrow-derived macrophage; DAMP, 3,2-dimirotoxoino)-3′-amino-N-methyl dipropylamine; EM, electron microscopy; GFP, green fluorescent protein; iNOS, inducible NO synthase; L-NMMA, N<sup>ω</sup>-monomethyl-L-arginine monooacetate; PL<sub>A</sub>, phospholipase A<sub>2</sub>; PLD, phospholipase D; WT, wild type.

---

Copyright © 2001 by The American Association of Immunologists 0022-1767/01/$02.00
human macrophages within 6 h, with a concomitant 50–70% reduction in the viability of intracellular mycobacteria. ATP treatment of a macrophage-free culture of BCG had no effect on bacterial viability, demonstrating this to be a cell-mediated mechanism (19). Virulent strains of tuberculosis were also found to be equally as susceptible to this mechanism (20).

The effects of ATP were shown to be mediated by P2X<sub>7</sub> receptors, as the cytotoxic and bactericidal activity of ATP were blocked by pretreatment of cells with P2X<sub>7</sub> antagonists, oxidized ATP, and KN62, and potentiated by the specific agonist benzoyl-benzyl ATP (19).

We have shown previously that the mycobactericidal effects of ATP are independent of both reactive oxygen and nitrogen intermediate generation. ATP was found to be equally effective on cells derived from patients with chronic granulomatous disease as against cells from healthy controls. Furthermore, BCG killing occurred even when such cells were additionally treated in vitro with NO inhibitors (19).

Electron microscopy (EM) studies have revealed that ATP-induced cell death of BCG-infected human macrophages is associated with the formation of cytoplasmic vacuoles into which the intracellular bacteria coalesce (21). This was thought to illustrate the promotion of phagosome-lysosome fusion and/or modulation of the phagosome pH within ATP-treated cells, resulting in the destruction of the mycobacteria.

In this study, we report the use of macrophages derived from various gene-disrupted (knockout) mice to further characterize the ATP effector mechanism involved and to confirm the selective involvement of the P2X<sub>7</sub> receptor in this mycobactericidal effect. Using EM and confocal fluorescent microscopy, we have further analyzed the multibacillary vacuole formation that occurs following ATP stimulation. Our findings suggest these play a critical role in mediating the death of mycobacteria as they progressively acidify and fuse with cell lysosomes. Finally, we also demonstrate a key role for phospholipase D (PLD) in the P2X<sub>7</sub> effector pathway, supporting similar findings reported by Kusner et al. (20).

Materials and Methods

Cells and bacteria

The murine macrophage cell line J774a.1 was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM (Life Technologies, Paisley, U.K.) supplemented with 10% FCS and 2 mM glutamine. The murine macrophage cell lines RAW 246.7, wild-type (WT) RAW 7.5R, and RAW 10S were a kind gift from J. M. Blackham (University of Cambridge, Cambridge, U.K.). The 7.5R and 10S lines are isogenic, Cambridge, U.K.). A. W. Segal (University College of London, London, U.K.), and E. F. Liew (University of Glasgow, Glasgow, U.K.), respectively, and have been previously described (23–25). The genetic backgrounds of the knockout mice were C57, and 129. WT C57BL/6 and 129 were used as controls, as appropriate. Bone marrow-derived macrophages (BMDMs) were obtained as follows. Femurs and tibias were cultured for 10 days, with changes of the medium on days 3 and 6. On day 9, the cells were adherent and morphologically looked like mature macrophages.

M. bovis BCG Evans strain was obtained from Evans Medical (Speke, U.K.) and maintained in log phase growth in 7H9 broth supplemented with 10% albumin dextrose catalase (ADC) enrichment medium and 0.2% Tween 80. Green fluorescent protein expressing BCG (GFP-BCG) was a kind gift from D. Young (Imperial College of London, London, U.K.). These contained the gene encoding a FACS-optimized GFP protein (26) constitutively expressed under the control of the mycobacterial heat shock protein 60 promoter in a pSM53 shuttle vector (27) construct. Stock aliquots, stored in glycerol at −70°C, were grown to log phase in 7H9 broth supplemented with 10% ADC enrichment medium, 0.2% Tween 80, and 50 µg/ml hygromycin (Sigma, St. Louis, MO).

BCG viability assay

Macrophages were grown to subconfluence in 200 µl/well cultures in 96-well round-bottom microtiter plates. This approximates to 5 × 10<sup>4</sup> cells in 200 µl/well. A total of 5 × 10<sup>5</sup> BCG Evans per well was added. To aid infection, plates were centrifuged at 700 × g for 10 min and incubated at 37°C for 60 min. Excess BCG were removed by washing. As assessed by CFU, −10% of BCG was taken up, giving a ratio of one viable BCG per macrophage. Where appropriate, the following metabolic inhibitors were then added for 60 min. N<sup>6</sup>-Monomethyl-L-arginine monoacetate (l-NMMA) (400 µM), superoxide dismutase (15 U/ml), catalase (0.1 mg/ml), and butan-1-ol (0.05–1%) were all purchased from Sigma. A 30 mM ATP (Boehringer Mannheim, Indianapolis, IN) solution was prepared in culture medium, adjusted to pH 7 with sodium bicarbonate, and passed through a 0.22-µm filter (Millipore, Bedford, MA) to sterilize. It was added to the macrophage cultures to give a final concentration 3 mM, and incubated at 37°C, 5% CO<sub>2</sub>. At appropriate time points, the plates were centrifuged at 700 × g for 10 min. A total of 150 µl supernatant was removed and replaced with 130 µl 7H9 medium, supplemented as above, plus 20 µl 2% saponin solution. Cell lysis was promoted by vigorous pipetting and then incubation at 37°C for 120 min. Fifty-microliter aliquots from each well were serially diluted in 7H9 supplemented with 10% ADC enrichment and 0.2% glycerol and plated in 96-well flat-bottom plates. The plates were then incubated at 37°C for 12 days. Microcolonies were counted using an inverted microscope (×100 magnification). All experiments were performed in quadruplicates.

Lucifer yellow uptake

Pore formation was assessed using a method adapted from that described by Steinberg et al. (28). Briefly, macrophages adherent to coverslips were incubated with 500 µg/ml lucifer yellow dye, 250 µM sulfipyrazine (Sigma), and 3 mM ATP in PBS for 5 min. They were washed in 250 µM sulfipyrazine in PBS and viewed immediately on a Zeiss confocal laser-scanning microscope (Zeiss, Oberkochen, Germany). Cells with pores larger than 463 Da exhibit intracellular staining.

Chromium-release assay

Mouse BMDMs were cultured in 96-well round-bottom microtiter plates, as previously described. Aliquots of 51Cr (ICN Pharmaceuticals, Costa Mesa, CA) were added at a concentration of 2 µCi/well, and the cells were incubated for 16 h. Cells were washed and incubated with 3 mM ATP for a further 16 h. Supernatants were removed, and their activity was counted on a Wallac 1260 Multi Gamma II gamma counter (PerkinElmer Wallac, Gaithersburg, MD). The cells themselves were dissolved in 1% Triton X-100 solution and also counted. Results are expressed as the percentage of the total activity in the supernatant.

Confocal fluorescent microscopy

Macrophages were grown to subconfluence on 13-mm glass coverslips in 24-well plates. This approximated to 5 × 10<sup>4</sup> cells/coverslip. GFP-BCG were resuspended in DMEM, and a single cell culture was prepared by water bath sonication for 5 min. Any remaining clumps were removed by slow speed centrifugation (100 × g for 5 min). The bacterial concentration and confirmation of a single cell suspension were determined using a thoma chamber. The BCG were then added to the macrophages to give a final concentration of 25 × 10<sup>3</sup>/coverslip. The plate was centrifuged at 700 × g for 10 min. The cells were incubated for 1 h at 37°C, 5% CO<sub>2</sub>, washed three times in DMEM to remove any extracellular bacteria, and incubated for an additional hour at 37°C, 5% CO<sub>2</sub>. Lysotracker (Molecular Probes, Eugene, OR) was added to the cells (final concentration of 50 nM) for 1 h before stimulation. ATP was added (final concentration of 3 mM), and the cells were incubated for the indicated times.

Before fixation, coverslips were washed three times in HBSS (Sigma). They were then fixed in 3% paraformaldehyde containing 10 mM lysine, 140 mM saline, and 40 mM HEPES (pH 7.3) for 15 min at room temperature. After washing in PBS plus 5% dextrose, the coverslip cultures were mounted on glass slides with Perma Fluor (Lipshaw Manufacturing, Detroit, MI), and the edges were sealed with nail varnish.

Slides were viewed with a Zeiss confocal laser-scanning microscope. Twenty fields were examined per slide, and representative pictures were taken.

The Journal of Immunology 3301
ATP-mediated pore formation and macrophage death are P2X7 receptor-free cultures (results not shown). Mediated function, as ATP had no effect on BCG grown in macrophage cell line (THP-1), and in murine BMDMs from various mouse strains (RAW), and with the human monocyte cell line (J774). ATP stimulation of BCG-infected J774 cells resulted in ~50% loss of mycobacterial CFU within 60 min posttreatment (Fig. 1). Loss of mycobacterial viability was detectable within 15 min and maximal at 2 h posttreatment. Subsequently, all viability studies were performed at 2 h post-ATP stimulation. Similar BCG killing kinetics was observed in another murine macrophage cell line (RAW), and with the human monocyte cell line (THP-1), and in murine BMDMs from various mouse strains (C57BL/6, BALB/c, and 129) (results not shown).

The ATP/P2X7 effector mechanism was confirmed as a cell-mediated function, as ATP had no effect on BCG grown in macrophage-free cultures (results not shown).

ATP-mediated pore formation and macrophage death are P2X7 receptor dependent

BMDMs from P2X7−/− mice were examined for their responses following ATP stimulation. Cells from WT mice were shown to rapidly take up the cell-impermeable dye lucifer yellow following treatment with ATP for 5 min, indicating P2X7 pore formation had occurred (Fig. 2, A and B), whereas cells from P2X7−/− mice failed to do so (Fig. 2, C and D). WT BMDMs were also shown to die in response to ATP stimulation over a 16-h period, as indicated by 51Cr release, whereas macrophages from P2X7−/− mice remained viable (Fig. 3a).

ATP-mediated killing of intracellular mycobacteria is P2X7 receptor dependent

Having demonstrated that macrophages from P2X7−/− mice had no P2X7−/−-associated cytotoxic activity, they were then assessed for their ability to kill intracellular mycobacteria following ATP stimulation. BMDMs were infected with BCG and exposed to ATP (3 mM) for 2 h. Compared with WT cells, macrophages from P2X7−/− mice were shown to be ineffective at killing BCG (Fig. 3b). The percentage of BCG killed was reduced from 50% in ATP-treated WT cells to <10% in P2X7−/− cells. Although some residual mycobactericidal activity was still occurring, the results show that P2X7 is the primary transducer of the intracellular mycobactericidal effector pathway activated following ATP stimulation of infected macrophages.

PLD activity is essential for ATP/P2X7-mediated BCG killing, but not macrophage death

PLD is involved in a number of signaling cascades via the production of phosphatidic acid from phosphorylcholine. If cells are pretreated with butan-1-ol, PLD preferentially uses the primary alcohol as a substrate producing the nonsignaling phosphatidyl butanol (30). Treatment with butan-1-ol after BCG infection, but before ATP stimulation, showed a concentration-dependent inhibition of BCG killing post-ATP (Fig. 4a), but had no effect on macrophage death, as measured by chromium release (Fig. 4b).

Radical oxygen intermediates and radical nitrogen intermediates are not required for BCG killing post-ATP/P2X7 stimulation

Experiments were performed to investigate whether the ATP-induced mycobactericidal effects observed in murine macrophages were attributable to the generation of reactive oxygen or reactive nitrogen intermediates.

The high levels of NO required for killing mycobacteria within murine macrophages are generated by the enzyme iNOS after appropriate priming and stimulation of the cells. Macrophages from iNOS−/− mice are therefore unable to up-regulate nitrogen radical production (25). p47phox is a key enzyme in the NADPH reductase pathway, and mice with a targeted disruption of this gene are unable to produce reactive oxygen radicals (24).

BMDMs from iNOS−/− and p47phox−/− mice and WT littermates were infected with BCG and treated with ATP (3 mM) for 2 h. The results (Fig. 5) reveal that ATP was equally effective in inducing bacterial death within iNOS−/− and p47phox−/− macrophages as in WT cells.

To confirm that production of neither oxygen or nitrogen radicals was required for the ATP effect, nitrogen radical production was blocked in the p47phox−/− macrophages using the NO inhibitor L-NMMA, and oxygen radical production was blocked in the iNOS−/− macrophages using superoxide dismutase and catalase (Fig. 5). ATP killing of BCG was shown to be equally as effective in all cell treatments. The data reveal that oxygen and nitrogen radicals do not have a role in the ATP effector mechanism.
Killing post-P2X<sub>7</sub> stimulation is not affected by Nramp1 genotype

The susceptibility of inbred mice strains to BCG is associated with a single point mutation of Nramp1 (31). Both the RAW and J774 macrophage cell lines are derived from BALB/c mice, which carry the susceptible mutation of Nramp1 (31).

To examine the possible influence of Nramp1 on the ATP-bacterial killing effect, RAW cells, stably transfected with either the resistant Nramp1 (RAW 7.5R) or the susceptible Nramp1 (RAW 10S), were used. ATP treatment of BCG-infected 7.5R and 10S cells was shown to be equally as effective in inducing killing of BCG, as was observed in the WT RAW cells (Fig. 6). This indicates that expression of Nramp1 does not affect the ability of RAW cells to kill BCG in response to ATP stimulation.

Further support for this finding is provided by the observation that BMDMs from BALB/c, C57BL/6, and 129 mice were all equally effective at killing intracellular mycobacteria following ATP stimulation. BALB/c and C57BL/6 mice are known to be susceptible to BCG infection and carry the susceptible Nramp1 mutation, whereas 129 mice are a resistant strain and carry the

**FIGURE 2.** Macrophage membrane permeabilization following ATP stimulation. Macrophages were cultured from the bone marrow of P2X<sub>7</sub><sup>/−</sup>/H<sup>−</sup> mice and WT littermates. They were stimulated with 3 mM ATP for 5 min in the presence of the cell-impermeable dye lucifer yellow. The cells were then washed and immediately viewed with confocal fluorescent (A and C) and transmission (B and D) microscopy. A and B demonstrate stimulated WT macrophages that are permeable to the dye, whereas C and D demonstrate stimulated macrophages from P2X<sub>7</sub><sup>/−</sup> mice whose membranes have remained impermeable. The pictures are representative of numerous fields from three experiments.

**FIGURE 3.**

a. Chromium release post-ATP stimulation of P2X<sub>7</sub><sup>/−</sup>-BMDMs. b. Intracellular BCG killing by ATP-stimulated P2X<sub>7</sub><sup>/−</sup>-BMDMs. BMDMs were cultured from P2X<sub>7</sub><sup>/−</sup> mice and WT littermates. a. Macrophages were labeled overnight with 51 Cr. Excess chromium was removed. Where indicated, they were then stimulated for 16 h with 3 mM ATP. γ Activity was counted in both the supernatant and cells. Results are expressed as percentage released over total. SDs for quadruplets are shown, and the results are representative of three experiments. After stimulation, the amount of chromium released by the WT macrophages increases, indicating that cell death had occurred. There was no response in the P2X<sub>7</sub><sup>/−</sup> macrophages. b. The macrophages were infected with BCG for 60 min, and extracellular BCG were washed off. Where appropriate, macrophages were stimulated with 3 mM ATP for 120 min. They were then lysed and assayed for BCG CFU. The percentage of intracellular BCG killed is calculated by comparing the CFU from stimulated with the CFU from unstimulated macrophages. SDs for four experiments, each done in quadruplet, are shown. WT, but not P2X<sub>7</sub><sup>/−</sup>, macrophages are effective at killing BCG.

**FIGURE 4.**

a. Effect of PLD inhibition on BCG killing by J774 macrophages post-ATP stimulation. J774 macrophages were infected with BCG and then treated for 60 min with the indicated concentrations of butan-1-ol to inhibit PLD activity. They were then stimulated with 3 mM ATP for 2 h. BCG viability was assessed and expressed as percentage of BCG killed. Inhibition of PLD activity completely inhibits BCG killing. SDs for quadruplets are shown, and the results are representative of three experiments. b. Effect of PLD inhibition on macrophage death post-ATP stimulation. 51 Cr-labeled macrophages were pretreated with the indicated concentrations of butan-1-ol for 60 min before 16-h stimulation with 3 mM ATP. Inhibition of PLD activity has no effect on macrophage death. SDs for quadruplets are shown, and the results are representative of three experiments.
Results are expressed as percentage of BCG killed. Where indicated, 400 μM L-NMMA, 15 U/ml superoxide dismutase, or 0.1 mg/ml catalase was added 60 min before stimulation. SDs for quadruplets are shown, and the results are representative of two to three experiments. In all scenarios, the macrophages remained effective at killing BCG, indicating that neither nitrogen nor oxygen radicals are required.

Intracellular BCG killing by BMDMs does not require oxygen or nitrogen radicals. BMDMs were cultured from p47phox−/−, iNOS−/−, and WT littermates. They were infected with BCG before being stimulated with 3 mM ATP for 2 h. BCG viability was assessed by CFU. Results are expressed as percentage of BCG killed. Where indicated, 400 μM L-NMMA, 15 U/ml superoxide dismutase, or 0.1 mg/ml catalase was added 60 min before stimulation. SDs for quadruplets are shown, and the results are representative of two to three experiments. In all scenarios, the macrophages remained effective at killing BCG, indicating that neither nitrogen nor oxygen radicals are required.

**FIGURE 5.** Intracellular BCG killing by BMDMs does not require oxygen or nitrogen radicals. BMDMs were cultured from p47phox−/−, iNOS−/−, and WT littermates. They were infected with BCG before being stimulated with 3 mM ATP for 2 h. BCG viability was assessed by CFU. Results are expressed as percentage of BCG killed. Where indicated, 400 μM L-NMMA, 15 U/ml superoxide dismutase, or 0.1 mg/ml catalase was added 60 min before stimulation. SDs for quadruplets are shown, and the results are representative of two to three experiments. In all scenarios, the macrophages remained effective at killing BCG, indicating that neither nitrogen nor oxygen radicals are required.

**BCG-containing phagosomes fuse to form acidified multibacillary vacuoles post-P2X7 stimulation: EM studies**

Mycobacteria survive within macrophages by inhibiting the maturation of their phagosomes, thus avoiding fusion with lysosomes (10). Lysosomes provide a potentially toxic environment for mycobacteria containing various hydrolases and cationic proteins in an acidic environment. The promotion of phagosome-lysosome fusion by ATP was therefore investigated as a potential mycobacterial-killing mechanism.

Confocal fluorescent microscopy was used to show that P2X7 stimulation of infected BMDMs resulted in the rapid fusion of the BCG-containing phagosomes with lysosomes (Fig. 7). The acidophilic dye LysoTracker (Molecular Probes) was used to prelabel the cell lysosomes red, and GFP-BCG were used to infect the BMDMs. When analyzed by confocal fluorescent microscopy, the BCG in non-ATP-treated cells (Fig. 7, A and B) appeared green, indicating that they were residing in nonacidic vesicles separate from the red-staining lysosomes. However, in ATP-treated cells (Fig. 7, C and D), an increasing percentage of the BCG appeared yellow (due to colocalization with the red LysoTracker), indicating that they were residing within acidic organelles that had fused with lysosomes. To quantify the colocalization of the BCG, serial observations were expressed as the percentage of colocalized BCG over total intracellular BCG (Fig. 8). The results illustrate the progressive acidification of the BCG-containing phagosomes. These experiments show that BCG-containing phagosomes rapidly fuse with lysosomes following ATP stimulation.

Experiments using UTP, GTP, and AMP failed to show phagosome-lysosome fusion (results not shown), suggesting that ATP was acting via the P2X7 receptor. In addition, phagosome-lysosome fusion was absent in ATP-stimulated P2X7−/− macrophages (Fig. 7, E and F). The data demonstrate that the phagosome-lysosome fusion that occurs following ATP stimulation is dependent on and is a function of P2X7 activation.

**Discussion**

In this study, loss of intracellular BCG viability was shown to occur within 15 min following ATP stimulation of infected murine macrophages. The rapidity of the effect excluded a role for either nutrient and/or iron deprivation as the antimycobacterial mechanism responsible. The speed of the bactericidal activity indicates the involvement of a preformed toxic mediator that does not require de novo synthesis. This is in agreement with our previous findings, in...
which we showed that the protein synthesis inhibitor cycloheximide was unable to block ATP-induced BCG killing (19).

The kinetics of ATP-induced BCG killing was more rapid than induction of cell death. However, although ATP-stimulated cell death and BCG killing were both shown to specifically P2X7-dependent, their effector signaling pathways were found to diverge downstream of P2X7 activation. Blocking of cellular PLD activity with butan-1-ol then inhibited BCG killing, but not macrophage death in response to ATP stimulation.

The P2X7 receptor (formally known as P2Z) is highly expressed on cells of the immune system, predominately cells of monocyctic lineage (33). However, macrophages are thought to express a number of additional purinergic receptors, including: P2Y1,2,4,5,6,10,11 and P2X1,4,5,7 (34), some of which can respond to ATP with varying sensitivity.

The fact that P2X7-/- macrophages are unable to effectively kill BCG in response to ATP demonstrates unequivocally that P2X7 is the major receptor involved in this specific effector pathway. However, we also observed a small, but nonsignificant level of residual BCG killing within ATP-treated P2X7-/- cells. This is in agreement with Kusner et al. (20), who noted that ATP was slightly more effective than the more specific P2X7 agonist benzoyl-benzyl ATP. Taken together, these findings indicate the possible involvement of an additional purinergic receptor that may contribute to the loss of bacterial viability seen in ATP-treated cells. We have provisionally characterized this additional purinergic receptor as P2Y11 (35).

Crossover studies using inhibitors to block both nitrogen radical production in p47phox-/- BMDMs and oxygen radical production in iNOS-/- BMDMs also demonstrated unequivocally that neither oxygen nor nitrogen radicals have a role in ATP killing of intracellular BCG. This confirms our previous findings, which showed that macrophage-derived macrophages from patients with chronic granulomatous disease are able to effectively kill BCG following ATP stimulation, and that the addition of the nitrogen radical inhibitor L-NMMA was also unable to inhibit ATP-mediated BCG killing in these cells (19).

Nramp1 is an important mechanism in murine defenses against various intracellular infections including BCG, and has been reported to be associated with human resistance to tuberculosis (36). Nramp1 is also known to be expressed on phagosome membranes (37), and has been proposed to play a role in both iron transport and phagosome acidification (16). In this study, Nramp1 was not found to be involved in the ATP-induced mycobactericidal effector process, as macrophages with either the susceptible or resistant form of the Nramp1 allele were shown to be equally capable of killing intracellular BCG following ATP stimulation.

The molecular probes LysoTracker and DAMP are weak bases that are used to study phagosome acidification because they are selectively taken up within cells by acidic organelles. In this study, both LysoTracker and DAMP preferentially targeted BCG-containing phagosomes following ATP stimulation of infected macrophages, indicating that phagosome-lysosome fusion had occurred. EM studies using DAMP labeling also illustrated that the BCG-containing phagosomes both acidified and fused with each other following ATP stimulation. This is in agreement with Molloy et al. (21), who also reported similar fusion of mycobacteria-containing phagosomes following ATP treatment.

Importantly, the phagosome acidification and phagosome fusion induced by ATP were not seen in BMDMs from P2X7 gene-disrupted mice. This illustrates that, like BCG killing, these fusion processes occur downstream of P2X7 activation.
There was an observed temporal relationship between ATP-induced phagosome acidification and BCG killing that supports the hypothesis that ATP-induced phagosome-lysosome fusion directly results in killing of the intracellular BCG. It is known that mycobacteria grow poorly in acidic environments (38) and the growth rate of intracellular *M. tuberculosis* is impaired when they enter large acidified vacuoles formed by coinfection with other intracellular organisms (39). Fusion with lysosomes would then expose the BCG to the detrimental effects of low pH and to the toxic actions of lysosomal proteases and antibacterial peptides. The rapid killing effect of ATP observed in this study would suggest that exposure to these lysosomal mediators results in swift mycobactericidal activity.

In confirmation of the findings reported by Kusner et al. (20), we also demonstrated that PLD activation is required for ATP-induced BCG killing. It is therefore of interest that PLD has been implicated in a number of organelle trafficking functions, e.g., degranulation by mast cells (40) and endosome fusion (41). PLD activation produces increased levels of phosphatidic acid, a known fusogen to endosome membranes (41). PLD may also activate additional phospholipases such as phospholipase A2 (PLA2) (42–45). Interestingly, PLA2 is known to be induced within ATP-treated macrophages (46). This in turn may then promote endosome fusion via the generation of arachidonic acid (47).

We hypothesize that BCG actively inhibit the fusigenicity of their phagosomes, thereby preventing phagosome maturation, i.e., lysosome fusion and acidification. One mechanism by which they...
may achieve this effect is by altering the lipid composition of their phagosome membranes. We propose that ATP might act to overcome this inhibition via the generation of fusinogenic mediators such as PLD and/or its downstream mediators, e.g., PLA₂ and arachidonic acid that act to promote organelle fusion.

Further investigation of this intracellular bacterial killing mechanism may help to elucidate a key macrophage-mediated anticytobacterial effector process. In addition, the signaling pathway(s) involved may prove to be amenable to modulation via targeted drug design for the treatment of multidrug-resistant tuberculosis.

References


29. Reference