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ATP-Induced Killing of Virulent Mycobacterium tuberculosis Within Human Macrophages Requires Phospholipase D

David J. Kusner and Juan Adams

The global dissemination of antibiotic-resistant Mycobacterium tuberculosis has underscored the urgent need to understand the molecular mechanisms of immunity to this pathogen. Use of biological immunomodulatory compounds to enhance antituberculous therapy has been hampered by the limited efficacy of these agents toward infected human macrophages and lack of information regarding their mechanisms of activity. We tested the hypotheses that extracellular ATP (ATP_e) promotes killing of virulent M. tuberculosis within human macrophages, and that activation of a specific macrophage enzyme, phospholipase D (PLD), functions in this response. ATP_e treatment of infected monocyte-derived macrophages resulted in 3.5-log reduction in the viability of three different virulent strains of M. tuberculosis. Stimulation of macrophage P2_X7 purinergic receptors was necessary, but not sufficient, for maximal killing by primary macrophages or human THP-1 promonocytes differentiated to a macrophage phenotype. Induction of tuberculocidal activity by ATP_e was accompanied by marked stimulation of PLD activity, and two mechanistically distinct inhibitors of PLD produced dose-dependent reductions in ATP_e-induced killing of intracellular bacilli. Purified PLD restored control levels of mycobacterial killing to inhibitor-treated cells, and potentiated ATP_e-dependent tuberculocidal activity in control macrophages. These results demonstrate that ATP_e promotes killing of virulent M. tuberculosis within infected human macrophages and strongly suggest that activation of PLD plays a key role in this process. The Journal of Immunology, 2000, 164: 379–388.

Tuberculosis is a preeminent world health problem, responsible for over 3 million deaths annually (1, 2). The health burden of tuberculosis is increasing due to the dissemination of antibiotic-resistant strains of Mycobacterium tuberculosis and the deleterious consequences of coinfection with HIV (3, 4). Advances in antituberculous therapies are urgently required both for treatment of the 8–12 million new cases of tuberculosis that occur each year, as well as for the 2 billion persons already infected with M. tuberculosis, who are at risk of developing the disease (1, 3).

The interactions between M. tuberculosis and macrophages are central to all stages of tuberculosis (5, 6), from the initial establishment of the organism’s protected intracellular niche during primary infection, through the containment of mycobacterial replication exerted by cell-mediated immune responses, to the reactivation stage in which mycobacterial virulence factors overwhelm host immunity. Two questions that are fundamental to understanding the pathogenesis of tuberculosis at the molecular level, and that are essential to the development of novel antituberculous therapies, are: 1) Can human macrophages kill M. tuberculosis? And, if so 2), what is the mechanism of tuberculocidal activity? Despite conflicting reports of the variable bactericidal effects of cytokines (such as TNF-α) and other immune modulators (e.g., vitamin D₃) (7–17), it has been difficult to unequivocally demonstrate physiologic activation of human macrophages to kill intracellular M. tuberculosis.

Extracellular ATP (ATP_e)³ stimulates a wide variety of cell types, including macrophages, via ligation of one or more classes of plasma membrane purinergic receptors (18). Treatment of human macrophages with ATP has recently been demonstrated to stimulate killing of the attenuated vaccine strain BCG (19, 20). Although the BCG strain is nonpathogenic, and is normally killed within human macrophages (21, 22), these observations suggest that ATP might also confer bactericidal activity toward virulent mycobacteria, and, perhaps, serve as a model for characterization of specific biochemical mechanisms required for antituberculous activity in human macrophages. ATP-induced killing of intracellular BCG required stimulation of macrophage ATP receptors of the P2_X7 class (19). To date, the postreceptor mechanisms responsible for bactericidal activity toward BCG are unknown. Similarly, and of considerably greater importance, the mechanisms that regulate killing of M. tuberculosis by human macrophages are also unknown.

Among the diverse classes of ATP receptors, the P2_X7 receptors (formerly designated P2Z) are notable for their restricted cellular distribution (macrophages, mast cells, and certain lymphocyte populations) and their stimulus-dependent formation of large, non-selective membrane pores, which, in macrophages, are permeable to hydrophilic molecules ≤900 Da (19, 23–26). Stimulation of macrophage P2_X7 receptors is associated with a marked increase in the activity of phospholipase D (PLD) (27, 28), an enzyme that has

³ Abbreviations used in this paper: ATP_e, extracellular ATP; BCG, bacillus Calmette-Guérin; BzATP, 3′-O-benzoylbenzoyl-ATP; CR, complement receptor; 2,3-DPG, 2,3-diphosphoglycerate; DPPC, [32P]dipalmitoylphosphatidylcholine; dTHP, differentiated THP; MDM, monocyte-derived macrophage; MOI, multiplicity of infection; oATP, oxidized ATP (2′,3′-didehydro-ATP); PA, phosphatidic acid; PC-PLC, phosphatidylcholine-specific phospholipase C; PEL, phosphatidylethanolamine; PI-PLC, phosphatidylinositol-specific phospholipase C; PLD, phospholipase D.

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been linked to several leukocyte antimicrobial mechanisms, including phagocytosis, generation of reactive oxidants, and granule secretion (29–36). The objectives of this study were to determine 1) whether ATP induces killing of virulent strains of \textit{M. tuberculosis} within infected human macrophages, and, if so 2), whether activation of PLD is required for this bactericidal activity.

### Materials and Methods

#### Chemicals and cells

HEPES, yozmosan, 2,3-DPG, trypan blue, leupeptin, aprotinin, bovine pancreatic trypsin, PMSE, IFN-γ, ATP, UTP, 3′-O-(4-benzoyl)benzozy-ATP (BzATP), EGTA, ethidium bromide, phosphatidylethanolamine, dimyristoylphosphatidylcholine, purified PLD preparations from \textit{Strepomyces chromofusca}, peanut, and cabbage, phosphatidylinositol-specific phospholipase C (PI-PLC), and phosphatidylcholine phospholipase C (PC-PLC) from \textit{Bacillus cereus} were obtained from Sigma (St. Louis, MO). RPMI 1640 medium with L-glutamine and PBS were purchased from Life Technologies Laboratories (Grand Island, NY). Middlebrook 7H9 broth was obtained from BBL Microbiology Systems (Cockeysville, MD), and 7H11 agar, oleic acid-albumin-dextrose-catalase enrichment medium, and auranine-rhodamine stain were from Difco Laboratories (Detroit, MI). Bactec 12B medium and PANTA PLUS kit were from Becton Dickinson (Spark, MD). Teflon wells were purchased from Savillex (Minnetonka, MN). Tissue culture plates were purchased from Linbro Flow Laboratories (McLean, VA). All organic solvents (HPLC grade) were obtained from Fisher (Fairlaw, NJ). Human serum albumin, genistein, and 1,25-dihydroxyvitamin VA were obtained from Calbiochem (San Diego, CA). [3H]Oleic acid was obtained from Amersham (Arlington Heights, IL). The THP-1 promonocytic cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The THP-1 cell line was purchased from Calbiochem (San Diego, CA). [3H]Oleic acid was obtained from Amersham (Arlington Heights, IL). TLD-1 promonocytic cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The THP-1 cell line was obtained from Calbiochem (San Diego, CA). [3H]Oleic acid was obtained from Amersham (Arlington Heights, IL).

#### Preparation of macrophages and differentiation of THP-1 cells

PBMC were isolated from healthy, purified protein derivative-negative, adult volunteers, and cultured in Teflon wells for 5 days in RPMI (pH 7.4) with 20% fresh autologous serum, as previously described (33). Macrophages (~2 × 10^6/sample) were purified by adhesion to 6-well plastic tissue culture plates or chronic acid-cleaned glass coverslips (25 mm diameter) for 2 h at 37°C in 5% CO₂. Mononuclear cells were washed repeatedly and then incubated in RPMI with 2.5% autologous serum, without antibiotics, for use in experiments. Effects of experimental manipulations on macrophage viability were assessed by exclusion of trypan blue, and monolayer density was determined by nuclei counting with napthol blue-black stain (33, 37). The human promonocytic THP-1 leukemia cell line was maintained in culture in RPMI, 10% FBS, 1% penicillin/streptomycin, 0.01% 2-ME at 37°C, 5% CO₂ in a humidified incubator (38, 39). THP-1 cells were differentiated to a macrophage phenotype by culture with 1000 µM poly(dI·dC)·poly(dI·dC), 5 µg/ml polymixin B, 20 µg/ml naldixic acid, 5 µg/ml trimethoprim, 5 µg/ml azlocillin. The content of [3H]C0₂ in the gas above the media was determined by on the day of inoculation of the Bactec bottles, and then once daily for 7 additional days, and expressed as the growth index on a linear scale. Determinations of mycobacterial viability by the Bactec method were in excellent agreement with the results of the CFU assay (Ref. 41 and data not shown).

#### Analysis of phagocytosis

Phagocytosis of \textit{M. tuberculosis} was determined as described (33, 42). Briefly, macrophage monolayers adherent to glass coverslips (~2 × 10^5 MDMS/coverslips) in 24-well tissue culture plates were incubated with \textit{M. tuberculosis} MOI 1:1 in RPMI, 20 mM HEPES, and 2.5% autologous nonimmune serum. Following incubation for various intervals, monolayers were washed repeatedly to remove nonadherent bacteria, fixed in 10% Formalin, and stained with auramine-rhodamine for 20 min (33, 42). Coverslips were washed with distilled water and incubated with acid alcohol for 3 min, washed, and incubated in KmnO₄ for 2 min. Adherent bacteria were quantitated by fluorescence microscopy of triplicate coverslips for each experimental condition (50–200 MDMS/coverslips), and results of a set of experiments were expressed as the mean (±SEM) number of adherent \textit{M. tuberculosis} per 100 macrophages (phagocytic index). Immunofluorescence microscopy demonstrated that all adherent mycobacteria are phagocytosed, both under control conditions, as well as in experiments in which phagocytosis is inhibited or augmented (Ref. 33 and data not shown).

#### Measurement of PLD activity

Macrophages were cultured in 6-well tissue culture plates at ~2 × 10^6 macrophages/well, and radioisotopically labeled with [3H]lyso-platelet-activating factor (3H]lyso-platelet-activating factor, 5 µCi/well) for 90 min at 37°C in RPMI, 20 mM HEPES, with 2.5% autologous serum. Following removal of unincorporated radioactivity, macrophages were incubated with ATP, nucleotide analogues, or buffer. In the majority of assays, 0.5% ethanol was added 2 min before stimulation, to permit detection of the specific transphosphatidylation product, [3H]phosphatidylethanol (PEt), as a metabolically stable index of PLD activity (32, 33). Production of phosphatidic acid (PA), the physiologic product of PLD-mediated catalysis, was monitored in samples supplemented with 5 µM leupeptin and 20 µM polymixin B. Following addition of substrates, reactions were terminated with 1.67 v/v of ice-cold methanol, macrophages were washed and transferred to polypropylene tubes, and 3.3 ml of chloroform was added. Chloroform/methanol extracts contained no viable \textit{M. tuberculosis}, as determined by absence of growth on 7H11 agar. Following phase separation, the chloroform layer was dried, and [3H]PA and [3H]PEt were isolated by TLC in an ethyl acetate/acetone/methanol/water (9:5:2) solvent system (33, 43), by comigration with pure phospholipid standards. Quantitation of [3H]cpm in PA and PEt was performed by liquid scintillation spectrophotometry, and counts were normalized for total cpm.
in phospholipid to correct for potential differences in labeling between experiments.

**Determination of the effects of metabolic inhibitors and purified phospholipases on the intracellular survival of **M. tuberculosis

To inhibit PLD-dependent generation of PA, infected macrophages were incubated with 2,3-DPG (0.1–3 mM) for 15 min or ethanol (0.03–15%) for 5 min at 37°C, before addition of ATP. After an additional 24-h incubation, macrophages were lysed and the viability of M. tuberculosis was quantitated, as noted above. In parallel samples, PLD activity was measured by determination of the levels of [3H]PHE or [3H]PA. In separate experiments, purified PLD from S. chromofuscus, peanut, or cabbage (0.1–100 U/ml) was added to ethanol- or 2,3-DPG-treated samples, and the effects on viability of M. tuberculosis and levels of [3H]PHE or [3H]PA were determined. Purified PI-PLC and PC-PLC from Bacillus cereus were utilized in parallel experiments. To test the requirement for enzymatic activity, phospholipases were inactivated by heating to 100°C for 10 min. One unit of PLD activity was defined as the production of 1 μmol PA/mg protein, utilizing [(32)P]dipalmitylophosphatidylcholine (DPPC)-labeled mixed phospholipid vesicles (phosphatidylethanolamine:phosphatidylinositol-4, 5-bisphosphate:DPPC (molar ratio of 16:1:4:1)) as substrate (44). Similar definitions were utilized for quantitation of the activities of PI-PLC (via production of inositol (1,4,5-trisphosphate) from phosphatidylinositol-4, 5-bisphosphate), and for PC-PLC (assayed as generation of [(32)P]choline from [(32)C]DPPC). All determinations of the sp. act. of purified phospholipases were conducted in RPMI 1640, 2.5% pooled human serum, at 37°C. 2,3-DPG, ethanol, and purified phospholipase preparations did not alter the viability of M. tuberculosis, either when incubated directly with mycobacteria in 7H9 media for 24 h at 37°C, or when added to infected macrophages in the absence of ATP (data not shown). These compounds also had no effect on macrophage viability or density of the monolayer.

**Data analysis**

Data from each experimental group were subjected to an analysis of normality and variance. Differences between experimental groups composed of normally distributed data were analyzed for statistical significance using Student’s t test. Nonparametric evaluation of other data sets was performed with the Wilcoxon rank sum test (45).

**Results**

**ATP induces killing of virulent M. tuberculosis within human macrophages**

To determine the effect of ATP on the viability of M. tuberculosis within infected human macrophages, we utilized a model in which blood monocytes are differentiated to a macrophage phenotype by in vitro cultivation for 5 days, followed by purification by adherence to tissue culture plates (33). Phagocytosis of the virulent H37Rv strain of M. tuberculosis by human macrophages was complete by 60 min (data not shown). At a bacterium/macrophage ratio of 1:1, 52% (range 48–57%) of the initial inoculum was ingested within 60 min, and 33% (range 30–36%) of the macrophages contained at least one intracellular bacillus. This low MOI has previously been shown to result in improved stability of the macrophage monolayer during prolonged in vitro culture, compared with higher levels of infection (13, 15, 40, 46). Following infection for 60 min, nonadherent bacilli were removed by repeated washings, and the infected macrophage monolayer was incubated in RPMI, 10% human serum, with or without ATP (3 mM). Infected macrophages were then cultured for 1 to 7 days, and the viability of intracellular M. tuberculosis was determined by quantitation of CFUs. Because prolonged culture of macrophages is accompanied by detachment of a minority of cells from the monolayer, we utilized a protocol based on the work of Kaplan and colleagues, in which the supernatant and adherent monolayer are combined in assessment of mycobacterial viability (40). Specific attention was directed at minimizing the clumping of M. tuberculosis at all stages of the experiment. As noted in Materials and Methods, the infecting inoculum contained >95% single bacilli, with a viability of ≥80%.

The growth of M. tuberculosis in untreated macrophages was characterized by a doubling time of 25.6 ± 1.6 h. Incubation of infected macrophages with 3 mM ATP resulted in significant reduction in the viability of intracellular M. tuberculosis, compared with control cultures treated with buffer alone (Fig. 1A). At 24 h, ATP-treated macrophages exhibited a 81 ± 5% reduction in viable bacilli (p < 0.001, n = 12). The tuberculocidal effect of ATP was most pronounced in those samples cultured for 7 days, in which ATP-treated samples exhibited a 3.5-log reduction in viable M. tuberculosis (p < 0.001, n = 5). The concentration dependence of ATP-induced killing of intracellular M. tuberculosis exhibited maximal efficacy at 3 mM ATP (data not shown). Compared with the initial level of infection (day 0), ATP-treated samples exhibited a 33-fold reduction in mycobacterial viability at day 7 (p < 0.001, represented as a 1.52-log reduction in Fig. 1A), consistent with a bactericidal, rather than a bacteriostatic, effect of ATP. Because the precision of the CFU assay was greatest at 24 h following addition of buffer or ATP, the majority of experiments were performed at this time point.

In addition to the H37Rv strain of M. tuberculosis, the effects of ATP on mycobacterial viability were evaluated utilizing two other well-characterized, highly virulent M. tuberculosis strains. The Erdman strain has been utilized extensively for both in vitro and in vivo studies of tuberculous pathogenesis (47, 48). The CSU#93 strain of M. tuberculosis has recently been established as a highly transmissible, virulent reference strain, following its identification as the cause of a large outbreak of infection in the southern United States (49). ATP treatment of macrophages infected with Erdman or CSU#93 M. tuberculosis resulted in significant killing of these virulent strains (Fig. 1B), to an extent similar to that noted with the H37Rv strain.

To establish a relevant phagocytic cell model in which ATP-dependent killing of M. tuberculosis could be characterized in greater detail, THP-1 human promonocytic leukocytes were differentiated to a macrophage phenotype by culture with IFN-γ, vitamin D3, and bacterial LPS (38, 39). dTHP-1 cells became adherent to tissue culture plastic or extracellular matrix proteins, demonstrated increased phagocytic capacity compared with undifferentiated cells, and formed numerous pseudopods (data not shown). Infection of dTHP-1 cells with the three virulent strains of M. tuberculosis (H37Rv, Erdman, and CSU#93), followed by treatment with ATP (0.1–5 mM), resulted in a level of killing of intracellular bacilli that closely approximated that demonstrated, above, in primary monocyte-derived human macrophages (Fig. 1C). Maximal tuberculocidal activity, induced by 3 mM ATP, resulted in a 78% decrease in viability of intracellular M. tuberculosis at 24 h (range 74–82%, p < 0.001, n = 21). The similarity in the ATP dose dependence and maximal efficacy of antituberculous activity expressed by primary macrophages and dTHP-1 cells supports the relevance of the latter as a model for in vitro characterization of the bactericidal activity of human macrophages toward M. tuberculosis.

To determine whether stimulation of P2X7 receptors (P2X7-R) was required for ATP-induced bactericidal activity toward virulent tubercle bacilli, macrophages were preincubated with the specific, irreversible P2X7-R inhibitor, 2′,3′-dialdehyde-ATP (oxidized ATP, oATP) (50). Exposure to oATP completely blocked ATP-induced killing of intracellular H37Rv M. tuberculosis (Fig. 2A). Further evidence for the involvement of P2X7-R consisted in reversible inhibition of tuberculocidal activity by elevation of extracellular Mg2+ (with 10 mM MgCl2), which decreases the concentration of ATP4− (the primary agonist of P2X7-R) (18, 23, 25, 26). Finally, the highly selective and potent P2X7-R agonist, BzATP (27, 28), significantly reduced the viability of H37Rv M. tuberculosis (reduction of 52%, range 48–57%, p < 0.001, n = 12). As

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with ATP, the tuberculocidal effect of BzATP was completely inhibited by oATP or MgCl₂ (Fig. 2A). In contrast, the P₂Y₂ receptor agonist, UTP, did not affect the viability of intracellular M. tuberculosis (data not shown). Taken together, these studies demonstrate that ATP-dependent killing of intracellular M. tuberculosis requires stimulation of P₂X₇-R.

ATP-induced tuberculocidal activity is dependent on activation of macrophage PLD

Stimulation of P₂X₇ receptors induces multiple, biochemically diverse changes in macrophages, including opening of a nonselective plasma membrane pore permeable to molecules <900 Da, rapid influx of Ca²⁺ and Na⁺, efflux of K⁺, plasma membrane depolarization, stimulation of PLD activity, and apoptosis (23, 27, 28). We hypothesized that activation of PLD is involved in ATP-dependent tuberculocidal activity, because PLD has been associated with several antimicrobial functions of phagocytes, including generation of reactive oxidants and secretion of microbicidal compounds (29–36). To test this hypothesis, we first compared the
effects of P₂X₇-R agonists and antagonists on macrophage PLD activity with their effects on tuberculocidal activity (Fig. 2A). In agreement with the work of Dubyak and colleagues (27, 28), stimulation of [³H]oleate-labeled macrophages with ATP or BzATP resulted in marked stimulation of PLD activity, which was inhibited by oATP or MgCl₂ (Fig. 2B). Comparison of Fig. 2, A and B, demonstrated that P₂X₇ receptor-mediated activation of PLD correlated closely with killing of intracellular M. tuberculosis.

To further evaluate the hypothesis that PLD functions in ATP-induced tuberculocidal activity, we determined the effects of two PLD inhibitors, 2,3-DPG and ethanol, on macrophage PLD activity and killing of intracellular M. tuberculosis. Although there is no specific inhibitor of PLD, 2,3-DPG exhibits several advantages as a PLD inhibitor, including: 1) a defined competitive mechanism of action, 2) low toxicity to intact cells, 3) inhibition of PLD-dependent phagocyte responses (including superoxide generation and phagocytosis), and 4) lack of inhibition of phosphatidylinositol-specific phospholipase C (the other major source of diglycerides in activated phagocytes) (33, 34). Preincubation of macrophage monolayers with 2,3-DPG (0.1–3 mM) for 15 min resulted in concentration-dependent reductions in ATP-stimulated PLD activity (Fig. 3A). The maximal concentration of 2,3-DPG, 3 mM, produced an 88% reduction (range 83–93%) in ATP-induced PLD activity, compared with macrophages treated with ATP alone (p < 0.001, n = 6), without affecting macrophage viability or monolayer density (33). 2,3-DPG-induced inhibition of ATP-stimulated PLD activity was closely paralleled by concentration-dependent reductions in killing of intracellular M. tuberculosis (Fig. 3B). Over the concentration range tested, 3 mM 2,3-DPG produced the greatest inhibition of tuberculocidal activity, 83% (range 78–89%, p < 0.001, compared with macrophages treated with ATP alone, n = 6). BzATP-induced killing of M. tuberculosis was inhibited to a similar extent by 2,3-DPG (data not shown). In the absence of ATP or BzATP, 2,3-DPG did not alter the viability of intracellular M. tuberculosis, nor did it affect the growth of extracellular tubercle bacilli (data not shown).

Short-chain primary alcohols, such as ethanol, inhibit PLD-mediated generation of PA by substituting for water as the nucleophilic acceptor of the phosphatidyl moiety (33, 36). In this way, such compounds inhibit PLD-dependent responses without blocking enzyme turnover, thus providing a complementary method of PLD inhibition that is mechanistically distinct from that of 2,3-DPG. Incubation of macrophages with ethanol (0.03–1%, v/v) for 2 min at 37°C, before addition of ATP, resulted in concentration-dependent inhibition of PA generation (Fig. 3D). Over the concentration range tested, 1% ethanol inhibited ATP-induced PA generation by 89% (range 85–93%, p < 0.001, n = 5). Ethanol also significantly inhibited killing of intracellular M. tuberculosis by ATP (Fig. 3D) or BzATP (data not shown). Inhibition of macrophage tuberculocidal activity by ethanol was concentration dependent, with 1% ethanol resulting in an 84% reduction in ATP-induced bactericidal activity (range 81–87%, p < 0.001, n = 5). In the absence of ATP or BzATP, 0.03–1%, ethanol did not alter the viability of M. tuberculosis within human macrophages, and it had no effect on macrophage viability.
or monolayer density (data not shown). In the absence of macrophages, these concentrations of ethanol also had no effect on the viability of extracellular *M. tuberculosis* in 7H9 media (data not shown).

Reconstitution of PLD-mediated signal transduction by purified PLD is associated with restoration of ATP-dependent tuberculocidal activity in 2,3-DPG- or ethanol-treated macrophages

The strong correlations between inhibition of ATP-stimulated PLD activity and reductions in mycobacterial killing support the hypothesis that PLD functions to promote macrophage tuberculocidal activity. However, it is possible that the PLD inhibitors promote intracellular growth of *M. tuberculosis* by mechanisms other than inhibition of PLD. Therefore, to further evaluate the role of PLD in ATP-dependent killing of intracellular *M. tuberculosis*, purified PLD from *S. chromofuscus* was utilized to reconstitute PLD-dependent signaling in macrophages in which endogenous PLD activity had been inhibited. Although administration of purified PLD preparations is limited in its ability to reconstitute the precise spatial and temporal determinants of PA production by endogenous phospholipase activity, this approach has repeatedly provided valuable information on PLD-mediated signal transduction in many cell types, including macrophages (29, 33, 52, 53).

To determine the approximate levels of *S. chromofuscus* PLD required to reconstitute PLD-dependent signaling in 2,3-DPG-treated macrophages, levels of PEt accumulation were determined at various concentrations of this purified PLD. Addition of *S. chromofuscus* PLD resulted in dose-dependent accumulation of PEt (Fig. 4A). Of note, incubation of 2,3-DPG-treated macrophages with 5 U/ml of *S. chromofuscus* PLD was associated with levels of PEt accumulation that closely approximated those produced in control cells stimulated by ATP (in the absence of 2,3-DPG). Addition of exogenous PLD to 2,3-DPG-treated macrophages also resulted in dose-dependent increases in tuberculocidal activity (Fig. 4B). Restoration of tuberculocidal activity to near normal levels occurred in samples in which PLD activity was essentially fully reconstituted by addition of 5 U/ml of *S. chromofuscus* PLD.

Heat inactivation of *S. chromofuscus* PLD (80°C, 30 min) eliminated both its ability to supplement endogenous PLD activity in 2,3-DPG-treated macrophages, and to augment tuberculocidal activity (data not shown). In the absence of ATP, addition of purified PLD to control or 2,3-DPG-treated macrophages did not result in decreased viability of intracellular *M. tuberculosis*. Similarly, *S. chromofuscus* PLD had no effect on the viability of extracellular *M. tuberculosis*, determined by growth in 7H9 media (data not shown). Because 2,3-DPG can inhibit the exogenous *S. chromofuscus* PLD (51), as well as the macrophage lipase (33), we characterized the interdependence of these two variables (2, 3-DPG and *S. chromofuscus* PLD) on macrophage tuberculocidal activity. In macrophages incubated with the maximally effective tuberculocidal concentration of ATP (3 mM), the IC_{50} for 2,3-DPG was 0.6 mM (Fig. 4C). Addition of 1 U/ml of *S. chromofuscus* PLD resulted in an increase in the IC_{50} of 2,3-DPG to 3 mM. A total of 5 U/ml of exogenous PLD eliminated the ability of 2,3-DPG (0.3–9 mM) to achieve 50% inhibition of ATP-stimulated tuberculocidal activity (Fig. 4C).

Similar studies utilizing *S. chromofuscus* PLD were performed with ethanol-treated macrophages. Addition of purified PLD resulted in concentration-dependent increases in the accumulation of [3H]PA in [3H]oleate-labeled macrophages. Incubation of ethanol (1%)- and ATP (3 mM)-treated macrophages with 10 U/ml of *S. chromofuscus* PLD resulted in a level of PA production (874 ± 72 cpm) that closely approximated that found in macrophages treated with 2,3-DPG (0–5 U/ml) and 3 mM ATP. A total of 5 U/ml of purified PLD eliminated the ability of 2,3-DPG to achieve 50% inhibition of ATP-stimulated tuberculocidal activity (Fig. 4C).

In macrophages incubated with 3 mM ATP and the indicated concentrations of *S. chromofuscus* PLD were added. The viability of intracellular *M. tuberculosis* was determined at 24 h and expressed as the growth index. Results in A—C represent mean ± SEM of four identical experiments, each performed in triplicate.
with ATP alone (885 ± 66 cpm) (Fig. 5A). In parallel with reconstitution of PA production, addition of *S. chromofuscus* PLD resulted in dose-dependent restoration of ATP-dependent mycobacterial killing (Fig. 5B). A total of 10 U/ml of exogenous PLD resulted in levels of tuberculocidal activity in ethanol- and ATP-treated macrophages that compared closely with those demonstrated in macrophages treated with ATP alone. In the absence of ATP, *S. chromofuscus* PLD did not alter mycobacterial viability in either control or ethanol-treated macrophages (data not shown). More complete analysis of the interactions of ethanol and *S. chromofuscus* PLD demonstrated that 1 U/ml of exogenous PLD increased the IC_{50} of ethanol from ~0.06% to 0.28% (Fig. 5C). A total of 5 U/ml of *S. chromofuscus* PLD resulted in a further increase of the IC_{50} for ethanol to ~0.9%. Thus, inhibition of ATP-induced tuberculocidal activity by 2,3-DPG or ethanol, and its reversal by purified PLD, strongly supports the hypothesis that endogenous PLD functions in ATP-dependent killing of intracellular *M. tuberculosis*.

The strong correlation between total PLD activity (endogenous + exogenous) and tuberculocidal activity suggested the additional hypothesis that *S. chromofuscus* PLD could augment ATP-induced tuberculocidal activity in control macrophages (i.e., in the absence of 2,3-DPG or ethanol). As demonstrated in Fig. 6, exogenous PLD increased both the potency and efficacy of ATP-dependent mycobacterial killing. Addition of 1 U/ml of *S. chromofuscus* PLD decreased the EC_{50} for ATP-induced tuberculocidal activity from 1 mM to ~25 μM. In addition, exogenous PLD potentiated the maximal level of bactericidal activity (at 3 mM ATP) from 78% (range 75–83%) to 94% (range 91–100%, p < 0.01, n = 4).

To determine whether the augmentation of ATP-induced tuberculocidal activity by *S. chromofuscus* PLD was a property common to this lipase class, we assessed the phospholipase specificity of this response. Purified preparations of PLD from cabbage and peanut, like *S. chromofuscus* PLD, were able to augment ATP-stimulated tuberculocidal activity. The mean (±SEM) growth index for untreated MDMs infected with H37Rv *M. tuberculosis* was 517 ± 46. Treatment with 3 mM ATP decreased this to 123 ± 11, and further decreases in the *M. tuberculosis* growth index resulted from addition of purified PLD from peanut (17 ± 3), cabbage (30 ± 4), or *S. chromofuscus* (24 ± 2). In contrast, purified phosphatidylinositol-specific phospholipase C (growth index 142 ± 22) or phosphatidylincholine phospholipase C (131 ± 16), at the concentrations utilized for the PLD enzymes, did not alter the level of killing of intracellular *M. tuberculosis* induced by ATP alone. The fact that evolutionarily diverse PLD enzymes all potentiated tuberculocidal activity provides further support for the hypothesis that endogenous PLD functions in ATP-stimulated killing of *M. tuberculosis* within infected human macrophages.

**Discussion**

An essential feature of the pathogenesis of tuberculosis is the bacilli’s survival within macrophages of the infected host (5, 6). The dramatically increasing incidence of antibiotic-resistant *M. tuberculosis* has compromised both the treatment and prevention of this global disease (3, 4). Our ability to control the enormous health burden of tuberculosis will most likely require development of novel therapeutic approaches. Improvements in both pharmacologic and vaccine-based therapies will benefit greatly from a more detailed understanding of the mechanisms that promote natural immunity to *M. tuberculosis*. At present, there is no physiologic agonist that can unambiguously stimulate the antituberculosis activity of human macrophages, although conflicting data regarding several cytokines and other inflammatory mediators have been presented in the discussion.
concentrations of ATP\textsubscript{e} (18, 54). The efficacy of ADP, AMP, and UTP; and 4) the requirement for mM
inhibitors, oATP and Mg\textsuperscript{2+}, in BCG, within human macrophages (19, 20), we tested the hypothesis that ATP\textsubscript{e} would exert a similar microbicidal
mechanism, which is not induced by BzATP. This revealed both similarities and differences compared with its effect
on intracellular BCG (19, 20). The most significant similarity was
the requirement for stimulation of macrophage cell surface P\textsubscript{2}X\textsubscript{7} receptors. In the case of M. tuberculosis, a necessary
role for P\textsubscript{2}X\textsubscript{7}-R was supported by several lines of evidence, including 1) inhibition of ATP\textsubscript{e}-dependent killing by the P\textsubscript{2}X\textsubscript{7}-R inhibitors, oATP and Mg\textsuperscript{2+};
2) stimulation of tuberculocidal activity by the selective P\textsubscript{2}X\textsubscript{7} receptor agonist, BzATP; 3) lack of
efficacy of ADP, AMP, and UTP; and 4) the requirement for mM
concentrations of ATP\textsubscript{e} (18, 54).

However, ATP-dependent killing of virulent M. tuberculosis
within human macrophages exhibited an important difference from
that previously documented with BCG (19, 20), namely, that ATP
was more efficacious than BzATP. Specifically, the maximal
BzATP-induced reduction in viability of M. tuberculosis (reduction of 52%) was consistently less than that induced by ATP (reduction of 78%). Because the efficacy of BzATP is greater than
that of ATP in stimulating P\textsubscript{2}X\textsubscript{7}-R-dependent cellular responses (25–27), these results suggest that ATP activates an additional tuberculocidal mechanism, which is not induced by BzATP. This

P\textsubscript{2}X\textsubscript{7}-R-independent effect of ATP is not due to stimulation of the other class of macrophage purinergic receptors, P\textsubscript{2}Y\textsubscript{2}, because the
P\textsubscript{2}Y\textsubscript{2}-R agonist, UTP, did not induce tuberculocidal activity, and did not enhance ATP- or BzATP-stimulated killing of M. tuberculosis. In summary, the nucleotide selectivity studies suggest that P\textsubscript{2}X\textsubscript{7}-R are necessary, but not sufficient, for maximal ATP-dependent
cutting of intracellular M. tuberculosis by human macrophages. Our findings parallel those of Bloom and colleagues, who recently demonstrated P\textsubscript{2}X\textsubscript{7}-R-independent killing of intracellular
BCG by murine macrophages (24). At present, we have no further information concerning the mechanism of this proposed P\textsubscript{2}X\textsubscript{7}-R-independent tuberculocidal effect of ATP.

Because the pathogenesis of tuberculosis involves multiple
mechanisms by which the bacilli can evade host immune defenses, it is likely that effective cell-mediated immunity to M. tuberculosis exhibits a similar level of complexity. This may be particularly true of ATP\textsubscript{e}-dependent tuberculocidal activity, because stimulation of P\textsubscript{2}X\textsubscript{7}-R results in multiple changes in macrophage physiolo
(19, 23, 25–27). We have focused on P\textsubscript{2}X\textsubscript{7}-R-dependent activation of PLD, due to the strong association of this phospholipase with the antimicrobial defenses of phagocytic leukocytes (29–36). Support for an important role of PLD in ATP\textsubscript{e}-induced tuberculocidal activity in human macrophages consisted of: 1) concordant inhibition of PLD activity and mycobacterial killing by the mechanistically distinct PLD inhibitors, 2,3-DPG and ethanol; 2) restoration of ATP\textsubscript{e}-induced killing by purified PLD, coincident with reconstitution of control levels of total PLD activity (exogenous + endogenous) in inhibitor-treated cells; and 3) potentiation of ATP-mediated tuberculocidal activity in control cells by exogenous PLD. Of note, purified PLD does not alter the viability of extracellular tubercle bacilli, and, in the absence of ATP, does not effect killing of intracellular M. tuberculosis. Thus, we hypothesize that the role of PLD is as a signal transduction intermediate that activates an, as yet, unidentified, bactericidal mechanism. Melen
dez et al. (55) have recently demonstrated that PLD regulates the intracellular trafficking of endocytosed immune complexes to lysosomes, suggesting that PLD may function in the mechanistically analogous maturation of phagosomes to microbicidal phagosomes. Experiments to evaluate this hypothesis are currently in progress.

Despite numerous demonstrations that purified PLD prepara
tions closely mimic the effects seen after activation of their endo
genous homologues (29, 33, 52, 53), several important questions
remain unresolved, including: 1) Does exogenous (extracellular)
PLD access phospholipid substrates and release products in a man
ner similar to that of endogenous (intracellular) PLD? 2) Does
addition of purified PLD result in increased hydrolysis of phos
holipids in intracellular membranes (Golgi, nuclei, granules,
etc.)? The recent cloning of two mammalian P2 X 7 receptors (56,
57) will permit more detailed evaluation of the requirement for
PLD in ATP\textsubscript{e}-induced tuberculocidal activity in human macrophages.

ATP accumulates in extracellular inflammatory fluid at concen
trations sufficient to stimulate P\textsubscript{2}X\textsubscript{7}-R and induced macrophage
tuberculocidal activity (18, 54). Potential sources of ATP\textsubscript{e}
include release from the cytosol of necrotic cells, secretory granules of
cytotoxic T cells and platelets, and export via plasma membrane
ABC transporters. Several recent reports demonstrate that macro
phages also secrete ATP, and that ATP\textsubscript{e} functions in an autocrine
pathway to promote macrophage activation and microbicidal ac
tivity (24, 54). At a focus of tuberculous inflammation, it is likely that both macrophages, as well as CD8\textsuperscript{+} T cells (and perhaps other

![FIGURE 6. Exogenous PLD augments ATP-dependent killing of intracellular M. tuberculosis in control macrophages. dTHP-1 macrophages were infected with H37Rv M. tuberculosis for 1 h, and then monolayers were washed and incubated for 24 h at 37°C. S. chromofuscus PLD (1 U/ml) or buffer (−PLD), and the indicated concentrations of ATP were added, followed by culture for an additional 24 h. Results represent mean ± SEM of three experiments, each performed in triplicate. The difference in the M. tuberculosis growth index between samples treated with either PLD or buffer was statistically significant (p < 0.002) at all ATP concentrations tested.](image-url)
lymphocytes), serve as sources of ATP. In addition, other components of CD8 T cells, specifically the granular proteins, perforin and granulysin (58, 59), can contribute to killing of tubercle bacilli.

A feature common to both the ATP- and perforin/granulysin-induced pathways of mycobacterial immunity is the induction of macrophage apoptosis (19, 20, 58, 59). We have noted similar ATP-induced apoptosis in both monocyte-derived macrophages and dTHP-1 cells (data not shown), but have no further information on the relation between apoptosis and mycobacterial killing.

This is a potentially rich area of investigation, because inhibition of the apoptosis of parasitized cells has been proposed as a virulence mechanism of a diverse range of intracellular pathogens, including viruses, bacteria, protozoa, and fungi (14, 20, 46). Indeed, Bal Acewicz-Sablinska et al. (14) have recently demonstrated that M. tuberculosis inhibits apoptosis of infected macrophages via inhibition of TNF-α-mediated autocrine signaling pathways.

Further characterization of the mechanism by which ATP, and PLD promote killing of M. tuberculosis within infected human macrophages may yield insights into the complement mechanisms of mycobactericidal activity induced by other inflammatory mediators, including cytokines. Advances in our understanding of the complex interactions between M. tuberculosis and the infected human host will form the foundation for therapeutic efforts to enhance immune defenses to this challenging and pervasive pathogen.

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