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ATP-Induced Killing of Virulent *Mycobacterium tuberculosis* Within Human Macrophages Requires Phospholipase D[1]

David J. Kusner[2]*†‡ 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 P_2X_7 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 D_3) (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 P_2X_7 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 P_2X_7 receptors (formerly designated P_2Z) are notable for their restricted cellular distribution (macrophages, mast cells, and certain lymphocyte populations) and their stimulus-dependent formation of large, nonselective membrane pores, which, in macrophages, are permeable to hydrophilic molecules $\leq$900 Da (19, 23–26). Stimulation of macrophage P_2X_7 receptors is associated with a marked increase in the activity of phospholipase D (PLD) (27, 28), an enzyme that has

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[3] Abbreviations used in this paper: ATP_e, extracellular ATP; BCG, bacillus Calmette-Guérin; BzATP, 3′-*O*-(-benzoyl)benzoyl-ATP; CR, complement receptor; 2,3-DPG, 2,3-diphosphoglycerate; DPPC, [^{32}P]dipalmitoylphosphatidylcholine; dTHP, differentiated THP; MDM, monocyte-derived macrophage; MOI, multiplicity of infection; oATP, oxidized ATP (2′,3′-dialdehyde-ATP); PA, phosphatidic acid; PC-PLC, phosphatidylcholine-specific phospholipase C; PEt, phosphatidylethanol; 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_e induces killing of virulent strains of *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, zymosan, 2,3-DPG, trypan blue, leupeptin, aprotinin, bovine pancreatic trypsin, PMSF, IFN-γ, ATP, UTP, 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP), EGTA, ethidium bromide, phosphatidylethanol, dimyristoylphosphatidic acid, purified PLD preparations from *Streptomyces chromofuscus*, peanut, and cabbage, phosphatidylinositol-specific phospholipase C (PI-PLC), and phosphatidylcholine phospholipase C (PC-PLC) from *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 auramine-rhodamine stain were from Difco Laboratories (Detroit, MI). Bactec 12B medium and PANTA PLUS kit were from Becton Dickinson (Sparks, MD). Teflon wells were obtained from Savillex (Minnetonka, MN). Tissue culture plates were purchased from Linbro Flow Laboratories (McLean, VA). All organic solvents (HPLC grade) were obtained from Fisher (Fairlawn, NJ). Human serum albumin, genistein, and 1,25-dihydroxyvitamin D_3 were purchased from Calbiochem (San Diego, CA). [^{3}H]Oleic acid was from Amersham (Arlington Heights, IL). The THP-1 promonocytic cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The H37Rv and Erdman strains of *M. tuberculosis* were obtained from the ATCC. The CSU#93 *M. tuberculosis* strain was generously provided by Dr. Thomas Shinnick (Centers for Disease Control, Atlanta, GA), and LPS from *Salmonella minnesota*, R5 strain, was a kind gift of Dr. Michael A. Apicella (University of Iowa, Iowa City, IA).

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 ($\sim 2 \times 10^6$/sample) were purified by adherence to 6-well plastic tissue culture plates or chromic acid-cleaned glass coverslips (25 mm diameter) for 2 h at 37°C in 5% CO_2. Monolayers 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% CO_2 in a humidified incubator (38, 39). THP-1 cells were differentiated to a macrophage phenotype by culture with 1000 U/ml IFN-γ, 100 nM 1,25-dihydroxyvitamin D_3, 1 ng/ml LPS in RPMI, 10% FBS, without antibiotics, for 48 h. Differentiated THP-1 cells (dTHP-1, 2×10^6/sample) were washed twice, and reconstituted in RPMI, 2.5% pooled human serum, before use in experiments.

Bacteria

Virulent strains of *M. tuberculosis* (H37Rv, Erdman, and CSU#93) were cultured and prepared for use in experiments, as noted previously (33). Briefly, aliquots of frozen *M. tuberculosis* stocks in 7H9 broth were thawed, cultured for 9 days on 7H11 agar at 37°C in 5% CO_2-95% air, scraped from agar plates, and suspended in RPMI by vortexing briefly. After settling, the supernatant was transferred to a new tube and allowed to settle once again. An aliquot of this final *M. tuberculosis* suspension was counted in a Petroff-Hauser chamber, and the concentration of bacteria was adjusted for use in experiments. *M. tuberculosis* preparations contained >95% single bacteria, with ≥80% viability by determination of CFUs (33). The effects of various experimental manipulations on the viability of *M. tuberculosis* were determined by analysis of CFUs.

Infection of macrophages with M. tuberculosis *and analysis of intracellular survival*

Primary macrophage monolayers or dTHP-1 macrophage-like cells in RPMI, 20 mM HEPES, 2.5% human serum were infected with *M. tuberculosis*, at a bacteria/macrophage ratio (multiplicity of infection, MOI) of 1:1, and then incubated for 1 h at 37°C. Monolayers were washed three times with RPMI at 37°C and incubated with RPMI, 10% serum for 24 h, before addition of ATP or buffer control. Following stimulation, infected macrophages were incubated at 37°C for 1 to 7 days, before quantitation of the growth of *M. tuberculosis* by determination of CFUs or by the Bactec method. The CFU assay was adapted from Kaplan and coworkers (40), with minor modifications. Because prolonged in vitro culture of macrophages is accompanied by detachment of a minority of cells from the monolayer, the viability of intracellular *M. tuberculosis* was determined by combining adherent and suspension macrophages for each sample. Supernatants (1 ml) from each sample were placed in 1.5 ml O-ring sealed microfuge tubes containing 290 μl of 7H9 media and 60 μl of 1% SDS in PBS. Samples were vortex mixed and incubated at room temperature for 10 min, before addition of 150 μl of 20% BSA in PBS. Cells remaining attached to the tissue culture wells were incubated with ice-cold sterile distilled H_2O for 10 min at 25°C, with intermittent gentle shaking. Following addition of 660 μl of 7H9 media and 60 μl of 1% SDS in PBS, wells were incubated for an additional 10 min, before addition of 300 μl of 20% BSA in PBS, and contents were transferred to O-ring sealed microfuge tubes. Samples derived from both supernatant and adherent fractions were centrifuged at $10{,}000 \times g$ for 10 min at 4°C, followed by removal of the supernatant and resuspension of the cell pellets in 100 μl of 7H9. Samples from initial supernatant and adherent fractions were combined, and the growth of *M. tuberculosis* was quantitated following incubation of triplicate dilutions on 7H11 agar.

Growth of *M. tuberculosis* by the Bactec method was determined by the level of mycobacterial-derived $^{14}CO_2$ from [^{14}C]palmitate. Samples were processed in the same manner as for the CFU determination, above, followed by addition of the 200-μl aliquot from each sample to 4 ml of Bactec 12B medium containing PANTA PLUS antimicrobial supplement (50 U/ml polymixin B, 5 μg/ml amphotericin, 20 μg/ml nalidixic acid, 5 μg/ml trimethoprim, 5 μg/ml azlocillin). The content of $^{14}CO_2$ in the gas above the media was determined 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 *M. tuberculosis* was determined as described (33, 42). Briefly, macrophage monolayers adherent to glass coverslips ($\sim 2 \times 10^5$ MDMs/coverslip) in 24-well tissue culture plates were incubated with *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 $KMnO_4$ for 2 min. Adherent bacteria were quantitated by fluorescence microscopy of triplicate coverslips for each experimental condition (50–200 MDMs/coverslip), and results of a set of experiments were expressed as the mean (±SEM) number of adherent *M. tuberculosis* per 100 macrophages (phagocytic index). Electron microscopic 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 42 and data not shown).

Measurement of PLD activity

Macrophages were cultured in 6-well tissue culture plates at $\sim 2 \times 10^6$ macrophages/well, and radioisotopically labeled with [^{3}H]lyso-platelet-activating factor ([^{3}H]lyso-platelet-activating factor, 5 μCi/well) for 90 min at 37°C in RPMI, 20 mM HEPES with 2.5% serum (33). Following washing to remove 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, [^{3}H]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 determined in samples lacking ethanol. Following a 30-min incubation, reactions were terminated with 1.67 vol of ice-cold methanol, macrophages were scraped and transferred to polypropylene tubes, and 3.3 vol of chloroform was added (33). Chloroform/methanol extracts contained no viable *M. tuberculosis*, as determined by absence of growth on 7H11 agar. Following phase separation, the chloroform layer was dried, and [^{3}H]PA and [^{3}H]PEt were isolated by TLC in an ethyl acetate/isooctane/acetic acid (9:5:2) solvent system (33, 43), by comigration with pure phospholipid standards. Quantitation of [^{3}H]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 (0.03–1%) 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 [^{3}H]PEt or [^{3}H]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 [^{3}H]PEt or [^{3}H]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]dipalmitoylphosphatidylcholine (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 [^{14}C]choline from [^{14}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. 1*A*). 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. 1*A*), 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. 1*B*), 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 D_3, 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. 1*C*). 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 P_2X_7 receptors (P_2X_7-R) was required for ATP-induced bactericidal activity toward virulent tubercle bacilli, macrophages were preincubated with the specific, irreversible P_2X_7-R inhibitor, 2′,3′-dialdehyde-ATP (oxidized ATP, oATP) (50). Exposure to oATP completely blocked ATP-induced killing of intracellular H37Rv *M. tuberculosis* (Fig. 2*A*). Further evidence for the involvement of P_2X_7-R consisted in reversible inhibition of tuberculocidal activity by elevation of extracellular Mg^{2+} (with 10 mM $MgCl_2$), which decreases the concentration of ATP^{4-} (the primary agonist of P_2X_7-R) (18, 23, 25, 26). Finally, the highly selective and potent P_2X_7-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

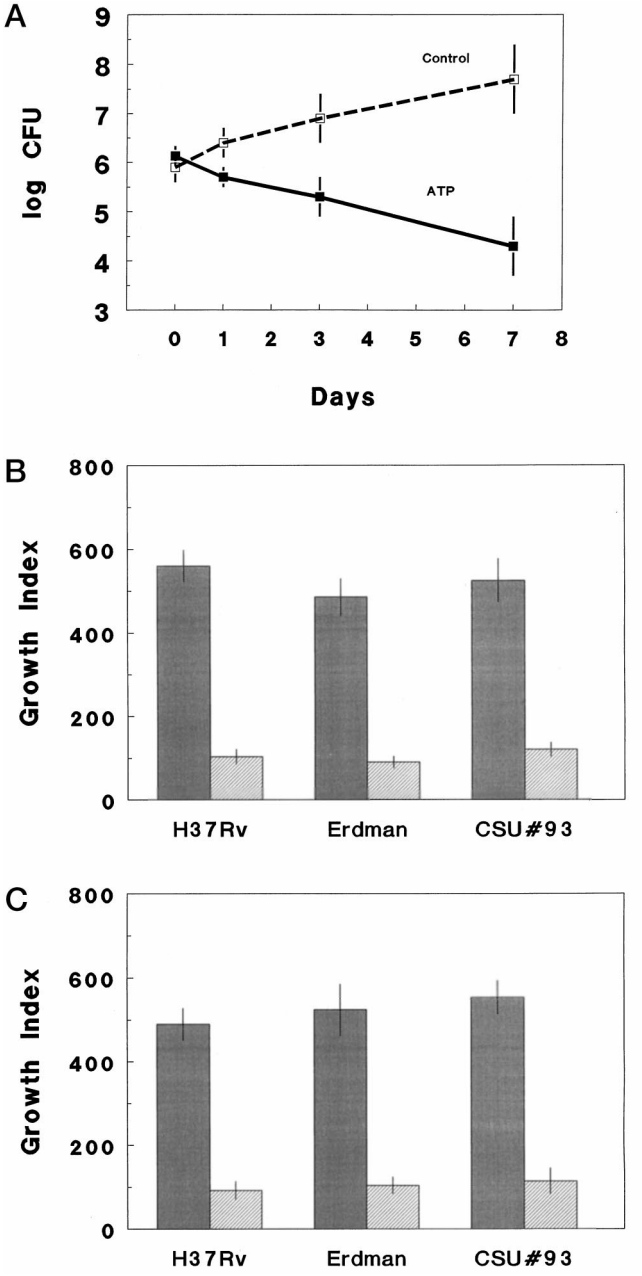

FIGURE 1. ATP induces the killing of virulent *M. tuberculosis* within human macrophages. *A*, Human monocyte-derived macrophages (MPs) were incubated with H37Rv *M. tuberculosis*, at an MOI of 1:1, for 60 min at 37°C, in RPMI, 2.5% autologous, nonimmune serum. Nonadherent bacilli were removed by washing, before treatment of samples with buffer alone (control, □) or 3 mM ATP (■). Infected macrophage monolayers were cultured for 1, 3, or 7 days at 37°C, 5% CO_2, followed by determination of CFUs. *B*, Primary monocyte-derived macrophages were infected with the indicated strains of *M. tuberculosis*, at an MOI of 1:1. Following addition of ATP (3 mM), infected macrophages were incubated for an additional 24 h. Survival of intracellular *M. tuberculosis* was determined following lysis of infected macrophages, utilizing the Bactec method, and is expressed on the abscissa as the growth index, determined by mycobacterial production of $[^{14}C]CO_2$ from [^{14}C]palmitate. *C*, The experiment was conducted as in *B* above, except that dTHP-1 macrophages were utilized, rather than primary macrophages. Data represent mean ± SEM of triplicate determinations from at least five identical experiments for each panel. The significance of differences in mycobacterial viability between control and ATP-treated samples was determined with the Wilcoxon rank sum test, and statistically significant differences existed at all time points in *A* and for each strain of *M. tuberculosis* in *B* and *C* ($p < 0.001$ for each).

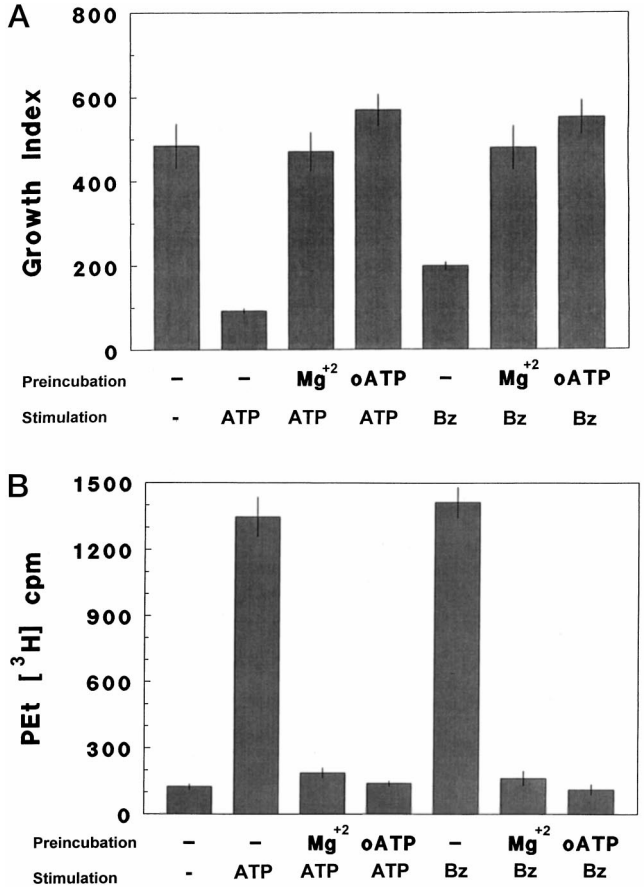

FIGURE 2. Selective P_2X_7 receptor agonists and antagonists modulate ATP-induced intracellular killing of *M. tuberculosis* and macrophage PLD activity. *A*, dTHP-1 macrophages were infected with H37Rv *M. tuberculosis*, and then cultured for 24 h, before preincubation with buffer (−), 10 mM $MgCl_2$ for 2 min (Mg^{2+}), or 300 μM oxidized ATP for 2 h (oATP). Infected MPs were stimulated with 3 mM ATP or 300 μM BzATP (Bz). The viability of intracellular *M. tuberculosis* was determined 24 h later by the Bactec method. *B*, [^{3}H]Oleic acid-labeled MPs were incubated with the indicated compounds, as in *A* above, with the addition of 0.5% ethanol to permit detection of the PLD-specific product, PEt, at 15 min, via TLC. PLD activity was expressed as normalized counts per 10^5 total cpm in phospholipid. Results represent mean ± SEM of four identical experiments performed in triplicate.

with ATP, the tuberculocidal effect of BzATP was completely inhibited by oATP or $MgCl_2$ (Fig. 2*A*). In contrast, the P_2Y_2 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 P_2X_7-R.

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

Stimulation of P_2X_7 receptors induces multiple, biochemically diverse changes in macrophages, including opening of a nonselective plasma membrane pore permeable to molecules <900 Da, rapid influx of Ca^{2+} 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

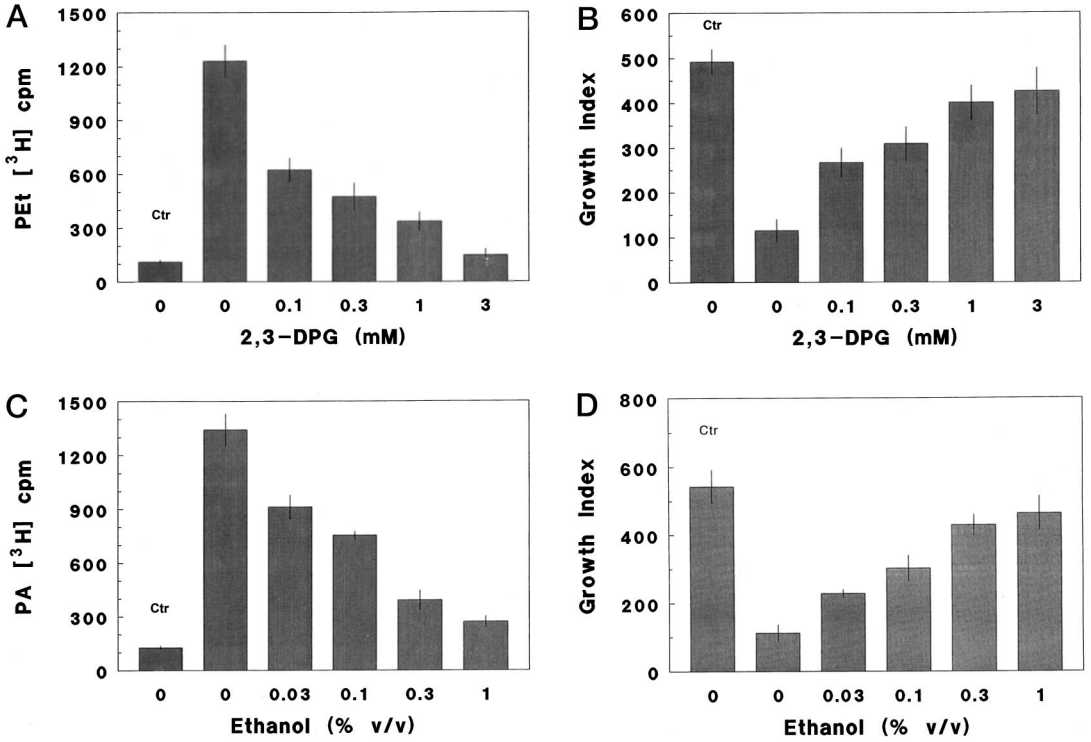

FIGURE 3. Inhibition of ATP-stimulated PLD activity by 2,3-DPG or ethanol is accompanied by concordant reductions in tuberculocidal activity. *A*, [^{3}H]Oleic acid-labeled macrophages were incubated with the indicated concentrations of 2,3-DPG, or buffer, for 15 min at 37°C. ATP (3 mM) was added to all samples, except the control (Ctr, 1st bar), and PLD activity was determined at 15 min by quantitation of [^{3}H]PEt in the presence of 0.5% ethanol. *B*, Macrophages infected with H37Rv *M. tuberculosis* were cultured for 24 h at 37°C, before addition of the indicated concentrations of 2,3-DPG. Fifteen minutes later, ATP was added to each sample, except the control (Ctr, 1st bar), and mycobacterial viability was determined 24 h later, by the Bactec method. *C*, Macrophages labeled with [^{3}H]oleic acid were incubated with the indicated concentrations of ethanol, or buffer, for 2 min at 37°C. ATP (3 mM) was added to all samples, except the control (Ctr, 1st bar), and the accumulation of [^{3}H]PA was determined at 15 min by TLC. *D*, Macrophages infected with H37Rv *M. tuberculosis* were cultured for 24 h at 37°C, before addition of the indicated concentrations of ethanol, or buffer. Two minutes later, ATP was added to all samples, except the control (Ctr, 1st bar). Viability of *M. tuberculosis* was determined 24 h after addition of ATP. Results represent mean ± SEM of triplicate determinations from at least five identical experiments for each panel.

effects of P_2X_7-R agonists and antagonists on macrophage PLD activity with their effects on tuberculocidal activity (Fig. 2*A*). In agreement with the work of Dubyak and colleagues (27, 28), stimulation of [^{3}H]oleate-labeled macrophages with ATP or BzATP resulted in marked stimulation of PLD activity, which was inhibited by oATP or $MgCl_2$ (Fig. 2*B*). Comparison of Fig. 2, *A* and *B*, demonstrated that P_2X_7 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, 51). 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. 3*A*). 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. 3*B*). 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 effect 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. 3*C*). One percent ethanol inhibited ATP-induced PLD activity by 89% (range 85–93%, $p < 0.001$, $n = 5$). Ethanol also significantly inhibited killing of intracellular *M. tuberculosis* by ATP (Fig. 3*D*) 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. 4*A*). 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. 4*B*). 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. 4*C*). 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. 4*C*).

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 [^{3}H]PA in [^{3}H]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

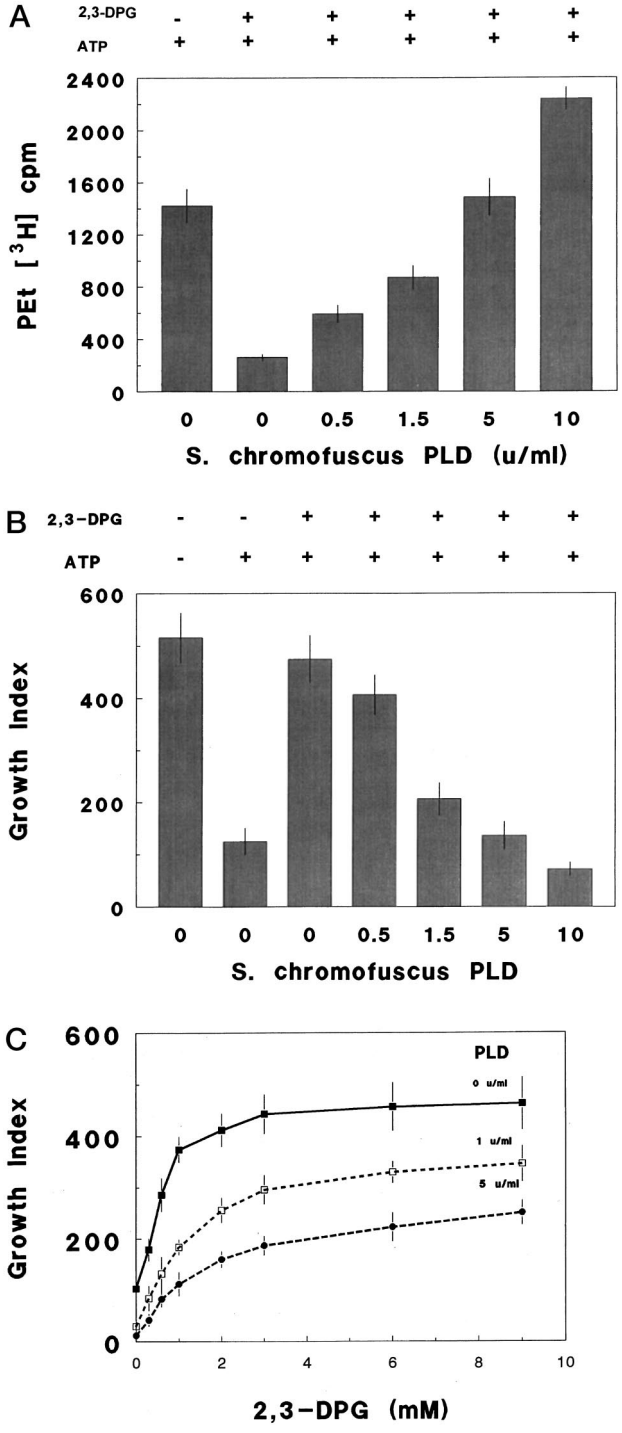

FIGURE 4. Purified PLD restores ATP-dependent tuberculocidal activity in 2,3-DPG- treated macrophages. *A*, dTHP-1 macrophages, radiolabeled with [^{3}H]oleic acid, were incubated with 3 mM 2,3-DPG, except for the control sample (*lane 1*). Fifteen minutes later, 3 mM ATP and the indicated concentrations of *S. chromofuscus* PLD were added concurrently. PLD activity was determined 15 min after the addition of ATP, as noted in the legend to Fig. 2. *B*, Macrophages, infected with H37Rv *M. tuberculosis*, were preincubated with 2,3-DPG (3 mM) or buffer, and then stimulated with 3 mM ATP and *S. chromofuscus* PLD. After incubation at 37°C for an additional 24 h, the viability of intracellular *M. tuberculosis* was determined. *C*, Monolayers were infected with H37Rv *M. tuberculosis*, and then incubated with buffer or the indicated concentrations of 2,3-DPG. Fifteen minutes later, 3 mM ATP and *S. chromofuscus* PLD (0–5 U/ml) 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. 5*A*). In parallel with reconstitution of PA production, addition of *S. chromofuscus* PLD resulted in dose-dependent restoration of ATP-dependent mycobacterial killing (Fig. 5*B*). 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. 5*C*). 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 phosphatidylcholine 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 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 pre-

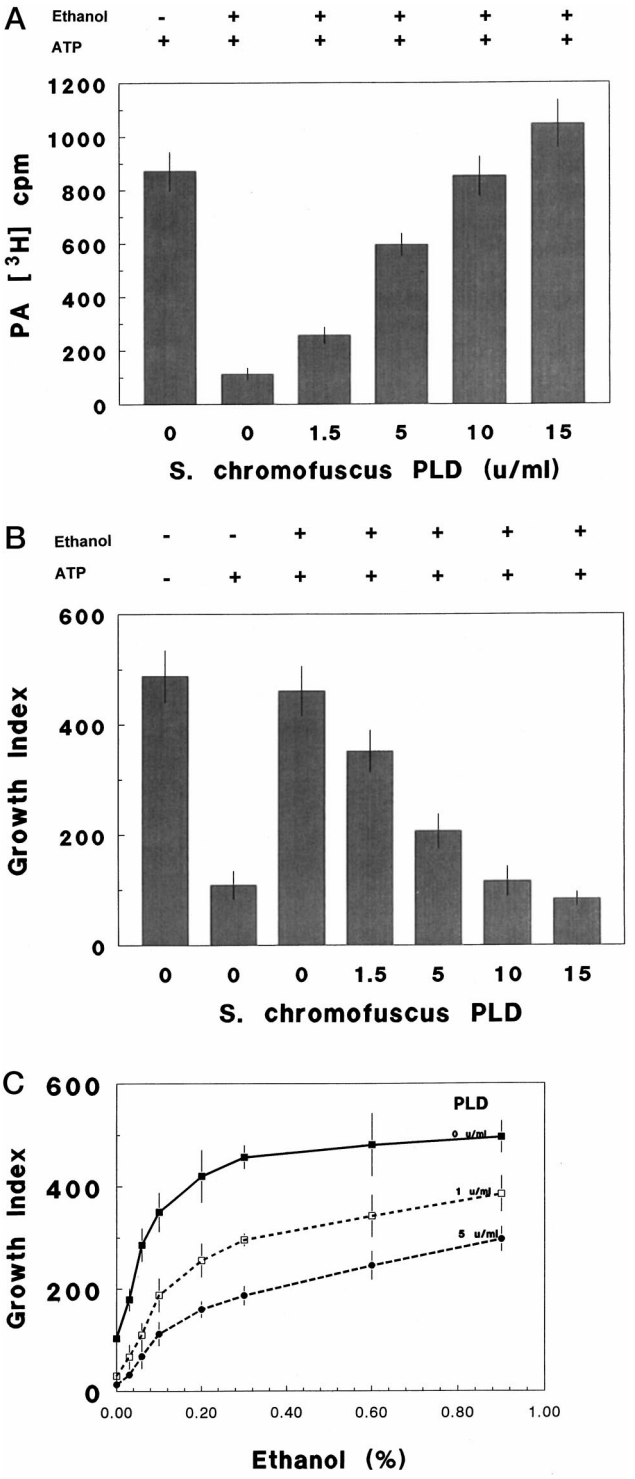

FIGURE 5. Exogenous PLD reverses ethanol-induced inhibition of PA generation and tuberculocidal activity. *A*, dTHP-1 macrophages, labeled with [^{3}H]oleic acid, were preincubated with buffer or 1% ethanol for 2 min, before addition of 3 mM ATP and the indicated concentrations of *S. chromofuscus* PLD. Generation of [^{3}H]PA was determined at 15 min, as noted in *Materials and Methods*. *B*, MPs infected with H37Rv *M. tuberculosis* were preincubated with ethanol (1%) and then stimulated with 3 mM ATP and *S. chromofuscus* PLD. Incubations were continued for an additional 24 h, followed by determination of mycobacterial viability. *C*, Macrophage monolayers were infected with H37Rv *M. tuberculosis*, and preincubated with buffer or the indicated concentrations of ethanol for 2 min at 37°C. ATP (3 mM) and *S. chromofuscus* PLD were then added, followed by determination of mycobacterial growth at 24 h. Results in *A–C* represent mean ± SEM of three identical experiments, each performed in triplicate.

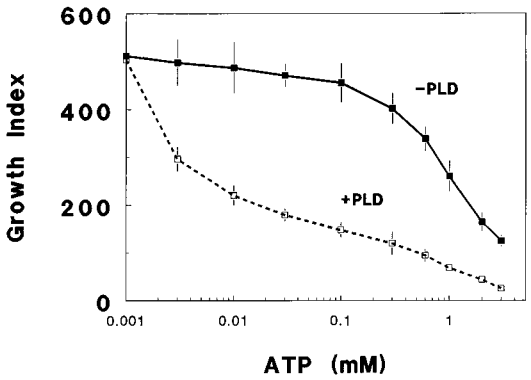

FIGURE 6. Exogenous PLD augments ATP-dependent killing of intracellular *M. tuberculosis* in control macrophages. dTHP-1 macrophages were infected with H37Rv *M. tuberculosis* for 1 h, and then monolayers were washed and incubated for 24 h at 37°C. *S. chromofuscus* PLD (1 U/ml) or buffer (−PLD), and 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.

sented (7–17). Furthermore, the specific molecular mechanisms that regulate and effect killing of intracellular *M. tuberculosis* are unknown.

Based on demonstrations that ATP_e induces killing of *Mycobacterium bovis*-BCG, within human macrophages (19, 20), we tested the hypothesis that ATP_e would exert a similar microbicidal activity toward virulent *M. tuberculosis* within infected human macrophages. This is not a trivial hypothesis, because BCG is not an intracellular pathogen unless host immunity is severely impaired (21, 22), a feature that both clearly distinguishes it from *M. tuberculosis*, and forms the basis for the use of BCG as a vaccine strain. ATP_e exerted a profound bactericidal effect against three highly virulent strains of *M. tuberculosis*, resulting in a 75–80% decrease in viability within macrophages at 24 h and a 3.5-log reduction at 7 days. Decreases in mycobacterial viability in ATP-treated macrophages, compared with the level of infection in the same cells before treatment, indicated that ATP induced a bactericidal activity toward intracellular *M. tuberculosis*.

Characterization of ATP-stimulated killing of *M. tuberculosis* 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_2X_7 purinergic receptors. In the case of *M. tuberculosis*, a necessary role for P_2X_7-R was supported by several lines of evidence, including 1) inhibition of ATP_e-dependent killing by the P_2X_7-R inhibitors, oATP and Mg^{2+}; 2) stimulation of tuberculocidal activity by the selective P_2X_7 receptor agonist, BzATP; 3) lack of efficacy of ADP, AMP, and UTP; and 4) the requirement for mM concentrations of ATP_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_2X_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_2X_7-R-independent effect of ATP is not due to stimulation of the other class of macrophage purinergic receptors, P_2Y_2, because the P_2Y_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_2X_7-R are necessary, but not sufficient, for maximal ATP-dependent killing of intracellular *M. tuberculosis* by human macrophages. Our findings parallel those of Bloom and colleagues, who recently demonstrated P_2X_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_2X_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_e-dependent tuberculocidal activity, because stimulation of P_2X_7-R results in multiple changes in macrophage physiology (19, 23, 25–27). We have focused on P_2X_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_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_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. Melendez 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 phagolysosomes. Experiments to evaluate this hypothesis are currently in progress.

Despite numerous demonstrations that purified PLD preparations closely mimic the effects seen after activation of their endogenous homologues (29, 33, 52, 53), several important questions remain unresolved, including: 1) Does exogenous (extracellular) PLD access phospholipid substrates and release products in a manner similar to that of endogenous (intracellular) PLD? 2) Does addition of purified PLD result in increased hydrolysis of phospholipids in intracellular membranes (Golgi, nuclei, granules, etc.)? The recent cloning of two mammalian PLD isoforms (56, 57) will permit more detailed evaluation of the requirement for PLD in ATP_e-induced tuberculocidal activity in human macrophages.

ATP accumulates in extracellular inflammatory fluid at concentrations sufficient to stimulate P_2X_7-R and induced macrophage tuberculocidal activity (18, 54). Potential sources of ATP_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 macrophages also secrete ATP, and that ATP_e functions in an autocrine pathway to promote macrophage activation and microbicidal activity (24, 54). At a focus of tuberculous inflammation, it is likely that both macrophages, as well as $CD8^+$ T cells (and perhaps other

lymphocytes), serve as sources of ATP_e. 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_e- 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, Balcewicz-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_e and PLD promote killing of *M. tuberculosis* within infected human macrophages may yield insights into complementary 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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