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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 Mycobacterium tuberculosis. Stimulation of macrophage P$_2$X$_7$ 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$_e$) 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$_2$X$_7$ 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$_2$X$_7$ receptors (formerly designated P$_2$Z) 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 $\leq$900 Da (19, 23–26). Stimulation of macrophage P$_2$X$_7$ receptors is associated with a marked increase in the activity of phospholipase D (PLD) (27, 28), an enzyme that has...
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 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, yozanos, 2,3-DPG, taypan blue, leupetin, aprotinin, bovine pancreatic trypsin, PMSF, IFN-γ, ATP, UTP, 3-O-(4-benzoyl)benzoyl-ATP (BzATP), EGTA, ethidium bromide, phosphatidylethanolamine, dimyristoylphosphatidylcholine, purified PLD preparations from Streptomyces chromofuscus, peanut, and cabbage, phosphatidylinositol-specific phospholipase C (PI-PLC), and phosphatidylinositol phosphate phospholipase C (PC-PLC) from Bacillus cereus were obtained from Sigma (St. Louis, MO). RPMI 1640 medium with 1-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 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 (Fairlawn, NJ). Human serum albumin, genistein, and 1,2-dihydroxyvitamin D₃ were purchased from Calbiochem (San Diego, CA). [³H]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 H37Ra 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 (≈2 × 10⁵/sample) were purified by adherence to 6-well plastic tissue culture plates or plastic cell culture vessels (25 mm diameter) for 2 h at 37°C in 5% CO₂. 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 with 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 U/ml IFN-γ, 100 nM 1,25-dihydroxyvitamin D₃, 1 ng/ml LPS in RPMI, 10% FBS, without antibiotics, for 48 h. Differentiated THP-1 cells (dTHP-1, 2 × 10⁶/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% CO₂, 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 on ice for 1 h. 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 Bactericidal 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 plated in 1.5 m l O-ring sealed microtubes 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 H₂O at 10 min at 25°C, with intermittent gentle shaking. Following addition of 600 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 microtubes. Samples derived from both supernatant and adherent fractions were centrifuged at 10,000 × 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 and final adherent fractions were combined, and the growth of M. tuberculosis was quantitated following incubation of triplicate dilutions on 7H11 agar.

**Analysis of phagocytosis**

Phagocytosis of M. tuberculosis was determined as described (33, 42). Briefly, macrophage monolayers adherent to glass coverslips (~2 × 10⁵ MDMS/cover slips) 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 KmnO₄ for 2 min. Adherent bacteria were quantitated by fluorescence microscopy of triplicate coverslips for each experimental condition (50–200 MDMS/cover slips), and results of a set of experiments were expressed as the mean (±SEM) number of adherent M. tuberculosis per cover slip (14). The number of macrophages per cover slip (14) microscopically 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 × 10⁸ 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, incubated in triplicate. Following incubation, unincorporated radioactive 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]lcephosphatidylethanol (PET), as a metabolically stable index of PLD activity (32, 33). Production of phosphatidic acid (PA), the physiologic product of PLD-mediated catalysis, was measured in samples incubated with [³H]lcephosphatidylethanol. Following the addition of reagents, reactions were terminated with 1.67 vol of ice-cold methanol, macrophages were scraped and transferred to polypolyene 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 [³H]PA and [³H]PET were isolated by TLC in an ethyl acetate/acetone/isoctane (9:5:2) solvent system (33, 43), by comigration with pure phospholipid standards. Quantitation of [³H]PET 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 (0.03–1.5%) 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 utilizing [32P]dipalmitoylphosphatidylcholine (DPPC)-labeled mixed 5-bisphosphate), and for PC-PLC (assayed as generation of [14C]choline from [3H]DPPC). All determinations of the sp. act. of purified phospholipases were inactivated by heating to 100°C for 10 min. One unit of PLD activity was defined as the production of 1 mol PA/mg protein, utilizing [32P]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 [3H]choline from [3H]DPPC). All determinations of the sp. act. of purified phospholipases were conducted in RPMI 1640, 2.5% pooled human serum, at 37°C. PLD activity was defined as the production of 1 mol PA/mg protein, utilizing [32P]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 [3H]choline from [3H]DPPC). All determinations of the sp. act. of purified phospholipases were conducted in RPMI 1640, 2.5% pooled human serum, at 37°C. PLD activity was defined as the production of 1 mol PA/mg protein, utilizing [32P]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 [3H]choline from [3H]DPPC). All determinations of the sp. act. of purified phospholipases were conducted in RPMI 1640, 2.5% pooled human serum, at 37°C.

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.

**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 differentiated 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 reduced 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.
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, 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. 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 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. 3C). 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. 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 [H]$^3$PA in [H]$^3$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 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 IC50 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 IC50 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 EC50 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 phosphatidylglycerol-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 [3H]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 [3H]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.

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 P2X7 purinergic receptors. In the case of M. tuberculosis, a necessary role for P2X7-R was supported by several lines of evidence, including (1) inhibition of ATPe-dependent killing by the P2X7-R inhibitors, αATP and Mg2+; (2) stimulation of tuberculocidal activity by the selective P2X7 receptor agonist, BzATP; (3) lack of efficacy of ADP, AMP, and UTP; and (4) the requirement for mM concentrations of ATPe (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 P2X7-R-dependent cellular responses (25–27), these results suggest that ATP activates an additional tuberculocidal mechanism, which is not induced by BzATP. This P2X7-R-independent effect of ATP is not due to stimulation of the other class of macrophage purinergic receptors, P2Y2, because the P2Y2-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 P2X7-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 P2X7-R-independent killing of intracellular BCG by murine macrophages (24). At present, we have no further information concerning the mechanism of this proposed P2X7-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 ATPe-dependent tuberculocidal activity, because stimulation of P2X7-R results in multiple changes in macrophage physiology (19, 23, 25–27). We have focused on P2X7-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 ATPe-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 ATPe-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 ATPe-induced tuberculocidal activity in human macrophages.

ATP accumulates in extracellular inflammatory fluid at concentrations sufficient to stimulate P2X7-R and induced macrophage tuberculocidal activity (18, 54). Potential sources of ATPe 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 ATPe 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 and PLD to promote 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 relationship 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, Balcicewicz-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 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.

Acknowledgments

We thank Dr. George R. Dubyak for his valuable advice, and Dr. Michael Pfaffer, Dr. Oyebode Olakanni, Mary Lindsey, and Anthony Chavez for recommendations and assistance with the assessments of mycobacterial viability. We also thank Dr. Thomas Shinick for his generous provision of CSU#93 M. tuberculosis, and Dr. Michael A. Apicella for his kind gift of purified LPS from Salmonella minnesota. We are very grateful to our colleagues, Drs. William M. Nauseef, Jerrold P. Weiss, Michael A. Apicella, Theresa Gionnioni, Lee-Ann Allen, and Gerene Denning, for their critique of these studies, and for promoting a highly interactive, challenging, and supportive research environment within the Inflammation Program at the University of Iowa.

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