Combined Effects of ATP on the Therapeutic Efficacy of Antimicrobial Drug Regimens against *Mycobacterium avium* Complex Infection in Mice and Roles of Cytosolic Phospholipase A<sub>2</sub>-Dependent Mechanisms in the ATP-Mediated Potentiation of Antimycobacterial Host Resistance

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Combined Effects of ATP on the Therapeutic Efficacy of Antimicrobial Drug Regimens against Mycobacterium avium Complex Infection in Mice and Roles of Cytosolic Phospholipase A2-Dependent Mechanisms in the ATP-Mediated Potentiation of Antimycobacterial Host Resistance

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ATP, which serves as a mediator of intramacrophage signaling pathways through purinoceptors, is known to potentiate macrophage antimycobacterial activity. In this study we examined the effects of ATP in potentiating host resistance to Mycobacterium avium complex (MAC) infection in mice undergoing treatment with a drug regimen using clarithromycin and rifampicin and obtained the following findings. First, the administration of ATP in combination with the clarithromycin and rifampicin regimen accelerated bacterial elimination in MAC-infected mice without causing changes in the histopathological features or the mRNA expression of pro- or anti-inflammatory cytokines from those in the mice not given ATP. Second, ATP potentiated the anti-MAC bactericidal activity of macrophages cultivated in the presence of clarithromycin and rifampicin. This effect of ATP was closely related to intracellular Ca\(^{2+}\) mobilization and was specifically blocked by a cytosolic phospholipase A\(_2\) (cPLA\(_2\)) inhibitor, arachidonyl trifluoromethylketone. Third, intramacrophage translocation of membranous arachidonic acid molecules to MAC-containing phagosomes was also specifically blocked by arachidonyl trifluoromethylketone. In the confocal microscopic observation of MAC-infected macrophages, ATP enhanced the intracellular translocation of cPLA\(_2\) into MAC-containing phagosomes. These findings suggest that ATP increases the host anti-MAC resistance by potentiating the antimycobacterial activity of host macrophages and that the cPLA\(_2\)-dependent generation of arachidonic acid from the phagosomal membrane is essential for such a phenomenon. The Journal of Immunology, 2005, 175: 6741–6749.

Clinical management of Mycobacterium avium complex (MAC)\(^3\) infections is difficult, because MAC infections are frequently encountered in immunocompromised hosts, particularly AIDS patients (1–3), and MAC organisms are highly or moderately resistant to common antituberculosis drugs, such as isoniazid, ethambutol, pyrazinamide, and rifampin (4, 5). Although some new drugs, including clarithromycin, azithromycin, and rifabutin, are fairly effective in controlling MAC bacteria in AIDS patients (4–7), treatment of pulmonary MAC infections is still difficult even with the use of multidrug regimens containing these drugs (5, 8). Therefore, the development of new antimicrobials and administration protocols that are potently efficacious against MAC infections is urgently needed. Although some new antimicrobial agents active against MAC, such as new ketolides (telithromycin and ABT-773), pyrimidine derivatives (SoRI 8890), resorcinomycin A, and pyrrole derivatives (BM212), are being developed (9, 10), few have been subjected to clinical studies. Thus, at present it appears that attempts to devise potent anti-MAC administration protocols using ordinary antitycobacterial drugs are more practical than awaiting the development of new anti-MAC drugs. In this context, it would be useful to devise regimens to treat MAC patients using ordinary anti-MAC agents in combination with immunomodulators.

Extracellular ATP is known to serve as a mediator of cell-to-cell communication by triggering a variety of biological responses in various cells, including hemopoietic cells, endothelial cells, and nerve cells, through ligation of plasma membrane purinergic receptors (11, 12). The biological activities of extracellular ATP are various and include mitogenic stimulation, gene expression, excitatory transmitter function, and induction of cell death. Two subfamilies of P2 purinoceptors have been described: ligand-gated ionotropic P2X receptors and G protein-coupled P2Y receptors (13, 14). Macrophages (Mc\(\phi\)) possess both P2X\(_1\) (formerly P2Z) and P2Y\(_1\) as major P2 receptors (15, 16). Ligation of P2X\(_1\) receptors with low concentrations of ATP (~100 \(\mu\)M) causes the influx of extracellular Ca\(^{2+}\) across the cell membrane (17, 18), whereas
prolonged activation with high concentrations of ATP (3 mM) results in the formation of large nonselective membrane pores permeable to hydrophobic molecules of up to 900 Da (19). These events are thought to cause subsequent changes in intracellular signaling and metabolic pathways leading to the activation of NF-κB and stress-activated protein kinase (SAPK)/JNK, caspases, and cell apoptosis (16, 20–22). P2Y2 receptors act via G proteins (Gi) to stimulate the phospholipase Cβ (PLCβ) signaling cascade, releasing Ca2+ from internal stores (16, 23).

It has recently been reported that treatment of human MoΦ with ATP (ATP4+−) potentiates MoΦ activity in killing of *Mycobacterium tuberculosis* (MTB) complex mycobacteria, such as MTB and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (24–28). ATP-induced killing of mycobacterial organisms within MoΦ is principally mediated by P2X7 receptors (24, 27), although it has also been reported that purinergic signaling regulates MoΦ activity in killing of BCG organisms via a P2X7-independent mechanism (29). In addition, ATP-mediated killing of mycobacterial organisms within MoΦ is mediated by phospholipase D, which is linked to leukocyte antimicrobial mechanisms dependent on the mobilization of intracellular Ca2+ and subsequent lysosomal fusion and acidification of mycobacteria-containing phagosomes (25–27).

These findings encouraged us to examine the effect of ATP administration to MAC-infected mice on the therapeutic efficacy of clarithromycin, a leading anti-MAC drug, in combination with rifampicin or the new benzoxazinorifamycin rifalazil (30). We found that ATP significantly potentiated the therapeutic efficacy of clarithromycin, leading to the killing of MAC-infected mice during the early stage of infection, principally by potentiating the anti-MAC activity of host MoΦ. In addition, we found that ATP-induced augmentation of MoΦ anti-MAC activity is dependent on type IV cytosolic phospholipase A2 (cPLA2), but not on reactive nitrogen intermediates (RNI) or reactive oxygen intermediates (ROI). It thus appears that arachidonic acid (AA), which results from cPLA2 activity, plays an important role in ATP-induced potentiation of MoΦ antimycobacterial activity.

### Materials and Methods

#### Organisms

MAC N-444 (serovar 8) and MAC N-260 (serovar 16) strains isolated from patients with MAC infection were used. These strains were identified as *Mycobacterium avium* complex *Mycobacterium* strains isolated from *Mycobacterium* strains isolated from patients with MAC infection were used. These strains were identified as *Mycobacterium avium* complex *Mycobacterium tuberculosis* (MTB) complex mycobacteria, such as MTB and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (24–28). ATP-induced killing of mycobacterial organisms within MoΦ is principally mediated by P2X7 receptors (24, 27), although it has also been reported that purinergic signaling regulates MoΦ activity in killing of BCG organisms via a P2X7-independent mechanism (29). In addition, ATP-mediated killing of mycobacterial organisms within MoΦ is mediated by phospholipase D, which is linked to leukocyte antimicrobial mechanisms dependent on the mobilization of intracellular Ca2+ and subsequent lysosomal fusion and acidification of mycobacteria-containing phagosomes (25–27).

#### Mice

Five-week-old BALB/c mice were purchased from Japan Clea.

#### Special agents

Special agents used in this study were as follows: clarithromycin (Taisho Pharmaceutical), rifalazil (Kaneka), rifampin (Sigma-Aldrich), ATP (ICN Biomedicals), benzoylbezoic ATP (BeATP; Sigma-Aldrich), calcium ionophore A23187 (Sigma-Aldrich), N5-monomethyl-l-arginine (NMMA; Dojindo), mannoalide (Wako Pure Chemical Industries), arachidonoyl trifluoromethylketone (a-TFMK; Sigma-Aldrich), indomethacin (Sigma-Aldrich), and colchicine (Sigma-Aldrich). 3H]oleic acid and [3H]oleic acid were purchased from American Radio-Labeled Chemicals.

### Experimental infection

Six-week-old BALB/c mice infected i.v. with 1 × 106 CFU of MAC were given, or not given, s.c. injections of clarithromycin (12 mg/kg) and rifampin (8 mg/kg) with or without simultaneous administration of ATP (40 mg/kg) s.c. once daily, five times per week, from day 1 after infection for up to 8 weeks. Doses of test drugs were fixed to be nearly equivalent to the clinical dosages by weight. At intervals, mice were killed and examined for bacterial loads in the lungs and spleens by counting the number of CFU in the homogenates of individual organs using Middlebrook 7H11 agar plates.

### Intragranular growth of MAC in MoΦ

MoΦ monolayer cultures prepared by seeding 1 × 105 mouse RAW264.7 MoΦ (RAW-MoΦ) or 5 × 104 7741.4 MoΦ (7741-MoΦ) on 96-well, round-bottom microculture wells were precultivated in 0.2 ml of RPMI 1640 medium containing 5% FBS and 25 mM HEPES at 37°C in a CO2 incubator (5% CO2–95% humidified air) for 18 h. After washing with HBSS containing 2% FBS, the MoΦ were incubated in 0.1 ml of 5% FBS-RPMI 1640 medium containing 2 × 107 CFU/ml MAC organisms (multiplicity of infection, 20) in a CO2 incubator for 2 h. The MAC-infected MoΦ were then washed with 2% FBS-HBSS to remove extracellular organisms and thereafter cultured in 0.2 ml of 5% FBS-RPMI 1640 medium with or without the addition of ATP, BeATP, A23187, or their combination in a CO2 incubator for up to 6 days. In some experiments CR were added to the culture medium at the concentrations equivalent to their C50 in the blood (clarithromycin, 2.3 μg/ml; rifampin, 6.2 μg/ml; rifalazil, 0.05 μg/ml) of humans who were given clinical dosages of these drugs. At intervals, the MoΦ were lysed with 0.07% SDS, followed by subsequent neutralization with 6% BSA. After collection of bacterial cells from the resultant MoΦ lysate by centrifugation at 2000 × g for 20 min and subsequent washing of recovered bacteria with distilled water by centrifugation, the number of CFU was counted on 7H11 agar plates.

### Intracellular translocation of MoΦ membranous AA and oleic acid to infected mycobacterial organisms

Profiles of intracellular translocation of membranous AA and oleic acid to mycobacterial organisms internalized in MoΦ phagosomes were examined as described previously (32). Briefly, zymosan A-induced mouse peritoneal exudate cells (2 × 106 cells) were cultured in 10 ml of 5% FBS-RPMI 1640 medium on an FBS-coated plastic culture dish (80-mm diameter; Falcon) at 37°C in a CO2 incubator for 2 h. After rinsing with 2% FBS-RPMI 1640, adherent cells consisting of >90% MoΦ were gently scraped off onto 20% FBS medium with a rubber policeman and collected by subsequent centrifugation at 250 × g for 5 min. Test MoΦ (6 × 106 cells) were incubated in 3.0 ml of 5% FBS-RPMI 1640 containing 4 μCi/ml [3H]AA or [3H]oleic acid (80 Ci/mmol) in polypropylene tubes (15 × 90 mm) at 37°C in a CO2 incubator for 24 h. After rinsing with 2% FBS-HBSS, the resultant MoΦ (2.5 × 106 cells) loaded with [3H]AA or [3H]oleic acid were suspended in 200 ml of culture medium containing 1.0 × 107/ml MTB organisms and then incubated at 37°C for 2 h. After washing with 2% FBS-HBSS by centrifugation (120 × g, 5 min) to remove extracellular organisms, infected MoΦ were cultivated in a CO2 incubator. After 12-h cultivation, MoΦ were collected, thoroughly washed with 2% FBS-HBSS, and lysed with 0.23% SDS solution for 10 min. Intramacrophage microorganisms were then collected by centrifugation (4000 × g, 10 min), and the radioactivity of recovered microorganisms was measured using toluene-based scintillant containing Triton X-100 using a Tri-Carb liquid scintillation spectrometer (Packard Instrument).

### Intracellular translocation of cPLA2

Mouse peritoneal MoΦ (1 × 106 cells) were seeded onto a 17-mm cover glass and cultured in 5% FBS-RPMI 1640 medium overnight using six-well culture dishes (Corning). The resultant MoΦ monolayer was then infected with MAC N-260 by incubation in medium (5 ml) containing 1 × 108 MAC (multiplicity of infection, 100) at 37°C for 2 h, then incubated in medium with or without addition of either ATP (3 mM) or BeATP (0.3 mM) at 37°C for 10 min. After fixation with 4% paraformaldehyde and then methanol, the MoΦ specimen was permeabilized with sequential treatments with 50 mM ammonium chloride for 10 min and 0.2% Triton X-100 for 10 min, and thereafter blocked with 2% BSA in PBS for 30 min, followed by subsequent treatment with anti-Fc mAb for 30 min. Immunolabeling was performed using the following Abs at 37°C for 2 h: 1) MAC staining (mouse anti-MAC mAb, primary Ab) and Alexa Fluor 568-conjugated goat anti-mouse IgG1 mAb (secondary Ab), each used at a dilution of 1/500; and 2) cPLA2 staining: mouse anti-cPLA2 mAb (primary Ab) and FITC-conjugated rat anti-mouse IgG2b mAb (secondary Ab), each used at
a dilution of 1/100. All specimens were viewed on an Olympus FV300 digital fluorescence confocal laser scanning microscope. The cPLA₂ colocalization index, in terms of the relative intensity of cPLA₂-fluorescence colocalizing with intracellular MAC organisms, was calculated as follows: cPLA₂ colocalization index = (FITC’s [cPLA₂] fluorescence intensity of area surrounding individual bacterium)/([Alexa Fluor 546]’s fluorescence intensity of area surrounding the same bacterium).

Expression of cytokine mRNA

RT-PCR analysis of cytokine mRNAs in lung tissues from mice infected with MAC was performed as follows. Total RNA was isolated from the lung tissues of MAC-infected mice with or without drug treatment harvested wk 4 after infection using the ISOGEN kit (Nippon Gene). After DNase I (Invitrogen Life Technologies) treatment (1 U of DNase/μg RNA sample) at room temperature for 15 min, the resultant RNA samples were reverse transcribed to the first chain of cDNA using random hexamer primers (Invitrogen Life Technologies) and 200 U of SuperScript II reverse transcriptase (Invitrogen Life Technologies) with the standard reaction mixture (20 μl): 1× reverse RT buffer (pH 8.3); 0.2 mM of each dNTP, 1 U of Taq polymerase (Takara Biomedicals), and 20 pmol of reaction at 42°C and subsequent heating at 72°C for 15 min, 1× Taq polymerase reaction mixture (50 ml) containing 1× PCR buffer (pH 8.3), 1 mM of each dNTP including dATP, dCTP, dGTP, and dTTP (Invitrogen Life Technologies); and 2.0 U of RNase inhibitor (Invitrogen Life Technologies). After 1-h reaction at 42°C and subsequent heating at 72°C for 15 min, 1-μl aliquots of resultant cDNA were amplified specifically by PCR in the standard reaction mixture (50 ml) containing 1× PCR buffer (pH 8.3), 0.2 mM of each dNTP, 1 U of Taq polymerase (Takara Biomedicals), and 20 pmol of sense and antisense primers for test cytokines (synthesized by Greiner Labotechnik) as follows (sense/antisense): TNF-α, AGCCCCAGCTGTCGACACACCAACACACAA/ACACCCATTCCCTTCACAGGACCA; IFN-γ, GAAAGCCCTAGAAGCTGCTAATACCTC/ACAGGATGATGATGCTACACAGAGCAAT; IL-10, TGACTGGCATGAGGATCAGCAG/ATCCTGAGGGGAAAGCCTAGAAAGTCTGAATAACT/ATCAGCAGCGACTCCTTTTGACCCTTTTCACACCCATTCCCTTCACAGGACCA; and TGF-β, ACCACTCGAGGTAGGTGAGGTG/TTGACATATCCTTCAAGACCGATACACACTATTGCTTCAGCTCCACAG/AGGGGCGGGGCGGGGCGCCGCTT; IL-4, TGACTGGCATGAGGATCAGCAG/ATCCTGAGGGGAAAGCCTAGAAAGTCTGAATAACT/ATCAGCAGCGACTCCTTTTGACCCTTTTCACACCCATTCCCTTCACAGGACCA; and TGF-β, ACCACTCGAGGTAGGTGAGGTG/TTGACATATCCTTCAAGACCGATACACACTATTGCTTCAGCTCCACAG/AGGGGCGGGGCGGGGCGCCGCTT; IL-13, TGACTGGCATGAGGATCAGCAG/ATCCTGAGGGGAAAGCCTAGAAAGTCTGAATAACT/ATCAGCAGCGACTCCTTTTGACCCTTTTCACACCCATTCCCTTCACAGGACCA; and TGF-β, ACCACTCGAGGTAGGTGAGGTG/TTGACATATCCTTCAAGACCGATACACACTATTGCTTCAGCTCCACAG/AGGGGCGGGGCGGGGCGCCGCTT; IL-2, TGACTGGCATGAGGATCAGCAG/ATCCTGAGGGGAAAGCCTAGAAAGTCTGAATAACT/ATCAGCAGCGACTCCTTTTGACCCTTTTCACACCCATTCCCTTCACAGGACCA; and TGF-β, ACCACTCGAGGTAGGTGAGGTG/TTGACATATCCTTCAAGACCGATACACACTATTGCTTCAGCTCCACAG/AGGGGCGGGGCGGGGCGCCGCTT; IL-6, TGACTGGCATGAGGATCAGCAG/ATCCTGAGGGGAAAGCCTAGAAAGTCTGAATAACT/ATCAGCAGCGACTCCTTTTGACCCTTTTCACACCCATTCCCTTCACAGGACCA; and TGF-β, ACCACTCGAGGTAGGTGAGGTG/TTGACATATCCTTCAAGACCGATACACACTATTGCTTCAGCTCCACAG/AGGGGCGGGGCGGGGCGCCGCTT. Reactions were conducted in a DNA Thermal Cycler (ASTEC) for 30 cycles, including denaturing at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min for each cycle. PCR products were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gels.

Statistical analysis

Statistical analysis was performed using a one-way ANOVA with a Bonferroni multiple comparisons post-test (StatView software; Hulinks).

Results

Effect of ATP administration on the therapeutic efficacy of CR against MAC infection

First, we examined the effects of ATP administration on MAC-infected mice, which were, or were not, given antimycobacterial CR drug regimens, on bacterial behavior at sites of infection (lungs and spleen). Fig. 1 shows the profiles of bacterial growth during the 4-wk period following infection in the lungs and spleens of MAC-infected mice, which were, or were not, treated with ATP, CR, or both. As shown in Fig. 1, A and B, in mice infected with the MAC N-444 strain (low virulence), persistent infection without bacterial growth was observed in the lungs, and gradual bacterial growth was noted in the spleen. ATP (50 mg/kg) alone did not affect the profiles of bacterial persistence in the lungs, whereas ATP in combination with CR (20 and 5 mg/kg, respectively) potentiated the bacterial elimination in the lungs due to the CR drug regimen (Fig. 1A). In the spleen, ATP alone slightly decreased the bacterial load at wk 4, whereas ATP in combination with CR enhanced CR-mediated bacterial elimination during wk 2–4 (Fig. 1B).

As shown in Fig. 1, C and D, in mice infected with the MAC N-260 strain (high virulence), significant levels of bacterial growth were observed in the lungs and spleen at wk 4 after infection. ATP alone did not affect bacterial growth in the lungs (Fig. 1C, □), but completely inhibited growth of MAC in the spleen (Fig. 1D, □). Administration of ATP in combination with CR caused more potent growth inhibition of the organisms in the lungs than that in mice given CR alone (Fig. 1C, ▪). As shown in Fig. 1D, bacterial elimination was clearly observed in the spleens of mice given ATP in combination with CR (□), whereas CR alone caused only growth inhibition of MAC (▪).

Fig. 2 shows histopathological examination of the lungs of infected mice given, or not given, ATP. In mice without ATP treatment, a number of tubercular lesions consisting of epitheloid-like giant cells surrounded by infiltrating lymphocytes were observed, but no caseous necrosis was noted (Fig. 2, A and B). As indicated in Fig. 2, E, in this case, the number of granulomas per square millimeter of field was 0.71 ± 0.06 (average of 100 fields). Notably, ATP treatment did not affect the histopathological profiles of MAC-infected mice even after 8-wk administration of ATP (Fig. 2, C and D), and the number of granulomas was 0.73 ± 0.05/mm² field (Fig. 2E). In contrast, CR significantly decreased the number of tubercular lesions (photo not shown), and the number of granulomas was 0.16 ± 0.02/mm² field (Fig. 2E). These findings suggest that ATP treatment affects neither the formation nor the progression of granulomatous lesions in MAC-infected mice, unlike antimicrobial drugs.

We next examined profiles of the mRNA expression of cytokines that up-regulate MoF antimycobacterial activity (TNF-α, IFN-γ, and IL-2) and those that down-regulate it (IL-4, IL-10, IL-13, and TGF-β) (33) in the lungs of infected mice given, or not given, CR with or without ATP at wk 4 after infection. As shown in Fig. 3, TNF-α mRNA expression was not changed due to MAC infection, but was markedly decreased by administration of CR. In contrast, IFN-γ mRNA expression was up-regulated due to MAC
infection, but was not affected by administration of CR. The expression of both IL-10 mRNA and IL-13 mRNA was markedly decreased by MAC infection. In these cases, administration of CR did not affect the mRNA expression of these cytokines. In contrast, TGF-β mRNA expression was slightly decreased in MAC-infected mice, but was not affected by administration of CR. Notably, ATP administration did not affect the profiles of mRNA expression of these cytokines in mice given CR alone. Neither IL-2 nor IL-4 mRNA expression was observed with any of the regimens tested (data not shown). These findings indicate that ATP administration did not modulate Th1 and Th2 cytokine gene expression in lungs of MAC-infected mice treated with CR.

Effects of ATP on antimicrobial activity of CR against intramacrophage MAC

To clarify the cellular mechanisms of the ATP-mediated increase in the in vivo efficacy of CR chemotherapy against MAC infection in mice, we examined the effects of high concentrations of ATP on the antimicrobial activity of Mφ (RAW-Mφ and J774-Mφ) against MAC organisms (N-444 and N-260 strains). As indicated in Fig. 4A, ATP at 10 mM, but not at 3 mM, weakly, but significantly, inhibited the growth of the low virulence MAC N-444 strain (31) in RAW-Mφ. Notably, both concentrations (3 and 10 mM) of ATP tested markedly potentiated the bactericidal activity of CR at the C<sub>max</sub> in blood against the MAC organisms residing within RAW-Mφ. As shown in Fig. 4B, in the case of RAW-Mφ infected with the high virulence MAC N-260 strain (31), ATP at 3 and 10 mM did not reduce, but, in fact, somewhat enhanced, the intramacrophage growth of organisms. These results, therefore, demonstrate that ATP exerts its antimicrobial activity against intramacrophage MAC mostly in the presence of antimycobacterial drugs. Nevertheless, both concentrations of ATP significantly augmented the bactericidal activity of CR against intramacrophage MAC. Fig. 4C shows the results of a similar experiment using J774-Mφ. In this case, ATP alone or in combination with CR exhibited similar effects on the behavior of intramacrophage MAC N-444 organisms, as in the case of RAW-Mφ shown in Fig. 4A. In this context, separate experiments showed that 10 mM ATP did not significantly augment the antimicrobial activity of CR against extracellular MAC when the organisms growing in 7H9SF medium were treated with CR (1/2 C<sub>max</sub> each) in combination with ATP during 4-day culture. In a representative experiment, log-unit values of the bacterial CFU after 4-day culture (n = 3) were as follows: day 0, 4.41 ± 0.01; [1], clarithromycin/rifampin, 2.08 ± 0.02; clarithromycin/rifampin plus ATP, 1.73 ± 0.03; and [2], clarithromycin/rifalazil, 1.63 ± 0.23; clarithromycin/rifalazil plus ATP, 1.80 ± 0.41. Therefore, it appears that ATP, even at 10 mM, does not affect the antimicrobial activity of CR against extracellular MAC.

Intracellular mechanisms of ATP-dependent potentiation of the antimicrobial activity of CR against intramacrophage MAC

Next we attempted to clarify the intracellular mechanisms of the ATP-mediated potentiation of antimicrobial activity of CR against MAC organisms residing inside Mφ. First, as shown in Fig. 5A, we found that the combination of ATP at a suboptimal concentration (0.3 mM) with calcium ionophore A23187 significantly potentiated Mφ anti-MAC activity, although ATP alone was ineffective in increasing this activity. Next, we examined the effects of ATP and BzATP (a potent P2X<sub>7</sub> receptor agonist) on the anti-MAC antimicrobial activity of Mφ cultivated in medium in the presence of ionophore A23187 with or without the addition of CR. In this experiment, RAW-Mφ were treated with IFN-γ (500 U/ml) for 24 h, and the same concentration of IFN-γ was added to the culture medium of MAC-infected Mφ. In this case, ATP and BzATP at a suboptimal concentration (0.3 mM) failed to potentiate Mφ anti-microbial activity against intracellular MAC, even after stimulation of Mφ with IFN-γ, which is known to increase Mφ sensitivity to ATP-mediated induction of Mφ apoptosis and cause concomitant expression of Mφ antimycobacterial activity (24) (data not shown). However, as shown in Fig. 5B, when the Mφ were treated with the Ca<sup>2+</sup> ionophore A23187 in combination with either ATP or BzATP at a suboptimal concentration (0.3 mM), significant bacterial killing of intramacrophage MAC was observed. This finding supports previous observations by Stober et al. (26) and Kusner and Barton (28) that ATP-mediated killing of mycobacteria within Mφ is dependent on intracellular Ca<sup>2+</sup> mobilization. In this case, the bactericidal activity of CR against intramacrophage MAC was further potentiated by BzATP, but not by ATP. This finding indicates that extracellular ATP augments the antimicrobial activity of CR against intramacrophage MAC activity organisms, specifically through P2X<sub>7</sub> receptors.

Next we examined the roles of ROI, RNI, and AA in the effector pathway(s) involved in the ATP-dependent increase in CR antimicrobial activity against intramacrophage MAC organisms, that is, ATP-mediated potentiation of Mφ anti-MAC activity with culture in the presence of CR. As shown in Fig. 5C (right columns), the potentiation by ATP at an optimal concentration (10 mM) of the antimicrobial activity of CR against intramacrophage MAC was markedly abrogated by a-TFMK (cPLA<sub>2</sub> inhibitor), but not by
superoxide dismutase/catalase (ROI scavengers) or NMMA (iNOS inhibitor). These findings suggest that cPLA₂-mediated release of AA into MAC-containing phagosomes may play an important role in ATP-mediated potentiation of Mφ anti-MAC activity in the presence of CR.

In this context, we previously found that in the case of [³H]AA-labeled Mφ, membranous radioactive component(s) (presumably [³H]AA) translocated to the microorganisms internalized within Mφ phagosomes (32). As shown in Fig. 5D, the translocation of the [³H]labeled membranous component(s) to intramacrophage mycobacteria was almost completely inhibited by α-TFMK and colchicine (an inhibitor of phagocytosis), but not by manoalide (a secretory PLA₂ inhibitor), NDGA (a lipoxygenase inhibitor), or indomethacin (a cyclooxygenase inhibitor). Moreover, AA directly into MAC-containing phagosomes may play an important role in the presence of CR.

Discussion

The major findings of this study can be summarized as follows. 1) Administration of ATP in combination with the CR drug regimen accelerated bacterial elimination in MAC-infected mice without causing changes in the histopathological features or the expression of pro- and anti-inflammatory cytokine genes from those in mice not given ATP. 2) ATP potentiated the anti-MAC bactericidal activity of host Mφ cultivated in the presence of clarithromycin and rifampycin. This effect of ATP was dependent on intracellular Ca²⁺ mobilization and cPLA₂, which generates AA from phospholipids. 3) Intramacrophage translocation of membranous AA molecules to MAC-containing phagosomes was also mediated by cPLA₂. Notably, ATP enhanced the intracellular translocation of cPLA₂ into MAC-containing phagosomes. Concerning these findings, the following discussion can be made.
FIGURE 4. ATP-mediated potentiation of the anti-MAC antimicrobial activity of Mφ cultured in the presence of CR. A, MAC N-444 (a low virulence isolate of M. avium)-infected RAW-Mφ were cultured in the medium with or without the addition of ATP or CR alone or both. C. None added; △, ATP (3 mM); ⊘, ATP (10 mM); ○, CR (clarithromycin, 2.3 μg/ml; rifalazil, 0.05 μg/ml); ▲, CR plus ATP (3 mM); ⬇, CR plus ATP (10 mM). B, MAC N-260 (a high virulence isolate of M. intracellulare)-infected RAW-Mφ were cultured in the medium with or without the addition of ATP or CR alone or together for 3 days. Each plot or bar indicates the mean ± SEM (n = 3). The asterisks denote a statistically significant difference between two specified groups (p < 0.01). These data represent one of two or three experiments that were performed with similar results.

First, it is somewhat strange that ATP treatment potentiated the ability of MAC-infected mice to decrease bacterial loads, especially when infected mice were given the CR drug regimen, without out affecting the formation/progression of granulomatous lesions and cytokine gene expression in the lungs of infected mice. Thus, it appears that ATP may principally enhance the host innate immunity that is much less dependent on Th1 cytokine-mediated granuloma formations than is the case for acquired cellular immunity against mycobacterial Ags.

Second, the present findings also indicate that ATP potentiates Mφ antimicrobial activity against not only MTB complex mycobacteria (MTB and M. bovis BCG) (24, 25), but also MAC mycobacteria, especially when infected Mφ are cultured in the presence of CR. However, it was noted that MAC organisms, particularly those of the high virulence N-260 strain, were much more resistant to the ATP-induced antimicrobial activity of Mφ than was MTB complex (Fig. 4). This may mean that the Mφ antimicrobial mechanisms required for the killing/inhibition of MAC are somewhat different from those required for the killing/inhibition of the MTB complex. As indicated in Fig. 5A, Mφ exhibited potentiation of anti-MAC activity in response to ATP signaling at suboptimal concentration (0.3 mM) only when Ca^{2+} ionophore A23187-mediated cytosolic Ca^{2+} mobilization was provided. In this context, an increased cytosolic Ca^{2+} concentration is known to promote the maturation of phagosomes containing mycobacterial organisms and thereby potentiate Mφ activity in killing/inhibiting intraphagosomal mycobacteria (40). It thus appears that ATP signaling at suboptimal concentrations via P2Y may mobilize intramacrophage signal transduction pathways independent of cytosolic Ca^{2+} mobilization.

In contrast, the intracellular signaling pathways induced by ATP signaling at optimal concentrations (3–10 mM) are known to

FIGURE 5. Dependence of the effects of ATP on Mφ anti-MAC activity on cytosolic Ca^{2+} mobilization. Mφ antimycobacterial effector molecules (ROI, RNI, and AA) and cPLA2 activities. A and B, Ca^{2+} mobilization-dependent expression of ATP’s effects in potentiating Mφ anti-MAC antimicrobial activity. A, MAC N-444-infected RAW-Mφ were cultured in the absence or the presence of ATP (0.3 mM) or A23187 (5 μM) alone or together for 5 days. B, RAW-Mφ were pretreated with IFN-γ (500 U/ml) for 24 h, then infected with MAC N-444, and cultured in the medium containing IFN-γ and A23187 (5 μM) in the absence or the presence of ATP (0.3 mM), BzATP (0.3 mM), or CR (clarithromycin, 2.3 μg/ml; rifamipin, 6.2 μg/ml), alone or together for 5 days. C, Influences of ROI scavengers (superoxide dismutase/catalase (SOD/CAT)), iNOS inhibitor (NMMA), and cPLA2 inhibitor (a-TFMK) on the effects of ATP in potentiating Mφ anti-MAC antimicrobial activity. MAC N-444-infected RAW-Mφ were cultured in the absence or the presence of ATP (10 mM) or CR (clarithromycin, 2.3 μg/ml; rifalazil, 0.05 μg/ml) alone or in combination with superoxide dismutase (1000 U/ml) plus catalase (900 U/ml), NMMA (0.5 mM), or a-TFMK (10 μM) for 5 days. D, Solute control (0.1% DMSO), SOD/CAT, NMMA, or a-TFMK alone exerted no influence on the intramacrophage growth of the organisms. D, cPLA2- and phospho-cytosine-dependent translocation of Mφ membrane AA to mycobacterial organisms internalized in phagosomal vesicles. [3H]AA-labeled mouse peritoneal Mφ were infected with MTB and then cultured in the medium with or without addition of mannoalide (20 μM), a-TFMK (100 μM), NDGA (20 μM), indomethacin (Indo; 20 μM), or colchicine (Colch; 2 μM) for 12 h. Radioactivity translocated to bacterial cells was measured as described in Materials and Methods. E, Profiles of time-dependent direct binding of [3H]AA to mycobacterial cells. Mφ organisms were incubated in the medium containing [3H]AA (12 μCi/ml) at 37°C for up to 12 h. Each bar or plot indicates the mean ± SEM (n = 4). The asterisks denote a statistically significant difference between two specified groups (*, p < 0.05; **, p < 0.01). These data represent one of two or three experiments that were performed with similar results.
RNI are reported to be the principal effectors of murine *M. Typhimurium*, their participation in killing and growth inhibition of MAC organisms, although it has been reported that such cellular antimicrobial functions against MTB (42, 44). Although more conventional signaling pathways did not fully support effective Mφ killing of MAC organisms, it has been reported that other cellular antimicrobial functions against MTB are mediated by a free fatty acid, AA, released by cPLA2 from phagosomal membrane phospholipids (48), in the antimycobacterial mechanisms of host Mφ are also supported by the following findings (32, 39). 1) MTB- or MAC-infected Mφ sequentially produced/released free fatty acid (AA) and subsequently RNI. 2) Sequential treatment of mycobacterial organisms with AA, then RNI, caused synergistic bactericidal activity against these pathogens. 3) In the case of [3H]AA-labeled Mφ, substantial amounts of [3H]oleic acid was found to translocate to intraphagosomal microorganisms during chase incubation after MTB infection. 4) Mφ antimicrobial activity against MTB was strongly inhibited by a-TFMK (cPLA2 inhibitor). Moreover, as indicated in Fig. 5D, the present study also revealed that the intramacrophage translocation of membranous AA molecules to intraphagosomal MAC organisms was specifically blocked by a-TFMK. These findings strongly suggest that free fatty acids (especially AA) produced by the enzymatic action of cPLA2 play an important role as antimycobacterial effectors in the expression of Mφ antimicrobial activity against mycobacterial pathogens. This concept is in part supported by the finding of Duan et al. (49) that the apoptosis-mediated expression of anti-MTB antimicrobial activity of human monocyte-derived Mφ was dependent on cPLA2, and its product, AA. In this context, separate experiments indicated that also in the case of [3H]oleic acid translocated to intraphagosomal MTB, although the translocation efficiency of [3H]oleic acid (1422 ± 100 cpm) was lower than that observed in the case of [3H]AA translocation (3069 ± 692 cpm). Notably, the [3H]oleic acid translocation was not affected by 100 μM a-TFMK (1392 ± 173 cpm), but was weakly inhibited by 20 μM manganese (1277 ± 176 cpm), indicating that intracellular oleic acid translocation to intraphagosomal MTB is mediated by PLA2 other than cPLA2, such as type IIA secretary PLA2, and type V secretory PLA2. These findings indicate that intramacrophage AA mobilization is mediated by cPLA2 in a specific fashion, although not only AA, but also oleic acid, play important roles in the expression of Mφ antimicrobial activity against intracellular mycobacterial organisms.

In the present study it was found that ATP-induced potentiation of Mφ anti-MAC activity in the presence of CR was almost completely abolished by a cPLA2 inhibitor, a-TFMK, but not by ROI scavengers or iNOS inhibitor (Fig. 5C). This indicates that the anti-MAC bactericidal activity of ATP-stimulated Mφ is primarily mediated by a free fatty acid, AA, released by cPLA2 from phospholipids of the phagosomal membrane. In addition, we found that

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Effects of ATP on the profiles of intracellular translocation of cPLA2 in MAC-infected Mφ. Confocal analysis was performed for peritoneal Mφs with or without ATP treatment to detect cPLA2 molecules, which were colocalized with internalized MAC organisms. a–c. Uninfected Mφ without ATP treatment. d–f. MAC-infected Mφ incubated in the absence of ATP. g–i. MAC-infected Mφ incubated in the presence of ATP (3 mM). j–l. MAC-infected Mφ incubated in the presence of BrATP (0.3 mM). MAC (a, d, g, and j), cPLA2 (b, e, h, and k), and merged images (c, f, i, and l) are indicated. Arrows indicate internalized MAC bacilli.

involve Ca²⁺ mobilization, followed by the activation of phospholipase D, leading to phagosomal-lysosome fusion and acidification of mycobacteria-containing phagosomes (25–28). In addition, a recent finding by Vergne et al. (41) indicated that Ca²⁺ and calmodulin are required for a Ca²⁺/calmodulin PI3K hVPS34 cascade essential for the production of phosphatidylinositol 3-phosphate on phagosomes and, consequently, maturation of phagosomes. As shown in Fig. 5A, mobilization of Ca²⁺-dependent signaling pathways did not fully support effective Mφ killing of MAC organisms, although it has been reported that such cellular events were sufficient for rapid killing of the MTB complex (24–28). Notably, we previously obtained evidence that the modes of interaction of MAC with the antimicrobial mechanisms of Mφ differ considerably from those of MTB. In bacterial killing experiments in a cell-free system, although RNI in combination with an unsaturated fatty acid, AA, play important roles in the antimycobacterial mechanisms of MAC-infected Mφ were subsequently more efficient in clearing the MAC than the former mice (45). This implies that RNI is not involved in the antimycobacterial mechanisms of MAC-infected Mφ. 2) It has been found that osteopontin-mediated facilitation of bacterial clearance of BCG organisms in infected mice was independent of RNI production (46). 3) As reported by Lammers et al. (24), neither RNI nor ROI played any critical role in the expression of antimycobacterial activity against BCG organisms by human monocyte-derived Mφ, which was given ATP signaling via P2X7 receptors. These findings strongly suggest the possibility that the RNI- and ROI-independent antimycobacterial mechanism(s) may be crucial for the antimycobacterial function of host Mφ.
in MAC-infected Mϕ, ATP signaling induced intracellular translocation/condensation of cPLA₂ molecules to phagosomes surrounding internalized MAC organisms (Fig. 6). To our knowledge, this is the first report that demonstrated intracellular translocation of cPLA₂ to intraphagosomal mycobacterial organisms and possible roles of cPLA₂ in intraphagosomal bacterial killing in ATP-treated Mϕ during the course of mycobacterial infection. Because serine phosphorylation of cPLA₂ molecules (especially of Ser505) is known to promote membrane penetration of hydrophobic amino acid residues in their active site rim (50), the cPLA₂ condensation to phagosomes membranes in response to ATP signaling (Fig. 6) also appears to be related to the serine phosphorylation of cPLA₂. Indeed, it was reported that ATP treatment of Mϕ resulted in the activation of cPLA₂ due to MAPK/ERK1,2-mediated phosphorylation of serine residues (51–53). In separate experiments, ATP-induced expression of potentiated anti-MAC activity was found to be associated with apoptotic cell death of ATP-treated Mϕ (H. Tomoeoka, K. Sato, C. Sano, and T. Shimizu, unpublished observation), as previously reported for BCG-infected Mϕ (24, 27). This is consistent with the finding by Duan et al. (49) that MTB induced Mϕ apoptosis through at least two types of signaling pathways, TNF-α or cPLA₂-dependent cascades, each of which is also crucial for Mϕ antimycobacterial defense mechanisms. In any case, these findings clearly indicate that ATP-mediated bacterial killing in MAC-infected Mϕ is strictly dependent on cPLA₂ functions and its metabolic product, AA, but is substantially independent of RNase and ROI. This finding strongly supports that concept that AA generated by the enzymatic action of cPLA₂ plays a critical role in the antimycobacterial mechanisms of host Mϕ.

Fourth, high concentrations of ATP (3–10 mM) were required to achieve significant levels of Mϕ anti-MAC activity even in the case of the low virulence MAC (N-444 strain)-infected Mϕ and to yield clear potentiation of Mϕ anti-MAC activity in the presence of CR (Fig. 4). This implies that ATP acts via P2X7 receptors to induce/potentiate Mϕ anti-MAC activity, because the active component of ATP interacting with P2X7 receptors is the fully ionized form of ATP (ATP³⁻), which is present as a small fraction of ATP at physiological pH. This conclusion is supported by the finding that these effects were mimicked by BzATP, a known agonist of P2X7 receptors (Fig. 5A). As shown in Fig. 5A, Ca²⁺ mobilization was required for Mϕ to display significant levels of anti-MAC activity when ligation of P2 receptors of MAC-infected Mϕ was provided by a low concentration of ATP (0.3 mM). Under these conditions, P2Y receptors (presumably P2Y₂ and/or P2Y₁₁ receptors) might participate in ATP-induced anti-MAC activity of Mϕ by activating PLCβ, which is coupled with PKC activation, and subsequent activation of MAPK-mediated cPLA₂ activation (53).

Fifth, another important finding of the present study is that ATP strongly potentiated Mϕ anti-MAC antimicrobial functions when Mϕ were treated with CR, a combination of anti-MAC antimicrobial drugs (clarithromycin plus rifampycin). It thus appears that the use of ATP in combination with certain antimycobacterial drugs, including macrolides and rifamycins, may be beneficial in achieving efficacious control of patients with intractable MAC infections. ATP is an essential compound for all living things and can be administered to humans without severe adverse effects. At present, ATP is safely used as a vasodilator for clinical control of ischemia in the coronary arteries and paroxysmal tachycardia (54, 55). It thus appears that ATP can be safely administered to MAC patients.

There have been a number of attempts to establish efficacious regimens of adjunctive immunotherapy for management of patients with mycobacterial infections involving administration of certain immunomodulators in combination with antimycobacterial drugs (33). Adjunctive clinical trials using IL-2 or GM-CSF found these agents to be efficacious to some extent in improving patients with tuberculosis or disseminated MAC infections (33). However, these immunomodulating cytokines as well as IFN-γ and IL-12 do not appear promising as therapeutic agents for mycobacterial infections because of the possibility of induction of immunosuppressive cytokines, such as TGF-β, IL-10, and IL-13, during adjunct therapy and, in some cases, severe adverse effects (33). Thus, the development of new classes of immunomodulators other than cytokines, particularly those with no severe adverse effects, is needed. In this context, ATP may be one of the most promising agents for clinical immunotherapy of mycobacterial infections in combination with anti-MAC chemotherapy for the following reasons. First, it is safe for humans, as described above. Second, ATP acts directly on Mϕ and rapidly causes not only potentiation of Mϕ antimycobacterial activity, but also concomitant apoptosis of Mϕ. It thus appears that ATP-stimulated Mϕ do not serve as a cell source of immunosuppressing cytokines, which suppress Mϕ antimycobacterial functions in an autocrine or paracrine fashion. Indeed, in the present study prolonged ATP administration (4 wk) did not increase the mRNA expression of these immunosuppressing cytokines (Fig. 3). It will be of interest to examine the therapeutic effects of regimens involving ATP in combination with macrolides (clarithromycin and azithromycin), rifamycins (rifampin, rifabutin, and rifalazil), and other drugs (ethambutol, streptomycin, and quinolones) against MAC infections over long observation periods at least 16 wk after infection. Additional studies are currently underway to elucidate the usefulness of ATP therapy of MAC infection in detail.

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Disclosures

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References


