Host-Directed Therapeutics: ATP as an Immunoadjunctive Agent for Chemotherapy against Mycobacterial Infections

Haruaki Tomioka

Department of Basic Medical Science for Nursing, Yasuda Women's University, Hiroshima, Hiroshima 731-0153, Japan

Adenosine triphosphate (ATP) treatment of human macrophages, which possess both $P2X_7$ and $P2Y_2$ receptors, potentiates the activity of macrophages (MΦs) to kill mycobacterial organisms in a $P2X_7$-dependent manner. ATP-mediated killing of mycobacterial organisms within MΦs is partly mediated by phospholipase D, which is linked to leukocyte antimicrobial mechanisms dependent on the mobilization of intracellular Ca^{2+} and subsequent lysosomal fusion and acidification of mycobacteria-containing phagosomes. ATP-mediated killing of intramacrophage mycobacteria is also attributable to MΦ apoptosis induced by $P2X_7$-mediated signaling. Furthermore, ATP signals transmitted through $P2X_7$ receptors up-regulate MΦ antimycobacterial activity in a cytosolic phospholipase A2-dependent manner. Recently, it has been demonstrated that ATP directly inhibits the growth of various bacteria, including *Staphylococcus*, *Pseudomonas*, and mycobacteria. The antibacterial activity of ATP is attributable to its iron-chelating ability. This chapter describes the possible usefulness of ATP-based immunoadjunctive therapy in the clinical management of intractable mycobacteriosis, using antimicrobial chemotherapeutics.

Keywords: ATP; mycobacterial infection; macrophages; adjunctive agent

1. Introduction

Tuberculosis (TB) is a growing international health concern, since it is the leading infectious cause of death in the world today. In particular, the appearance of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* (MTB), which exhibit in vitro resistance to at least two major antituberculous drugs, usually isoniazid (INH) and rifampin (RMP), and cause intractable TB, has contributed greatly to the increase in the incidence of TB [1]. Because of the global health problems associated with TB, the increasing cases of MDR-TB and high rate of co-infection with HIV, the development of potent new antituberculous drugs without cross-resistance with known antimycobacterial agents is urgently needed [1]. Moreover, antituberculous chemotherapy is unable to kill/eliminate all organisms over a short period of time. Thus, effective treatment is prolonged and costly, and may be complicated by drug toxicities. On the other hand, the clinical management of MAC infection is difficult, since it is frequently encountered in immunocompromised hosts, particularly AIDS patients, and MAC organisms are moderately to highly resistant to common antituberculosis drugs such as INH, ethambutol (EMB), pyrazinamide (PZA), and RMP. Although some drugs including clarithromycin (CAM), azithromycin (AZI), and rifabutin (RBT) are moderately effective in controlling MAC bacteremia in AIDS patients, the treatment of pulmonary MAC infections is still difficult even with the use of multi-drug regimens containing these drugs [2].

Although some new antimicrobial agents active against MTB (especially MDR-MTB or dormant MTB), such as bedaquiline, delamanid, pretomanid, and oxazolidinones, SQ109, and those active against MAC, such as new ketolides and pyrrole derivatives, have been or are being developed, only limited numbers of new drugs, including delamanid and bedaquiline, have been approved for clinical use or have been subjected to phase clinical trials for chemotherapy against mycobacterial infections (PA-824, SQ109, sutezolid, and AZD5847: all drugs are under phase II studies) [3, 4]. Thus, at present, it appears that devising potent anti-MTB or anti-MAC administration protocols using ordinary antimycobacterial drugs is more practical than awaiting the development of new antimycobacterial drugs. The relative reduction of antimycobacterial immunity coexists with active TB and MAC infections. Therefore, the enhancement of host immune responses by adjunctive immunotherapy may decrease the duration of chemotherapy needed by TB patients or increase the overall efficacy of antimycobacterial regimens for patients with intractable TB or MAC infection. The modulation of specific host immune responses, including reactions that impact inflammatory events and immunopathology, seem to limit mycobacterial replication in hosts and related pathologies. Therefore, adjunctive host-directed therapy (HDT) is expected to promote the anti-MTB effector functions of macrophages (MΦs), such as autophagy and the production of antimicrobial molecules and proinflammatory cytokines [5-8]. Moreover, HDT is efficacious in preventing lung injury due to the overexpression of anti-TB immunity by modifying specific mechanisms causing lung inflammation and tissue damage [5, 7, 9]. However, it appears that adjunctive immunotherapy is still associated with serious problems and dilemmas, such as high cost, occasionally strong side effects, and, in many cases, only modest efficacy in potentiating host defense mechanisms against mycobacterial pathogens, primarily because of the spontaneous induction of MΦ-deactivating cytokines during the course of long-term administration [5]. Thus, it is important to synthesize or screen out new low-cost and safe drugs with mild immunopotentiating activity, which do not induce immunosuppressing cytokines, such as TGF-β, IL-10, and IL-13, during administration for long periods of time.

In this context, adenosine 5'-triphosphate (ATP), which is classically associated with cellular energy metabolism, may be promising as a low-cost and safe immunoadjunctive drug for the clinical treatment of intractable mycobacterioses. ATP exhibits various biological activities, such as mitogenic stimulation and gene expression, through the ligation of P2 purinoceptors [10, 11]. MΦs possess both $P2X_7$ and $P2Y_2$ receptors [10-12]. It is well-known that ATP treatment of human MΦs potentiates their activity in killing mycobacterial organisms in a $P2X_7$-dependent manner [13]. These situations suggest the usefulness of ATP as an immunoadjunctive drug for mycobacterial chemotherapy. In this review article, I have highlighted the possible usefulness of ATP-based immunoadjunctive therapy in the clinical control of certain refractory bacterial infections, especially TB and MAC infection.

2. Immunoadjunctive agents for TB therapy

According to Hawn et al. [8], host-directed therapeutics (HDTs) are divided into the following major groups by focusing on general mechanistic strategies: (1) HDTs invalidating MTB pathogenesis in host macrophages, (2) HDTs facilitating the host's protective immune responses against MTB organisms, and (3) HDTs reducing the host's deleterious responses that cause exacerbation of TB disease. The authors proposed the mechanisms of HDT action against MTB, as follows: (1) Inhibition of host-related factors required for MTB pathogenesis, (2) augmentation of the host's innate immune responses including autophagy, (3) suppression of the host's deleterious immune reactions such as hyperinflammation, facilitating MTB pathogenesis, (4) induction of novel immune response, against MTB, (5) recovery of immune responses that are suppressed by MTB, and (6) modulation of mycobacterial functions, increasing the susceptibility of MTB pathogens to the host's defenses. In their review, the candidate HDTs with small molecular sizes, which have already been approved by the FDA or are under late-stage clinical evaluation for other disease conditions, are listed as adjunctive therapy against TB disease [8]. The lists provided by Hawn et al. [8], Wallis and Hafner [6], and Rayasam and Balganesh [9] include a number of promising agents that target the following cellular events of hosts: (1) protein kinase-mediated signaling pathways of host cells (e.g., imatinib, gefitinib), (2) cAMP-mediated signaling axes (e.g., CC-3052, cilostazol, pentoxifylline, and sildenafil), (3) eicosanoid-mediated signaling pathways (e.g., aspirin, zileuton, prostaglandin E_2, ibuprofen, and statin), (4) autophagy and related signaling cascades (e.g., rapamycin, metformin, vadimezan, nitazoxanide, gefitinib, fluoxetine, valproic acid, prochlorperazine, and lithium), and (5) lipid metabolism (e.g., mepenzolate, thiazolidinedione, and statin).

According to classical studies, adjunctive therapy using immunopotentiating cytokines, including IL-2, IL-18, IL-12, IFN-γ, and GM-CSF, is fairly efficacious in improving patients with intractable TB or MAC infection. Notably, a recent randomized control trial with aerosolized and subcutaneously administered IFN-γ performed by Gao et al. [14] demonstrated that both adjunctive therapies were significantly efficacious in reducing symptoms of fever, wheeze, and night sweats in TB patients. However, it appears that these cytokines are not promising for clinical use against mycobacterial infections because of the possible induction of immunosuppressive cytokines during adjuvant therapy and severe side effects. Moreover, therapy using these cytokines is very expensive. This is a serious disadvantage to clinical treatment of mycobacterial infections using these cytokines. Thus, the development of new classes of immunomodulators, particularly those with no severe adverse effects and a low cost, is desired. As described in previous reviews and reports [5, 6, 8, 9, 15, 16], immune response modifiers which appear to be useful in potentiating host resistance to mycobacterial infections include: (1) vitamin D/calcitriol, (2) nonsteroidal anti-inflammatory drugs including diclofenac sodium, (3) autophagy-inducing agents, (4) galactosylceramide, (5) picolinic acid, (6) poloxamer CRL-1072, (7) DNA vaccine expressing mycobacterial components, (8) inhibitors of immunosuppressing cytokines, (9) the traditional Chinese medicine Mao-Bushi-Saishin-To, (10) heat-killed *Mycobacterium vaccae*, (11) mycobacterial muramyl dipeptide (MDP) derivative glucosaminyl MDP, (12) recombinant BCG secreting IL-2, IFN-γ, or GM-CSF, (13) imidazoquinoline S28463, (14) levamisole, (15) diethyldithiocarbamate, (16) dibenzopyran FCE 20696, (17) secretory leukocyte protease inhibitor (SLPI), and (18) ATP and its analogues as described below.

3. ATP as a promising immunoadjunctive agent against mycobacterial infection

ATP is noteworthy because of its peculiar effects on the function of host MΦs. Extracellular ATP is known to serve as a mediator of cell-to-cell communication by triggering a variety of biological responses in various cells, such as hematopoietic, endothelial, and nerve cells, through the ligation of plasma membrane purinergic receptors [17]. The biological activities of extracellular ATP are diverse and include mitogenic stimulation, gene expression, an excitatory transmitter function, and the induction of cell death. The following two subfamilies of P2 purinoceptor have been described: ligand-gated ionotropic P2X receptors and G protein-coupled P2Y receptors [17, 18]. MΦs possess both $P2X_7$ and $P2Y_2$ as major P2 receptors [17, 18]. While P2Y receptors have metabotropic functions, P2X receptors are ionotropic and form trimeric structures, causing the transmission of positive electrical ions through the cellular cytoplasmic membranes [17, 18]. Because of the low affinity of $P2X_7$ receptors for ATP, the $P2X_7$ receptor's function is inhibited by Mg^{2+} ions due to the decrease in the extracellular concentration of ATP^{4-}, a natural agonist of P2X receptors [19].

Although recent studies indicated that the $P2X_7$ receptor also acts as a scavenger receptor for bacteria and apoptotic cells in the absence of extracellular ATP [20], it is known that the ligation of $P2X_7$ receptors with low concentrations of ATP (~100 μM) causes the influx of extracellular Ca^{2+} across the cell membrane, while prolonged activation with high concentrations of ATP (>1 mM) results in the formation of large nonselective membrane pores permeable to hydrophilic molecules up to 900 Da in size [13, 18]. These events are thought to cause subsequent changes in intracellular signaling and metabolic pathways leading to the activation of NF-κB, SAPK/JNK, and caspases. Notably, ATP is also known to induce the recruitment and activation of the NOD-like receptor-family protein 3 (NLRP3)-inflammasome, leading to the release of IL-1β but not IL-1α or IL-6 release, via the $P2X_7$ receptor-related signaling events, especially the localized cytoplasmic Ca^{2+} ion changes causing $P2X_7$ receptor recruitment [22]. On the other hand, $P2Y_2$ receptors act via G proteins (Gq) to stimulate the phospholipase Cβ (PLCβ) signaling cascade releasing Ca^{2+} ions from internal stores [17]. It has been reported that the treatment of human macrophages with ATP (ATP^{4-}) causes the potentiation of macrophage activity in killing mycobacterial organisms, including MTB and BCG [13]. ATP-induced killing of mycobacterial organisms within MΦs is principally mediated by $P2X_7$ receptors [13, 22], although it has been reported that purinergic signaling also regulates MΦ activity in killing BCG organisms via a $P2X_7$-independent mechanism [23]. In this context, the following situations with $P2X_7$ receptors are noteworthy: In MΦs, the $P2X_7$ receptors are coupled with apoptosis, the production of ROIs, membrane blebbing, regulation of various protein kinases and phosphatases, including protein kinase C, protein kinase D, MAPK, tyrosine phosphatase, and certain lipases, such as phospholipase C, phospholipase D, and phospholipase A_2 [24]. Furthermore, the activation of $P2X_7$ receptors in LPS-stimulated MΦs, inducing Ca^{2+} influx, non-selective large pore formation, and activation of MAPK pathways, triggers caspase-1 activation followed by the subsequent increase in the generation of IL-1β and IL-18 and up-regulates the expression of inducible nitric oxide synthase (iNOS), and ROI-dependent apoptosis [24, 25]. In this context, it has also been reported that the ATP-induced activation of $P2X_7$ receptors caused an increase in the release of IL-1β and prostaglandin E_2 but down-regulation of MHC class I expression in Kupffer macrophages via Ca^{2+} influx and large pore formation [26]. With special reference to macrophage apoptosis related to $P2X_7$ receptor activation, recent studies indicated an interesting situation whereby ATP-mediated activation of the $P2X_7$ receptor induces membrane blebbing and cellular apoptosis dependent on anoctamin 6 (a protein with Ca^{2+}-dependent phospholipid scramblase and Ca^{2+}-activated Cl^- channels), but independent of pannexin-1, proinflammatory caspase-1, and TLR signaling [27, 28]. Notably, it has been reported that the $P2X_7$ receptor forming an intact $P2X_7$-nonmuscle myosin IIA membrane complex up-regulates MΦ phagocytosis of nonopsonized bacteria, including *Staphylococcus aureus* and *Escherichia coli*, while extracellular ATP attenuates the phagocytosis of bacteria by MΦs [29]. It is therefore thought that an excessively increased level of $P2X_7$-mediated phagocytosis of bacteria is limited by extracellular ATP, being followed by subsequent promotion of the release of pro-inflammatory cytokines, that cause autocrine up-regulation of the antimicrobial functions of host MΦs [24].

3.1 ATP-mediated potentiation of antimycobacterial activity of host MΦs

As shown in Fig. 1, ATP signaling via the $P2X_7$ and $P2Y_2$ receptors causes Ca^{2+} influx, large pore formation, and the activation of G protein-coupled signaling cascades [13, 18]. $P2Y_2$ receptors act via G proteins (Gq) to stimulate the phospholipase Cβ (PLCβ) signaling cascade, releasing Ca^{2+} from internal stores [18, 30]. These events thereafter upregulate the down-stream signaling pathways including calmodulin-, NF-κB-, stress-activated protein kinase (SAPK)/JNK-mediated signaling axes, and various kinds of caspase linked to cell apoptosis [18, 31, 32].

It has been reported that treatment of human MΦs with ATP (ATP^{4-}) potentiates MΦ activity to kill MTB complex mycobacteria, such as MTB and BCG [13, 22, 33-35]. ATP-induced killing of mycobacterial organisms within MΦs is principally mediated by $P2X_7$ receptors, since the effects of ATP are mimicked by benzoylbenzoyl-ATP (BzATP), a potent $P2X_7$ receptor agonist, while they are inhibited by oxidized ATP, an irreversible inhibitor of the $P2X_7$ receptor [13, 22, 33, 34]. Notably, it has been reported that purinergic signaling also regulates MΦ activity to kill BCG organisms via a $P2X_7$-independent mechanism [23]. With special reference to the participation of down-stream signaling pathways of the $P2X_7$ receptor, Stober et al. [34] and Kusner and Barton [35] demonstrated that ATP-mediated killing of mycobacteria within MΦs is intracellular Ca^{2+} mobilization-dependent. In addition, ATP-mediated killing of mycobacterial organisms within MΦs is mediated by phospholipase D, which is linked to leukocyte antimicrobial mechanisms dependent on the mobilization of intracellular Ca^{2+} and subsequent lysosomal fusion and acidification of mycobacteria-containing phagosomes [22, 33, 34].

In this context, subsequent studies concerning the effect of ATP on MΦ activity against MAC organisms revealed the following [36]. (1) ATP significantly potentiates the therapeutic efficacy of the drug regimen, CAM in combination with rifamycin (CR), in treating MAC-infected mice during the early stage of infection in terms of reduction of bacterial loads in the spleens and lungs of host mice during the first four weeks after infection, principally by potentiating the anti-MAC activity of host MΦs. (2) The mobilization of intracellular Ca^{2+} is crucial for the ATP (or BzATP)-mediated potentiation of MΦ anti-MAC activity, because the combination of ATP with the calcium ionophore A23187 significantly potentiated MΦ antimicrobial activity against the pathogen. (3) ATP-induced augmentation of macrophage

anti-MAC activity is dependent on cytosolic phospholipase A_2 ($cPLA_2$) but not on RNIs or ROIs. This is supported by the following evidence: The potentiation by ATP of the antimicrobial activity of CR against intramacrophage MAC was markedly abrogated by arachidonyl trifluoromethyl ketone (a-TFMK), a specific $cPLA_2$ inhibitor, but not by superoxide dismutase/catalase (ROI scavengers) or NG-monomethyl L-arginine (NMMA), an inducible nitric oxide synthase (iNOS) inhibitor [36]. These findings suggest that the $cPLA_2$-mediated release of arachidonic acid from phospholipids of the phagosomal membrane into MAC-containing phagosomes plays an important role in the ATP-mediated potentiation of MΦ anti-MAC activity in the presence of CR. In this context, the translocation of membranous arachidonic acid to intramacrophage mycobacteria was markedly inhibited by a-TFMK and colchicine (an inhibitor of phagocytosis), but not by manoalide (a secretory PLA_2 inhibitor), NDGA (a lipoxygenase inhibitor), or indomethacin (a cyclooxygenase inhibitor) [36]. Furthermore, separate experiments indicated that, in MAC-infected MΦs, ATP signaling via $P2X_7$ receptors induces $cPLA_2$ translocation to the phagosomal membranes surrounding internalized MAC organisms [36]. Taken together, it is thought that ATP signaling causes $cPLA_2$ activation via the intracellular Ca^{2+} mobilization pathway, thereby enhancing the $cPLA_2$-mediated release of arachidonic acid, one of the major antimycobacterial effectors of MΦs [37], into MAC-containing MΦ phagosomes. In this context, a recent study by Ouaalti et al. [38] demonstrated that, in LPS-stimulated MΦs, $P2X_7$ receptors caused the activation of Ca^{2+}-insensitive PLA_2 ($iPLA_2$) and increased the release of unsaturated fatty acids via activation of the MEK-1 pathway. This suggests possible roles of $iPLA_2$ in addition to $cPLA_2$ in the potentiation of antimycobacterial activity of MΦs responding to the ATP signals transmitted through $P2X_7$ receptors.

Although it is commonly thought that RNIs, and not ROIs, play central roles in the expression of the antimycobacterial activity of MΦs, a series of studies by Akaki et al. [37] indicated that ROI molecules are markedly effective in exhibiting antimycobacterial activity when they act on mycobacterial pathogens in the presence of halide and ferrous ions, because they generate extremely toxic hypohalite ions and consequently promote halogenation reactions on bacterial target components. Therefore, ROIs may also play important roles in the manifestation of the antimicrobial activity of MΦs against mycobacterial organisms. Notably, MΦ production of ROIs is enhanced in response to $P2X_7$ receptor activation [39], thereby indicating that up-regulation of the antimycobacterial functions of MΦs in response to ATP signaling via $P2X_7$ receptors is partly attributable to increased ROI production after $P2X_7$ receptor activation. In this context, it has recently been demonstrated that $P2X_7$-mediated Ca^{2+} influx causes ROI generation by MΦs by up-regulating c-Src/Pyk2 and ERK1/2 pathways [40]. In contrast, this signaling cascade involves neither PI3K, p38 MAPK, nor calmodulin-mediated signaling events [40]. Alternatively, the increased ROI production in response to $P2X_7$ receptor activation may be important for the up-regulation of MΦ signaling pathways involving MAPKs and PI3K rather than for the direct antimicrobial action of ROI molecules [25].

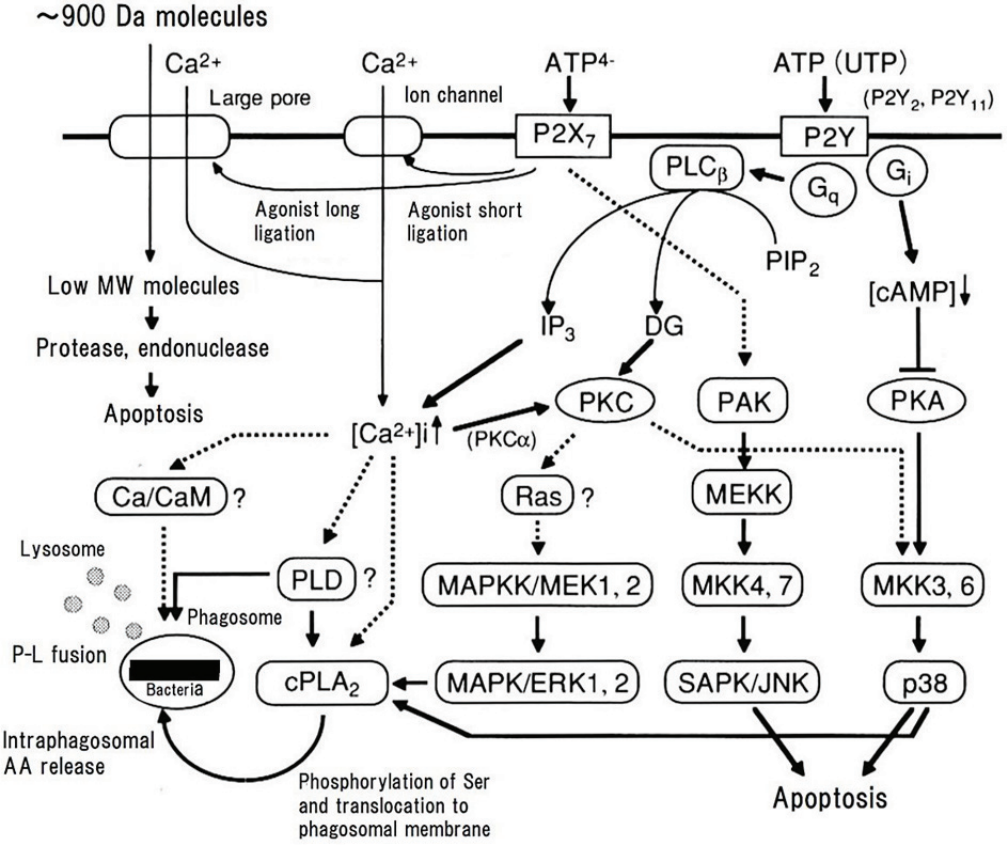

Fig. 1 Mechanisms of ATP-induced potentiation of the antimycobacterial activity exerted by host macrophages.

In relation to the roles of the ATP-$P2X_7$ receptor signaling system in the potentiation of MΦ antimycobacterial functions, Th17 cell expansion and differentiation in response to the activation of $P2X_7$ receptors may be important for host defense against mycobacterial infections, since Th17 cells are known to play significant roles in the expression of host resistance to bacterial pathogens, including mycobacteria. It has been demonstrated that the ATP released by activated Tregs triggers a decrease in the Treg-specific transcription factor Foxp3 protein, and induces the conversion of Tregs to Th17 cells through the activation of $P2X_7$ receptors [41]. This indicates a role of the ATP-$P2X_7$ signaling cascade in modulating Treg-mediated immunosuppression. In addition, the $P2X_7$ receptor is effectively involved in T-cell activation since the ATP release was required for TCR-mediated Ca^{2+} ion influx and IL-2 production [42].

3.2 ATP exhibits antimicrobial activity against some kinds of pathogenic bacteria through deprivation of ferric ions

It is unknown which antimicrobial molecules act as effectors in intracellular mycobacterial killing by ATP-stimulated MΦs. One candidate is long chain free fatty acids, which are generated in the intraphagosomal milieu by the enzymatic action of cytosolic phospholipase A_2. MΦ treatment with high concentrations of extracellular ATP is thought to cause the influx and accumulation of ATP in MΦ cytoplasm through large membrane pores generated in response to $P2X_7$-mediated ATP signaling. Indeed, the long C-terminal tail of $P2X_7$ allows its conformational change, causing the formation of a large pore, which permits transmembrane fluxes of small hydrophilic molecules, including ATP.

On the basis of this working hypothesis, Tatano et al. [43] recently examined the possibility that intracellularly condensed ATP molecules participate in the expression of MΦ antimycobacterial activity. They found that ATP inhibited the growth of various bacteria, such as mycobacteria (*Mycobacterium intracellulare*, *M. avium*, *Mycobacterium kansasii*, and MTB), *S. aureus* including methicillin-resistant *S. aureus* (MRSA), and *Pseudomonas aeruginosa*, without damaging bacterial surface structures, including the cytoplasmic membrane and cell wall. On the other hand, other bacterial species, including *Listeria monocytogenes*, *E. coli*, and *Klebsiella pneumoniae*, are essentially resistant to ATP. Notably, anti-*M. intracellulare* activity was shared by ATP, benzoylbenzoyl ATP ($P2X_7$ agonist), and UTP (P2Y agonist), but not by AMP (P2Y agonist). In addition, oxidized ATP ($P2X_7$ antagonist), and suramin, MIA, and DIDS ($P2X_7$ inhibitors) did not block ATP activity. Therefore, ATP signaling in MAC organisms through bacterial P2 purinoceptor-like molecules is not required for the expression of ATP's bacteriostatic action.

ATP is known to chelate metal ions, such as Mg^{2+} and Mn^{2+}, causing the modulation of various enzymatic reactions *in vivo*. Therefore, ATP may exhibit antimicrobial effects on certain microorganisms by depriving them of essential metal ions. This was confirmed by Tatano et al. as follows [43]. Firstly, ATP's anti-*M. intracellulare* activity was significantly reduced by $MgCl_2$ and $FeCl_3$. Secondly, all test metal chelators exhibited antimicrobial activity against *M. intracellulare* in the order of EDTA > EGTA > ATP > pyrophosphate (iron-chelator), and the excess free Mg^{2+} ion-occupying chelating ability of these agents partly blocked their antibacterial effects. Indeed, it has been demonstrated that EDTA exhibits strong antimicrobial activity against *P. aeruginosa*, and the antibacterial activity of certain antibiotics which inhibit bacterial protein synthesis was reported to be significantly potentiated when used in combination with EDTA, presumably due to the chelation of Ca^{2+} and Mg^{2+} ions.

Ferric ions are essential for the growth and survival of most living organisms. During infection in host animals, bacterial organisms are obligated to compete with host iron-chelating substances, such as transferrin and lactoferrin, to acquire ferric ions. Using siderophores, bacterial high-affinity ferric chelators, bacteria maintain their intracellular iron concentrations at sufficient levels for survival and growth in hosts [44]. Tatano et al. [43] indicated that ATP exhibits significant levels of ferric ion-chelating activity. The combined use of $FeCl_3$ markedly reduced the antibacterial activity of ATP as well as EDTA against *M. intracellulare* and *P. aeruginosa*, strongly suggesting that the antibacterial activities of ATP and EDTA are principally based on their ferric ion-chelating ability. In addition, ATP-resistant *E. coli* and *K. pneumoniae*, but not ATP-susceptible *S. aureus*, were found to produce significant levels of siderophores when cultivated in the presence of high concentrations of ATP. In *P. aeruginosa*, siderophore production was only observed in ATP-resistant strains. These findings strongly suggest that the ATP-induced production of siderophores is limited to ATP-resistant bacteria.

To confirm the above, using gene technology, Tatano et al. [43] newly established an enterobactin-deficient (*entB*$^-$) mutant from ATP-resistant *K. pneumoniae*, and observed the recovery of ATP susceptibility in the enterobactin-deleted mutant. Therefore, ATP's antibacterial activity is attributable to its ferric ion-chelating ability. Since intracellular ATP is distributed in the cytosol of macrophages at high concentrations, as indicated by the author's experiments and as previously described by Morandini et al. [24], ATP appears to augment MΦ antimicrobial activity by directly attacking intracytosolic and intra-autophagosomal pathogens. ATP induces autophagy in macrophages and causes rapid bacterial killing within autophagosomes [45, 46]. Therefore, it is likely that cytosolic ATP is accumulated in autophagosomes together with mycobacterial organisms and exhibits an antimicrobial action against bacteria residing within autophagolysosomes. In this context, Tatano et al. [43] reported that the intracellular ATP concentration was maintained at around 1 mM for up to 4 h after bacterial infection, showing that the intracellular paucity of ATP did not occur in MΦs because of bacterial invasion. Importantly, this finding indicates that, in macrophages engulfing bacteria, sufficient intracellular ATP concentrations for the efficacious expression of ATP antibacterial activity are maintained

during the course of infection. In addition, the pretreatment of MΦs with a representative MΦ-activating cytokine, IFN-γ, with or without subsequent triggering with phorbol myristate acetate (PMA), did not cause a significant increase in the intracellular ATP concentration [43]. This finding suggests that ATP's antimicrobial action against intramacrophage bacteria is not potentiated in connection with MΦ activation elicited by IFN-γ-priming and PMA-triggering. It thus appears that intracytosolic ATP only partially contributes to the manifestation of the antibacterial activities of macrophages as a basic intramacrophage antimicrobial system. Notably, PMA-triggering caused a marked decrease in the intramacrophage ATP concentration, suggesting the rapid consumption of intracytosolic ATP, presumably due to the PMA-induced potentiation of intracellular metabolic pathways related to inflammatory reactions elicited by PMA-mediated signaling.

Finally, Tatano et al. [43] demonstrated that ATP exhibited combined in vitro antimicrobial effects with some chemotherapeutics, including vancomycin against MRSA and CAM plus EMB against *M. intracellulare*, suggesting its usefulness as an adjunctive drug in the chemotherapy of certain intractable infections, in addition to its immunoadjunctive effects through the potentiation of antimicrobial functions of host phagocytes, as described above.

3.3 Usefulness of ATP for clinical treatment of intractable mycobacteriosis, particularly MAC infection

The clinical management of MAC infections is difficult, since such infections are frequently encountered in immunocompromised hosts, particularly AIDS patients [1], and MAC organisms are highly or moderately resistant to common antituberculosis drugs such as INH, EMB, PZA, and RMP [47]. Although some new drugs including CAM, AZI, and RBT are fairly effective in controlling MAC bacteremia in AIDS patients, the treatment of pulmonary MAC infections is still difficult even with the use of multi-drug regimens containing these drugs [47]. 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, pyrimidine derivatives, resorcinomycin A, and pyrrole derivatives, are being developed [47], few have undergone clinical studies. Thus, at present, attempts to devise potent anti-MAC administration protocols using ordinary antimycobacterial drugs may be 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. With respect to the usefulness of ATP for the clinical treatment of MAC infections, the following aspects are noteworthy. In previous studies using MTB and MAC as target organisms, while ATP at 3 to 5 mM up-regulated the antimicrobial activity of MΦs against MTB, this was not noted for macrophages infected with MAC organisms, although intramacrophage MAC growth was weakly inhibited in ATP-treated MΦs under certain conditions, especially when macrophages were stimulated with Ca^{2+} ionophores, causing the mobilization of Ca^{2+} from its intracellular pool [36]. In addition, ATP potentiated macrophage anti-MAC antimicrobial functions under conditions in which MΦs were treated with anti-MAC antimicrobial drugs including a macrolide (CAM) and rifamycin (RMP and rifalazil). When MAC-infected mice were subcutaneously given ATP at 40 mg/kg, once daily, 5 times per week, for up to 8 weeks after infection, bacterial growth in the lungs was significantly inhibited during the first 2 weeks compared to that in control mice without ATP administration [48]. This effect on intrapulmonary MAC growth was no longer observed after week 2, and MAC grew much more rapidly thereafter in mice given ATP than in control mice. These findings show that the efficacy of ATP in potentiating MΦ anti-MAC activity is less marked than that for macrophage anti-MTB activity [48]. The finding that ATP administration inhibited MAC growth in the lungs of infected mice in the early stages of infection suggests that ATP may be useful in treating MAC infection when given in combination with anti-MAC antimicrobial drugs. In any case, it can be expect that ATP has some potential as an immunoadjunctive drug for the clinical treatment of MAC infections.

4. Concluding Remarks

In the period before the establishment and application of antimycobacterial chemotherapy, symptomatic treatment of TB, including rest, nutrition, and sunlight exposure was successful to some extent in arresting TB, indicating that there is a significant capacity for self-cure in hosts with MTB infection. This suggests the possible usefulness of HDT using immunoadjunctive drugs for the clinical management of TB and other intractable mycobacterioses, especially MAC infections. From this viewpoint, a number of HDTs have been or are being developed for clinical use in the treatment of mycobacterial infections, as described above. Notably, ATP may be one of the most promising agents for clinical immunotherapy of mycobacterial infections in combination with chemotherapy using antimycobacterial drugs, for the following reasons: Firstly, ATP is an essential compound in all cells and is therefore a safe agent for human beings. In fact, ATP is safely used as a vasodilator for the clinical control of coronary artery ischemia and paroxysmal tachycardia. It thus appears that ATP can be safely administered to patients with mycobacterioses, especially TB and MAC infections, for long periods of time. Secondly, ATP directly acts on MΦs and rapidly causes not only the potentiation of MΦ antimycobacterial activity but also the concomitant apoptosis of MΦs. Thus, ATP-stimulated MΦs do not appear able to serve as a cellular source of immunosuppressing cytokines (IL-10, IL-13, and TGF-β), which suppress

macrophage antimycobacterial functions in an autocrine or paracrine fashion. Thus, ATP is suitable for use as an adjunctive agent in combination with antimycobacterial drugs for the clinical treatment of intractable MTB and MAC infections. In further studies to develop ATP as an adjunct for the clinical treatment of mycobacterioses, assessment of ATP must include the evaluation of the benefit of ATP therapy in accelerating bacterial elimination and reducing excess levels of inflammatory reactions and the resulting tissue damage at sites of infection in hosts. To achieve this, it is necessary to develop and establish peripheral markers of the clinical effects of ATP therapy on patients and mycobacterial pathogens.

References

[1] Tomioka H. Current status of some antituberculosis drugs and the development of new antituberculous agents with special reference to their in vitro and in vivo antimicrobial activities. Curr Pharm Des. 2006;12:4047-70.

[2] Benson CA. Disseminated *Mycobacterium avium* complex infection: implications of recent clinical trials on prophylaxis and treatment. AIDS Clin Rev 1997; 98: 271-87.

[3] Tomioka H. New approaches to tuberculosis - novel drugs based on drug targets related to toll-like receptors in macrophages. Curr Pharm Des. 2014; 20:4404-17.

[4] Olaru ID, von Groote-Bidlingmaier F, Heyckendorf J, Yew WW, Lange C, Chang KC. Novel drugs against tuberculosis: a clinician's perspective. Eur Respir J. 2015; 45:1119-31.

[5] Tomioka H. Adjunctive immunotherapy of mycobacterial infections. Curr. Pharm. Design 2004; 10: 3297-312.

[6] Wallis RS, Hafner R. Advancing host-directed therapy for tuberculosis. Nat Rev Immunol. 2015; 15:255-63

[7] Tobin DM. Host-Directed Therapies for Tuberculosis. Cold Spring Harb Perspect Med. 2015. pii: a021196.

[8] Hawn TR, Shah JA, Kalman D. New tricks for old dogs: countering antibiotic resistance in tuberculosis with host-directed therapeutics. Immunol Rev. 2015; 264:344-62..

[9] Rayasam GV, Balganesh TS. Exploring the potential of adjunct therapy in tuberculosis. Trends Pharmacol Sci. 2015; pii: S0165-6147(15)00095-4.

[10] Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. Pharamcol. Rev. 2006; 58:58–86.

[11] Idzko M, Ferrari D, Eltzschig HK. Nucleotide signalling during inflammation. Nature 2014; 509:310–7.

[12] Di Virgilio F, Vuerich M. Purinergic signaling in the immune system. Auton Neurosci. 2015;. pii: S1566-0702(15)00047-8.

[13] Lammas DA, Stober C, Harvey CJ, Kendrick N, Panchalingam S, Kumararatne DS. ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X$_7$) receptors. Immunity 1997; 7:433–44.

[14] Gao XF, Yang ZW, Li J. Adjunctive therapy with interferon-gamma for the treatment of pulmonary tuberculosis: a systematic review. Int J Infect Dis. 2011; 15:e594-600.

[15] Ralph AP, Kelly PM, Anstey NM. L-arginine and vitamin D: novel adjunctive immunotherapies in tuberculosis. Trends Microbiol. 2008; 16:336-44.

[16] Schön T, Lerm M, Stendahl O. Shortening the 'short-course' therapy- insights into host immunity may contribute to new treatment strategies for tuberculosis. J Intern Med. 2013; 273:368-82.

[17] Abbracchio MP, Burnstock G. 1998. Purinergic signalling: pathophysiological roles. Jpn. J. Pharmacol. 1998; 78:113-45.

[18] Di Virgilio F, Ferrari D, Falzoni S, Chiozzi P, Munerati M, Steinberg TH, Baricordi OR. P2 purinoceptors in the immune system. Ciba Found. Symp. 1996; 198:290-302.

[19] Steinberg TH, Silverstein SC. Extracellular ATP^{4-} promotes cation fluxes in the J774 mouse macrophage cell line. J Biol Chem. 1987; 262:3118-22.

[20] Wiley JS, Gu BJ. A new role for the P2X$_7$ receptor: a scavenger receptor for bacteria and apoptotic cells in the absence of serum and extracellular ATP. Purinergic Signal. 2012; 8:579-86.

[21] Franceschini A, Capece M, Chiozzi P,et al. The P2X$_7$ receptor directly interacts with the NLRP3 inflammasome scaffold protein. FASEB J. 2015; 29:2450-61.

[22] Fairbairn IP, Stober CB, Kumararatne DS, Lammas DA. ATP-mediated killing of intracellular mycobacteria by macrophages is a P2X7-dependent process inducing bacterial death by phagosome-lysosome fusion. J. Immunol. 2001; 167:3300–7.

[23] Sikora A, Liu J, Brosnan C, Buell G, Chessel I, Bloom BR. Purinergic signaling regulates radical-mediated bacterial killing mechanisms in macrophages through a P2X$_7$-independent mechanism. J. Immunol. 1999; 163:558-61.

[24] Morandini AC, Savio LE, Coutinho-Silva R The role of P2X$_7$ receptor in infectious inflammatory diseases and the influence of ectonucleotidases. Biomed J. 2014; 37:169-77.

[25] Noguchi T, Ishii K, Fukutomi H, Naguro I, Matsuzawa A, Takeda K, Ichijo H. Requirement of reactive oxygen species-dependent activation of ASK1-p38 MAPK pathway for extracellular ATP-induced apoptosis in macrophage. J Biol Chem. 2008; 283:7657-65.

[26] Toki Y, Takenouchi T, Harada H, Tanuma S, Kitani H, Kojima S, Tsukimoto M. Extracellular ATP induces P2X$_7$ receptor activation in mouse Kupffer cells, leading to release of IL-1β, HMGB1, and PGE$_2$, decreased MHC class I expression and necrotic cell death. Biochem Biophys Res Commun. 2015; 458:771-6.

[27] Hanley PJ, Kronlage M, Kirschning C, et al. Transient P2X$_7$ receptor activation triggers macrophage death independent of Toll-like receptors 2 and 4, caspase-1, and pannexin-1 proteins. J Biol Chem. 2012; 287:10650-63.

[28] Ousingsawat J, Wanitchakool P, Kmit A, Romao AM, Jantarajit W, Schreiber R, Kunzelmann K. Anoctamin 6 mediates effects essential for innate immunity downstream of P2X$_7$ receptors in macrophages. Nat Commun. 2015; 6:6245.

[29] Gu BJ, Saunders BM, Jursik C, Wiley JS. The P2X$_7$-nonmuscle myosin membrane complex regulates phagocytosis of nonopsonized particles and bacteria by a pathway attenuated by extracellular ATP. Blood. 2010; 115:1621-31.

[30] Dubyak GR, Cowen DS, Meuller LM. Activation of inositol phospholipids breakdown in HL60 cells by P2-purinergic receptors for extracellular ATP: evidence for mediation by both pertussis toxin-sensitive and pertussis toxin-insensitive mechanisms. J.

Biol. Chem. 1988; 263: 18108-17.
[31] Ferrari D, Wesselborg S, Bauer MKA, Schluze-Osthoff K. Extracellular ATP activates transcription factor NF-κB through the P2Z purinoreceptor by selectively targeting NF-κB p65 (RelA). J. Cell. Biol. 1997; 139:1635-43.
[32] Humphreys BD, Rice J, Kertesy SB, Dubyak GR. Stress-activated protein kinase/JNK activation and apoptotic induction by macrophage $P2X_7$ nucleotide receptor. J. Biol. Chem. 2000; 275:26792-8.
[33] Kusner DJ, Adams J. ATP-induced killing of virulent *Mycobacterium tuberculosis* within human macrophages requires phospholipase D. J. Immunol. 2000; 164:379–88.
[34] Stober CB, Lammas DA, Li CM, Kumararatne DS, Lightman SL, McArdle CA. ATP-mediated killing of *Mycobacterium bovis* bacille Calmette-Gue´rin within human macrophage is calcium dependent and associated with the acidification of mycobacteria-containing phagosomes. J. Immunol. 2001; 166:6276–86.
[35] Kusner DJ, Barton JA. ATP stimulates human macrophages to kill intracellular virulent *Mycobacterium tuberculosis* via calcium-dependent phagosome-lysosome fusion. J. Immunol 2001; 167:3308-15.
[36] Tomioka H, Sano C, Sato K, Ogasawara K, Akaki T, Sano K, Cai SS, Shimizu T. 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_2-dependent mechanisms in the ATP-mediated potentiation of antimycobacterial host resistance. J. Immunol. 2005; 175:6741–9.
[37] Akaki T, Sato K, Shimizu T, Sano C, Kajitani H, Dekio S, Tomioka H. Effector molecules in expression of the antimicrobial activity of macrophages against *Mycobacterium avium* complex roles of reactive nitrogen intermediates, reactive oxygen intermediates, and free fatty acids. J. Leukoc. Biol. 1997; 62:795-804.
[38] Ouaaliti M, Seil M, Dehaye JP. Activation of calcium-insensitive phospholipase A_2 ($iPLA_2$) by $P2X_7$ receptors in murine peritoneal macrophages. Prostaglandins Other Lipid Mediat. 2012; 99:116-23.
[39] Lenertz LY, Gavala ML, Hill LM, Bertics PJ. Cell signaling via the $P2X_7$ nucleotide receptor: linkage to ROS production, gene transcription, and receptor trafficking. Purinergic Signal. 2009; 5:175-87.
[40] Martel-Gallegos G, Casas-Pruneda G, Ortega-Ortega F, et al. Oxidative stress induced by $P2X_7$ receptor stimulation in murine macrophages is mediated by c-Src/Pyk2 and ERK1/2. Biochim Biophys Acta. 2013; 1830:4650-9.
[41] Schenk U, Frascoli M, Proietti M, et al. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. Sci Signal. 2011; 4:ra12.
[42] Woehrle T, Yip L, Elkhal A, et al. Pannexin-1 hemichannel-mediated ATP release together with $P2X_1$ and $P2X_4$ receptors regulate T-cell activation at the immune synapse. Blood. 2010; 116:3475-84.
[43] Tatano Y, Kanehiro Y, Sano C, Shimizu T, Tomioka H. ATP exhibits antimicrobial action by inhibiting bacterial utilization of ferric ions. Sci Rep. 2015; 5:8610.
[44] Chu BC, Garcia-Herrero A, Johanson TH, et al. Siderophore uptake in bacteria and the battle for iron with thehost; a bird's eye view. Biometals 2010; 23:601–11.
[45] Biswas D, Qureshi OS, Lee WY, Croudace JE, Mura M, Lammas DA. ATP-induced autophagy is associated with rapid killing of intracellular mycobacteria within human monocytes/ macrophages. BMC Immunol. 2008; 9:35.
[46] Bradfute SB, Castillo EF, Arko-Mensah J, Chauhan S, Jiang S, Mandell M, Deretic V. Autophagy as an immune effector against tuberculosis. Curr. Opin. Microbiol. 2013; 16:355–65.
[47] Tomioka H. Type II pneumocytes in the evaluation of drug antimycobacterial activity. Exp Opin Pharmacother 2003; 4: 127-39.
[48] Sano C, Sano K, Sato K, Ogasawara K, Shimizu T, Tomioka H. Effects of ATP on the in vitro and in vivo antimicrobial activity of murine macrophages against *Mycobacterium avium* complex. Jpn J Chemother 2002; 50:848-53.

