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Mycobacterium tuberculosis Induces the Production of Tumor Necrosis Factor-α, Interleukin-6, and CXCL8 in Pulmonary Epithelial Cells Through Reactive Oxygen Species-dependent Mitogen-activated Protein Kinase Activation

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Upon contact with airway epithelial cells, mycobacteria activate several signal transduction events that are required for induction of inflammatory cytokines/chemokines. In this study, we found that *Mycobacterium tuberculosis* (Mtb)-induced reactive oxygen species (ROS) production is essential for the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and CXC-chemokine ligand (CXCL) 8 through the activation of mitogen-activated protein kinases [MAPKs; extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK] in A549 cells representing alveolar epithelial cells. We observed that Mtb rapidly enhanced ROS production after stimulation in a toll-like receptor (TLR) 2-dependent manner. In addition, Mtb triggered ERK1/2 and p38 MAPK signaling pathways which were dependent on ROS generation in A549 cells. Moreover, Mtb stimulation significantly increased the secretion of TNF-α, IL-6, and CXCL8 over that in untreated controls. Pretreatment of A549 cells with the antioxidant, *N*-acetylcysteine and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, diphenylene iodonium, substantially inhibited Mtb-induced production of TNF-α, IL-6, and CXCL8. Studies using inhibitors selective for ERK1/2 and p38 MAPK pathways showed that both pathways play an essential role in the induction of TNF-α, IL-6, and CXCL8 at transcriptional levels in A549 cells. Collectively, our findings indicate the critical role of TLR2-dependent ROS in the Mtb-induced inflammatory cytokine/chemokine production in alveolar epithelial cells through MAPK-dependent signaling pathways.

Key Words: *Mycobacterium tuberculosis*, Tumor necrosis factor-α, Interleukin-6, CXCL8, Alveolar epithelial cells, Reactive oxygen species

INTRODUCTION

Human tuberculosis (TB) is one of the leading causes of death and the most aggressive infectious diseases worldwide (1). Although one-third of population in the world is thought to be infected with *Mycobacterium tuberculosis* (Mtb) (2), only 5~10% of infected individuals will develop overt disease during their lifetime (3). The protective immunological mechanisms that provide the majority of individuals to be successfully containing these organisms are yet to be unknown. Nevertheless, a delicate coordination between innate and adaptive immune responses through the production of cytokines and chemokines may play a crucial role in mobilizing the host defense system against this extremely successful pathogen (4). Elucidating the regulatory pathways of the cytokines/chemokines induction after Mtb infection will provide the key molecular mechanisms

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activated by this important human pathogen, and help develop a rational design of more effective vaccine adjuvants (5).

The majority of previous studies have focused on alveolar macrophages in host defense against TB. However, alveolar epithelial cells may not be a simple barrier, but respond to their microenvironment, induce various cytokines and chemokines, thus may contribute to the innate immune response during pulmonary TB (6). Indeed, infection of A549 cells with Mtb induced significant levels of nitric oxide (NO) and inducible NO synthase mRNA (6). A large body of literatures indicates that reactive oxygen species (ROS) can mediate intracellular signaling pathways through regulating protein tyrosine kinases/phosphatases, and play essential roles in the fine control of certain pathophysiological conditions (7~9). Our recent studies using macrophages have reported the significant roles of intracellular ROS in modulating toll-like receptor (TLR)-dependent innate signaling pathways (10~12). However, little is known about the role of ROS in airway epithelial cells in response to Mtb or its components in terms of TLR-dependent signaling.

Recent studies also have revealed the intracellular signaling pathways of A549 cells in response to mycobacteria. Mendez-Samperio *et al*. have shown that phosphatidylinositol 3-kinase (PI3K)/Akt and the p38 mitogen-activated protein kinase (MAPK) signaling pathways are involved in the regulation of *M. bovis* BCG-induced interleukin (IL)-10 secretions in human lung epithelial cells (13). In addition, MEK1/2 and p38 MAPK signaling pathways are critically involved in the transcription of LL-37 mRNA expression in A549 cells (14). Although MAPK activation appears to be important in the enhancement of antimycobacterial activity and cytokine production following a mycobacterial infection (5, 15), the precise role of MAPK signaling in the regulation of cytokines/chemokines in A549 cells in response to Mtb has been largely unknown.

In this study, we examined the role of ROS in A549 cells representing alveolar epithelial cells during the secretion of tumor necrosis factor (TNF)-α, IL-6, and CXC-chemokine ligand (CXCL) 8 expression in response to Mtb. Our results showed that Mtb activated a rapid TLR2-dependent superoxide generation in A549 cells. In addition, ROS generation was required for Mtb-mediated MAPK activation and the expression of TNF-α, IL-6, and CXCL8. Furthermore, we found that both extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK pathways were necessary in the induction of TNF-α, IL-6, and CXCL8 at the transcriptional levels in A549 cells.

MATERIALS AND METHODS

Preparation of Mtb

Cultures of Mtb H37Rv (kindly provided by Dr. Richard L. Friedman, University of Arizona, Tucson, AZ, USA) were prepared as described previously (16). Briefly, Mtb H37Rv was grown at 37℃ on Middlebrook 7H10 agar (Difco, Detroit, MI, USA) medium supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase; Becton & Dickinson, San Jose, CA, USA) and 0.05% Tween 80 (Sigma-Aldrich, St Louis, MO, USA). Stock strains were grown in roller bottles, to late log phase in liquid Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% OADC enrichment. Then, the bacterial culture was divided into 1 ml aliquots in cryovials, and stored at -70℃ until needed. Representative vials were thawed, and viable CFU was enumerated on Middlebrook 7H10 agar. Single-cell suspensions of mycobacteria were prepared as described previously (17).

Cell culture and infection of Mtb

The human alveolar epithelial cell line A549 (ATCC CCL-185^{TM}; American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL), sodium pyruvate, nonessential amino acids, penicillin G (100 IU/ml), and streptomycin (100 μg/ml). A549 cells were infected with Mtb (MOI = 5) and incubated for the indicated times at 37℃ under 5% CO_2. After the time allowed for phagocytosis, cells were washed three times with fresh phosphate-buffered saline to remove extracellular bacteria and then incubated again with complete DMEM

Table 1. Primer sequences

Gene	Primer Sequences
hTNF-α	forward : 5'-CAGAGGGAAGAGTTCCCCAG-3'
	reverse : 5'-CCTTGGTCTGGTAGGAGACG-3'
hIL-6	forward : 5'- TGACCCAACCACAAATGC-3'
	reverse : 5'-CGAGCTCTGAAACAAAGGAT-3'
hCXCL8	forward : 5'-CATGACTTCCAAGCTGGCCG-3'
	reverse : 5'-TTTATGAATTCTCAGCCCTC-3'
β-actin	forward : 5'- ATCTGGCACCACACCTTCTACAATGAGCTGCG-3'
	reverse : 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGCG-3'

without antibiotics for indicated times. Cultures of uninfected cells were maintained under the same conditions during the entire time of the assays.

Antibodies and reagents

A nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, diphenylene iodonium (DPI), an antioxidant, *N*-acetyl-*L*-cysteine (NAC), a specific inhibitor of p38 MAPK, SB203580, and a specific inhibitor of MEK, U0126, were purchased from Calbiochem (San Diego, CA, USA). Dimethyl sulfoxide (DMSO; Sigma) was added to cultures at 0.1% (vol/vol) as a solvent control. Rotenone was purchased from Sigma. Specific antibodies (Abs) against phospho-(Thr202/Tyr204)-ERK1/2, and phospho-(Thr180/Tyr182)-p38 were purchased from Cell Signaling (Danvers, MA, USA). Mouse anti-human TLR2 monoclonal Ab (mAb; T2.5, immunoglobulin G1 [IgG1]) and mouse anti-human TLR4 mAb (clone HTA 125, IgG2a) were purchased from eBioscience (San Diego, CA, USA). The Ab to α-actin was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

Enzyme-linked immunosorbent assay (ELISA), Western blot analysis, and reverse transcriptase-polymerase chain reaction (RT-PCR)

A549 cells were treated as indicated and processed for analysis by sandwich ELISA as previously described (16). For the sandwich ELISA, cell culture supernatants were analyzed for TNF-α, IL-6, and CXCL8 using Duoset antibody pairs (BD PharMingen, San Diego, CA, USA) as described previously (16). Assays were performed as recommended by the manufacturers. Western analysis was performed as previously described (16). Primary antibodies were used at a 1:1,000 dilution. Membranes were developed using a chemiluminescence assay (ECL; Amersham-Pharmacia, Freiburg, Germany) and subsequently exposed to chemiluminescence film (Amersham-Pharmacia). For semi-quantitative RT-PCR analysis, RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primer sequences were shown at Table 1.

Detection of ROS production

Intracellular ROS levels were measured by dihydroethidium (DHE) assays, as previously described (16). The cells were examined under a laser-scanning confocal microscope (model LSM 510; Zeiss, Thornwood, NY, USA). Five groups of cells were randomly selected from each sample, and the mean relative fluorescence intensity for each group of cells was measured with the Zeiss vision system (LSM510, version 2.3) and then averaged for all the groups.

Statistical analyses

For statistical analysis, the data obtained from independent experiments were presented as the mean ± standard deviation (SD); they were analyzed using a paired *t*-test with Bonferroni adjustment or ANOVA for multiple comparisons. Differences were considered significant at $p < 0.05$.

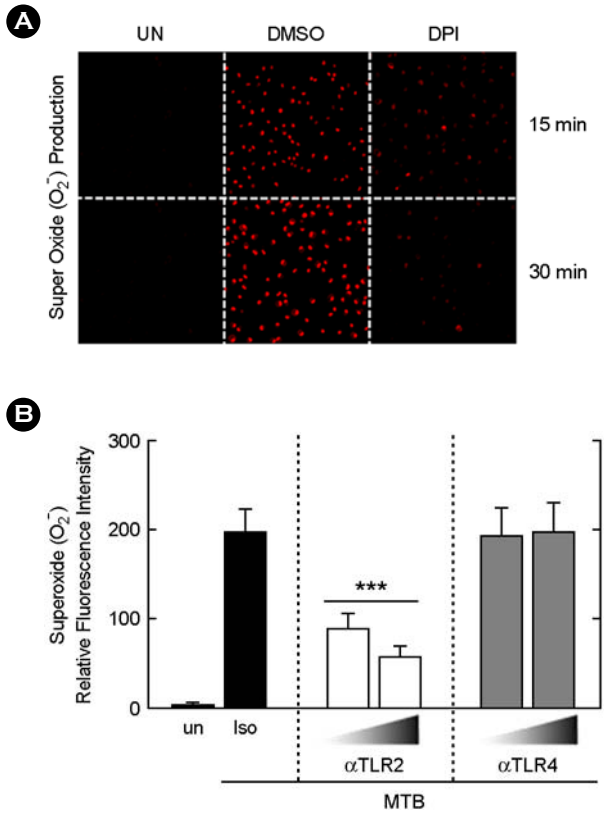

Figure 1. Mtb-induced ROS generation is dependent on the TLR2, but not TLR4, in A549 cells. **(A)** The A549 cells were stimulated with Mtb (MOI = 5) for 15 to 30 min and then DHE assays were performed. Representative immunofluorescence images are shown. The data shown are representative of three experiments. **(B)** The experimental conditions follow the same pattern as outlined in Panel A. The A549 cells were pre-incubated with an anti-TLR2 (αTLR2), anti-TLR4 (αTLR4; both 5, 10 μg/ml) or an isotype control mAb (10 μg/ml), followed by the stimulation with Mtb (MOI = 5) for 30 min. The quantitative data for DHE (for superoxide) fluorescence shown are the mean ± SD of three experiments. Significant differences (***, $p < 0.001$). UN, unstimulated; Iso, isotype control.

RESULTS

Mtb induces ROS generation in a TLR2-dependent manner

Our previous study showed that intracellular ROS can regulate intracellular signal transduction cascades in monocytes/macrophages during mycobacterial infection (10, 11). However, ROS formation in response to Mtb is poorly characterized in A549 cells. We examined whether Mtb stimulation led to ROS generation using oxidative fluorescent dye DHE to detect superoxide production in A549 cells. The chemiluminescent signal intensities attributable to superoxide production were markedly elevated in A549 cells when stimulated with Mtb for 15 to 30 min (Fig. 1A). The NADPH oxidase inhibitor DPI (Fig. 1A, right) significantly attenuated Mtb-induced superoxide production in A549 cells.

Then, we examined whether Mtb-mediated ROS generation was dependent on TLR2. Mtb-induced superoxide generation in A549 cells was significantly attenuated by the pretreatment with an anti-TLR2 Ab, which was measured by fluorescence microscopy (Fig. 1B). No significant difference was observed in Mtb-induced ROS production in cells pretreated with an anti-TLR4 Ab or an isotype control Ab. These results indicate that TLR2 plays an important role for Mtb-mediated ROS generation in A549 cells.

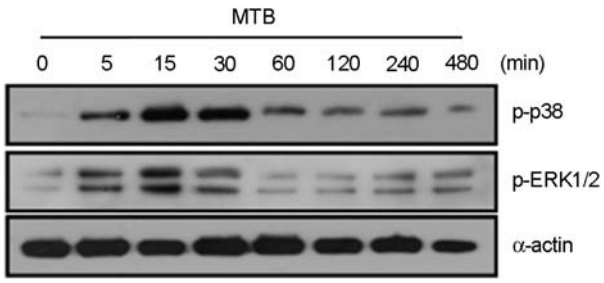

Figure 2. Mtb rapidly induces the phosphorylation of MAPKs by A549 cells. The A549 cells were stimulated with Mtb (MOI = 5) for the indicated times (0~480 min). The cells were harvested and subjected to Western blot analysis for phosphorylated ERK1/2 and p38 MAPK. The same blots were washed and blotted for α-actin as the loading controls. Data are representative of five independent experiments with similar results.

Mtb leads to the phosphorylation of MAPKs in A549 cells

MAPKs play a crucial role for mediating innate immune responses to Mtb and mycobacterial components (5). Therefore, we examined the MAPK activation in response to Mtb in A549 cells. A549 cells were stimulated with Mtb at an MOI of 5, and the phosphorylation of p38 MAPK and ERK1/2 was analyzed at various time courses (Fig. 2). Mtb stimulation resulted in a strong phosphorylation of p38 MAPK at 15~30 min in A549 cells. The total inhibition of ERK1/2 and p38 MAPK phosphorylation by their respective inhibitors at 15 min after stimulation with

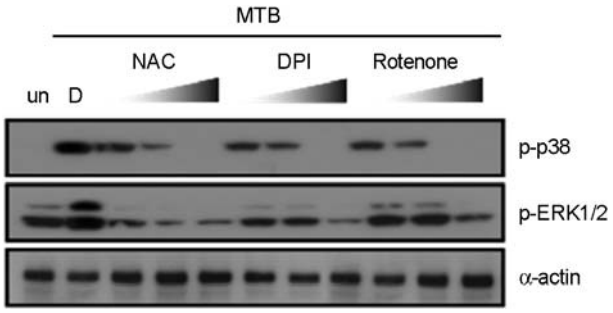

Figure 3. Intracellular ROS production is essential for Mtb-induced MAPK activation by A549 cells. The A549 cells were pretreated with or without NAC (10, 50, 100 mM), DPI (5, 10, 20 μM) or rotenone (5, 10, 20 μM) for 45 min before stimulation with Mtb (MOI = 5). The cells were harvested after 15 min and subjected to Western blot analysis for phosphorylated ERK1/2 and p38 MAPK. The same blots were washed and blotted for α-actin as the loading controls. Data are representative of three independent experiments with similar results. un, unstimulated; D, solvent control (0.1% DMSO).

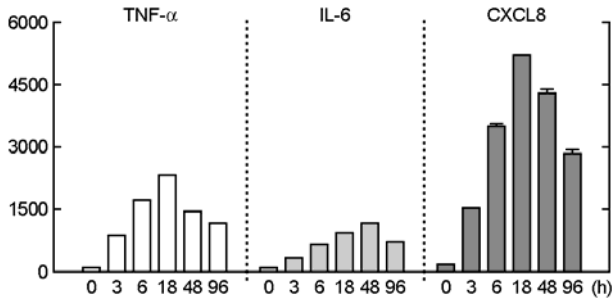

Figure 4. Mtb induces pro-inflammatory cytokines and chemokine production by A549 cells. The A549 cells were stimulated with Mtb (MOI = 5) for the indicated times and then performed ELISA analysis (for TNF-α, IL-6 and CXCL8). Data are the mean ± SD of three experiments.

Mtb was confirmed on Western blots (data not shown).

Intracellular ROS formation is involved in Mtb-induced MAPK signaling in A549 cells

To evaluate whether ROS are involved in Mtb-mediated MAPK activation, A549 cells were pre-treated with or without ROS scavengers. As shown in Fig. 3, pretreatment with various antioxidants, such as the general ROS scavenger, NAC, an NADPH oxidase inhibitor DPI, and a mitochondrial electron transfer chain subunit I inhibitor, rotenone, for 45 min attenuated Mtb-mediated p38 MAPK and ERK1/2 activation in A549 cells in a dose-dependent manner. These results suggest that NADPH oxidase-derived intracellular ROS are mainly involved in the Mtb-induced MAPK activation in A549 cells.

Mtb stimulation induces pro-inflammatory cytokine release in A549 cells

In order to examine the kinetics and dose-dependency of Mtb-induced pro-inflammatory cytokine responses, A549 cells were stimulated with various MOIs (0.5~10) or various times (3~96 h) of Mtb stimulation. Then, cytokine ELISA was performed to assess TNF-α, IL-6, and CXCL8 secretions to the culture supernatants. Mtb stimulation induced relatively low levels of TNF-α, IL-6, and CXCL8 at MOI of 0.5 to 1 (data not shown). When A549 cells were exposed to Mtb at an MOI of 5, the secretion of cytokines significantly increased in the culture medium compared to those by Mtb stimulation at an MOI of 1 ($p < 0.01$, data not shown). Therefore, we used the MOI of 5 in this study. In addition, the highest peak of TNF-α and CXCL8 production in response to the Mtb stimulation was always detected 8~24 h after stimulation (Fig. 4). The peak production of IL-6 was around 18~48 h after Mtb stimulation. However, the IL-6 release at 48 h was not significantly different from that at 18 h. Hence, supernatants were harvested at 18 h after stimulation for additional experiments.

ROS release was responsible for the secretion of TNF-α, IL-6, and CXCL8 in A549 cells

Previous studies have suggested that ROS generation was required for the 30-kDa antigen-induced pro-inflammatory chemokines release in human monocytes (10). However, the roles of ROS formation for pro-inflammatory cytokine production in A549 cells had not been determined. Thus, we examined whether ROS played a role in the secretion of TNF-α, IL-6, and CXCL8, from A549 cells. As shown in Fig. 5, Mtb-induced TNF-α, IL-6, and CXCL8 levels were substantially modulated by pre-treatment with NAC, DPI, and rotenone, in a dose-dependent manner, in A549 cells. These results suggest that ROS-dependent signaling plays a central role for pro-inflammatory responses to Mtb in pulmonary epithelial cells.

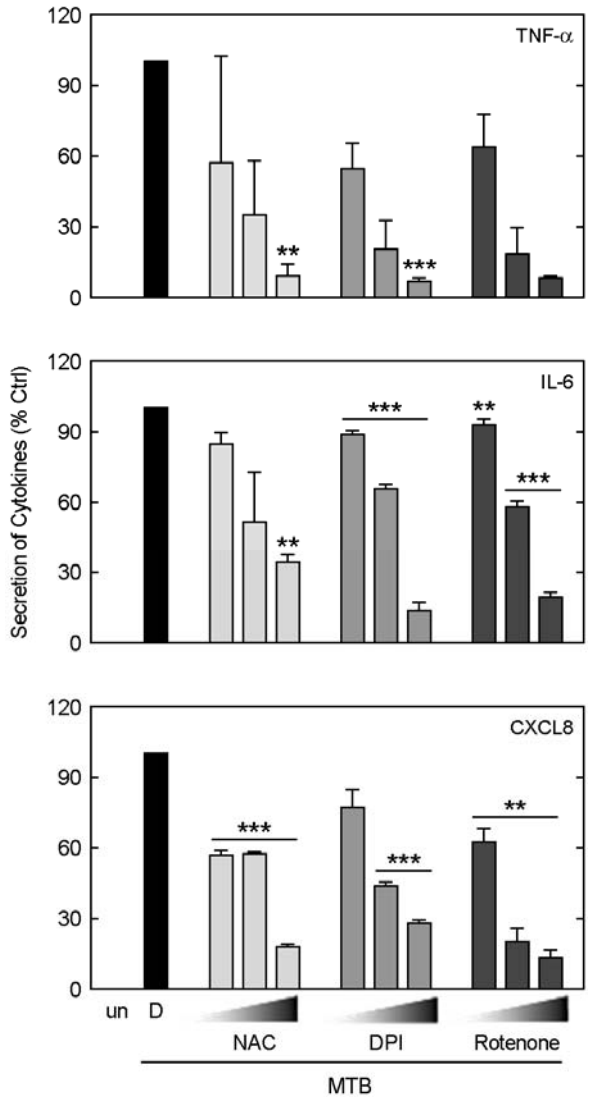

Figure 5. The roles of ROS in pro-inflammatory cytokine and chemokine production in response to Mtb stimulation in A549 cells. The A549 cells were pretreated with NAC (10, 50, 100 mM), DPI (5, 10, 20 μM) or rotenone (5, 10, 20 μM) for 45 min, followed by stimulation with Mtb (MOI = 5) for 18 h. The supernatants were harvested at 18 h, and the production of pro-inflammatory cytokines (for TNF-α and IL-6) and chemokine (for CXCL8) was measured by ELISA. Data are the mean ± SD of three experiments. un, unstimulated; D, solvent control (0.1% DMSO). Significant differences (**, $p < 0.01$;***, $p < 0.001$)

The ERK1/2 and p38 MAPK pathways are critical for Mtb-induced TNF-α, IL-6, and CXCL8 formation in A549 cells

ERK1/2 and p38 MAPK are both pivotal to the cytokine formation induced by mycobacterial antigen in human monocytes (18). To further understand the functional roles

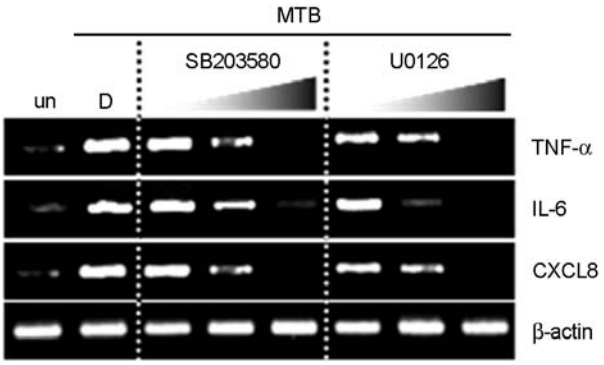

Figure 6. The roles of MEK1/2 or p38 MAPK on the mRNA expression of TNF-α, IL-6, and CXCL8 in A549 cells. The A549 cells were pretreated in the presence or absence of U0126 (5, 10, 20 μM) or SB203580 (1, 5, 10 μM) for 45 min, and then stimulated with Mtb (MOI = 5) for 6 h. The mRNA expression of TNF-α, IL-6, and CXCL8 was detected by semi-quantitative RT-PCR analysis by using the specific primers. The data shown are representative of three experiments. un, unstimulated; D, solvent control (0.1% DMSO).

of these kinases in the activation of A549 cells induced by Mtb stimulation, we used highly specific inhibitors of the kinases and measured the resultant cytokine formation. The cells were pretreated with a p38 inhibitor (SB203580) or an MEK inhibitor (U0126) for 45 minutes before being exposed to Mtb (MOI = 5). Both U0126 and SB203580 were added to the cell culture at a final DMSO concentration of 0.1%, and DMSO was used as a control.

The specific inhibitors for p38 MAPK and MEK significantly decreased the mRNA expression levels of TNF-α, IL-6, and CXCL8 in A549 cells in response to Mtb stimulation (Fig. 6). The inhibition was not attributable to solvent control because DMSO did not exhibit any inhibitory effects at this concentration.

DISCUSSION

The innate immune system is the first line of host defense mechanism against pathogens before the development of adaptive immune responses. ROS are mainly produced by leukocytes and by the respiratory mitochondrial chain, and serve as an important second messenger to control a broad range of physiological and pathological processes (11, 19, 20). Now it is being recognized that the epithelium is no longer an "innocent bystander," but may play a central role

in innate and inflammatory responses thus determining the balance between a state of homeostasis and mucosal injury (21). In particular, airway epithelial cells express various types of TLRs and produce cytokines in response to TLR ligation. For example, *Streptococcus pneumoniae* enhanced TLR1 and TLR2 mRNA expression in BEAS-2B cells, and induced CXCL8 release (22). It was also reported that A549 cells express functional TLR4, predominantly in the intracellular compartment (23). Other studies have shown that human primary type II alveolar epithelial cells express functional TLR2 and TLR4, which can be modulated by treatment with lipopolysaccharide and TNF-α (24). Although our recent data have shown a robust production of ROS in response to Mtb or its components play an essential role in the activation of TLR2-dependent signaling pathway by macrophages (10~12), little is known about the role of TLRs and/or ROS-dependent signaling pathway that can activate and enhance the Mtb-mediated innate immune responses in A549 cells. The present data demonstrate that Mtb-induced inflammatory responses are exclusively dependent on the TLR2-mediated ROS generation and MAPK pathways.

Our data demonstrate that Mtb actively induced TNF-α, IL-6, and CXCL8 secretions from A549 cells. TNF-α is mainly secreted by activated macrophages, and plays a key role in granuloma formation and host defense against TB (25). TNF-α also contributes to the prevention of reactivation of chronic persistent TB, because neutralization of this cytokine induced fatal reactivation of TB, characterized by an increased tissue bacillary burden and severe histopathological deterioration of the host (26). IL-6 is a pro-inflammatory cytokine produced by various cell types, including monocytes, fibroblasts, T- and B-cells. In addition, it has been thought to play a role in mycobacterial infection through the activation of adaptive immune responses and immunoglobulin productions (27). Earlier studies showed that IL-6 plays a role in the infectious process, and it was reported that lower levels of IL-6 inhibit mycobacterial growth in murine macrophages (28). On the contrary, significant amounts of IL-6 by macrophages after mycobacterial infection inhibited T-cell responses (29), suggesting that constitutively increased amounts of IL-6 may lead to a depression of the macrophage activities to induce T-cell stimulation. In addition, an extensive body of literatures suggests a role of chemokines in mycobacterial infection, to support monocyte differentiation and trafficking, thus promoting antimycobacterial responses (30). Both CXCL8 and CCL2 are important chemokines produced by Mtb-infected A549 cells, thus may contribute to the local inflammatory response in human TB (31). Of note, CXCL8 is known to function as a strong chemo-attractant for neutrophils (32), T lymphocytes (33), and basophils (34), thus serving to attract inflammatory cells to the site of pathogen invasion (35). In human cells, recombinant CXCL8 enhances the intracellular killing of *M. fortuitum* by human granulocytes through oxygen-independent mechanisms (36). Moreover, this CXC chemokine is critical for granuloma formation *in vivo*, which is an important local response to control TB (37). Together with our data, TNF-α, IL-6, and CXCL8 may play an important role in host defense against human TB.

The current data clearly demonstrate that the intracellular ROS signaling is essential for the activation of MAPK pathways and subsequent pro-inflammatory responses to Mtb in A549 cells. MAPKs include Ser/Thr kinases such as p38 MAPK, c-Jun N-terminal kinase, and ERK1/2 (38). The MAPK pathways are one of the most significant targets of heavy metals and ROS in the cellular signal transduction (11, 39). The results demonstrated here that A549 cells showed an oxidative burst and enhanced phosphorylation of ERK1/2 and p38 MAPK, which mediated increased pro-inflammatory responses during Mtb infection. Similarly, previous findings demonstrated that mycobacterial purified protein derivatives markedly activated ROS generation by monocytes/macrophages, and this was required for the inflammatory feedback mechanism through the activation of apoptosis signal-regulating kinase 1/p38/p47phox pathways (11). In addition, a recent study has shown that Rac1, a small GTPase of the Rho family, modulated TNF-α-induced ROS generation, which affected the monocyte adhesion to A549 cells as well as the up-regulation of intercellular adhesion molecule-1 expression in these cells (40). Other

recent study has also demonstrated that bronchial epithelial cells induce cyclooxygenase-2 expression and IL-6 production in response to particulate matter in ambient air through a ROS-dependent NF-κB pathway (41). Taken together, ROS generation plays an influential role in the regulation of innate and inflammatory responses through a modulation of downstream signaling pathways including MAPKs.

Although this study has focused on the roles of epithelial cells during Mtb infection, many studies reported the roles of macrophages in innate immune responses to Mtb infection. In our preliminary experiments, we found that Mtb (MOI = 1) did not significantly induce the cytokine production, ROS generation, and MAPK phosphorylation (data not shown). In contrast, others reported that Mtb induced a robust innate immune responses in macrophages at MOI = 1 (11, 16). Another study showed that macrophages and epithelial cells showed differential cytotoxic responses to infection with *Pseudomonas aeroginosa* (42). Further comparative studies will provide the precise functions between macrophages and epithelial cells in innate immune responses to Mtb infection. In this study, we present a novel insight of TLR2-ROS signaling pathway through the activation of MAPK pathways, responsible for the induction of pro-inflammatory responses in A549 cells upon the Mtb infection. The evaluation of the molecular mechanisms which drive innate immune responses in airway epithelial cells would provide a new insight on the development for potential vaccines or therapeutics against TB.

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