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Lithium Inhibits Growth of Intracellular *Mycobacterium kansasii* through Enhancement of Macrophage Apoptosis[§]

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Mycobacterium kansasii **(Mk) is an emerging pathogen that causes a pulmonary disease similar to tuberculosis. Macrophage apoptosis contributes to innate host defense against mycobacterial infection. Recent studies have suggested that lithium significantly enhances the cytotoxic activity of death stimuli in many cell types. We examined the effect of lithium on the viability of host cells and intracellular Mk in infected macrophages. Lithium treatment resulted in a substantial reduction in the viability of intracellular Mk in macrophages. Macrophage cell death was significantly enhanced after adding lithium to Mk-infected cells but not after adding to uninfected macrophages. Lithium-enhanced cell death was due to an apoptotic response, as evidenced by augmented DNA fragmentation and caspase activation. Reactive oxygen species were essential for lithium-induced apoptosis. Intracellular scavenging by N-acetylcysteine abrogated the lithium-mediated decrease in intracellular Mk growth as well as apoptosis. These data suggest that lithium is associated with control of intracellular Mk growth through modulation of the apoptotic response in infected macrophages.**

Keywords: apoptosis, lithium, macrophage, *Mycobacterium kansasii*, reactive oxygen species

Introduction

Mycobacterium kansasii (Mk), a nontuberculous mycobacterium (NTM), is frequent cause of NTM lung disease such as chronic bronchopulmonary disease in immunocompetent patients and pulmonary, extrapulmonary, or disseminated disease in immunocompromised patients (Arend *et al.*, 2004; Field and Cowie, 2006).

Macrophages constitute the first line of defense against mycobacteria in the lung and provide important intracellular niches within which mycobacteria survive and replicate (Schluger and Rom, 1998). Therefore, infected cells must eliminate intracellular mycobacteria to protect the host. Different types of cell death are associated with microbial infections. Bacteria manipulate host defense mechanisms to survive and replicate (Song, 2013).

It has recently been suggested that macrophage apoptosis represents a crucial and alternative host innate defense mechanism against mycobacteria (Keane *et al.*, 2000). In infected macrophages, apoptotic death reduces the viability of intracellular mycobacteria (Molloy *et al.*, 1994; Fratazzi *et al.*, 1997; Keane *et al.*, 2000). In contrast, virulent mycobacteria suppress apoptosis and induce further necrosis in macrophages, which is related to the spread of the bacteria. We reported previously that clinical Mk isolates with higher growth rates in macrophages induce higher levels of necrosis (Sohn *et al.*, 2010). Although various apoptotic stimuli have been reported, including adenosine triphosphate, Fas ligand, and tumor necrosis factor (TNF) (Molloy *et al.*, 1994; Fratazzi *et al.*, 1997), little is known about the mechanisms underlying the modulation of mycobacteria-induced apoptotic death in macrophages.

Lithium is used extensively as a mood stabilizer for treatment of bipolar disorders (Manji and Lenox, 1998). Although the mechanism underlying its actions is not understood fully, several biologic effects of lithium have been reported. There is growing evidence that lithium induces neuroprotection against a variety of injuries in cultured cells and animal models (Wei *et al.*, 2001; Ren *et al.*, 2003; Noble *et al.*, 2005). In addition, lithium greatly reduces the production of major proinflammatory cytokines following stimulation of Toll-like receptor (TLR) agonists in monocytes by inhibiting glycogen synthase kinase (GSK) 3; this suggests that lithium may help regulate inflammation (Martin *et al.*, 2005). Immune-stimulating and antimicrobial properties of lithium have also been reported (Lieb, 2004). Moreover, lithium has been shown to significantly enhance TNF-induced apoptosis in tumor cells (Beyaert *et al.*, 1989; Hoeflich *et al.*, 2000; Schotte *et al.*, 2001) and hepatocytes (Schwabe and Brenner, 2002). However, there is no report of the effect of lithium on mycobacteria-induced cell death.

In the present study, we evaluate the effect of lithium on the viability of intracellular mycobacteria through modu-

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lation of the death of Mk-infected macrophages. Lithium reduced the viability of intracellular Mk and significantly enhanced apoptosis of infected macrophages, which occurs by caspase activation and oxidative stress. Furthermore, blocking of the lithium-induced apoptotic response failed to reduce intracellular Mk growth.

Materials and Methods

Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committees of Chungnam National University (Permit Number: 2010-3-9) and performed in accordance with Korean Food and Drug Administration guidelines.

Reagents

Lithium chloride (LiCl), potassium chloride (KCl), and sodium chloride (NaCl) were purchased from Sigma (USA). Anti-caspase-3, anti-caspase-9, anti-phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), anti-phosphorylated p38 mitogen activated protein kinase (MAPK), anti-phosphorylated stress activated protein kinase/cJun N-terminal kinase (SAPK/JNK), and anti-β-actin antibodies were obtained from Cell Signaling Technology (USA). SB216763 and N-acetylcysteine (NAC), and specific inhibitors of ERK1/2 (U0126), p38 MAPK (SB203580), and SAPK/JNK (SP600125) were obtained from Calbiochem (USA). 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, USA). Enzyme-linked immunosorbent assay (ELISA) kits for the detection of TNF, IL-12p40, and IL-10 were from eBioscience (USA).

Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were obtained from 6- to 8-week-old, female C57BL/6 mice. Bone marrow cells from the femur and tibia were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, USA) containing 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, and 25 ng/ml recombinant mouse M-CSF (R&D Systems, USA) at 37°C in the presence of 5% CO_2. After 4 days, non-adherent cells were removed and differentiated macrophages were incubated in antibiotic-free DMEM until use.

Mycobacterium kansasii

A *Mycobacterium kansasii* (Mk) isolate from a patient with pulmonary disease was used. The clinical isolate SM-3 was identified, and then cultured and prepared for use as described previously (Sohn *et al.*, 2010).

Survival of mycobacteria in BMDM

To measure intracellular mycobacterial viability, the infected cells were washed with serum-free medium and lysed in distilled water containing 0.01% Triton X-100 (Sigma) for 30 min, then the colony forming unit (CFU) content of lysates were determined. Colonies were counted 12 days after plating.

Cell viability assay

Cell viability was assessed using a Cell Counting Kit-8 (Dojindo Laboratories, Japan). BMDMs were grown in 96-well plates prior to treatment and infection. After treatment with various reagents and infection with mycobacteria according to the experimental design, aliquots of kit reagent were added and incubated for a further 1.5 h. Cell viability was determined using a microplate reader at 450 nm. The results were subjected to statistical analysis, and are expressed relative to the values of the control specified in each experiment.

Apoptosis assay

Apoptosis was measured by nucleosomal fragmentation (Cell Death Detection ELISA^{plus}, Roche Applied Science, USA), as recommended by the manufacturers. Briefly, differentiated BMDMs were plated in 96-well plates, incubated with or without reagents, and challenged with mycobacteria. Cell lysates were subjected to antigen capture ELISA to measure free nucleosomes, and the optical density at 405 nm (OD_{405}) was read in a microplate reader. Absorbance values were normalized to those of control cells to derive the nucleosomal enrichment factor of all concentrations, as per the manufacturer's protocol.

Immunoblot analysis

Cell lysates were prepared in RIPA buffer (50 mM Tris, 100 mM NaCl, 0.1% SDS) (pH 7.4) supplemented with the Complete Protease Inhibitor mixture (Roche Applied Science). Protein concentrations were determined using a BCA assay (Pierce, USA). Aliquots (30 μg) of proteins from total lysates were electrophoresed on a SDS-PAGE gel and then transferred to Immobilon-P membranes (Millipore, USA). The membranes were reacted serially with primary and HRP-conjugated secondary antibodies, as described previously (Kwon *et al.*, 2009). The blots were visualized by enhanced chemiluminescence according to the manufacturer's protocol (Pierce).

Cytokine measurement by ELISA

Supernatants from the mycobacteria-infected BMDM in the presence or absence of lithium chloride were collected 24 h post-infection, sterile-filtered, and then stored at -80°C until use. The levels of TNF-α, IL-12p40, and IL-10 were determined by ELISA using a commercial kit (eBioscience) according to the manufacturer's instructions.

Measurement of reactive oxygen species

Cellular oxidative stress was assessed by monitoring the formation of reactive oxygen species (ROS) using DCFH-DA. BMDMs were plated in 24-well plates, preincubated with LiCl, and infected with mycobacteria. Cells were harvested with trypsin/EDTA and incubated with 2.5 mM DCFH-DA in PBS for 15 min. Green fluorescence intensities were quantified using a FACS Canto II (BD Biosciences, USA).

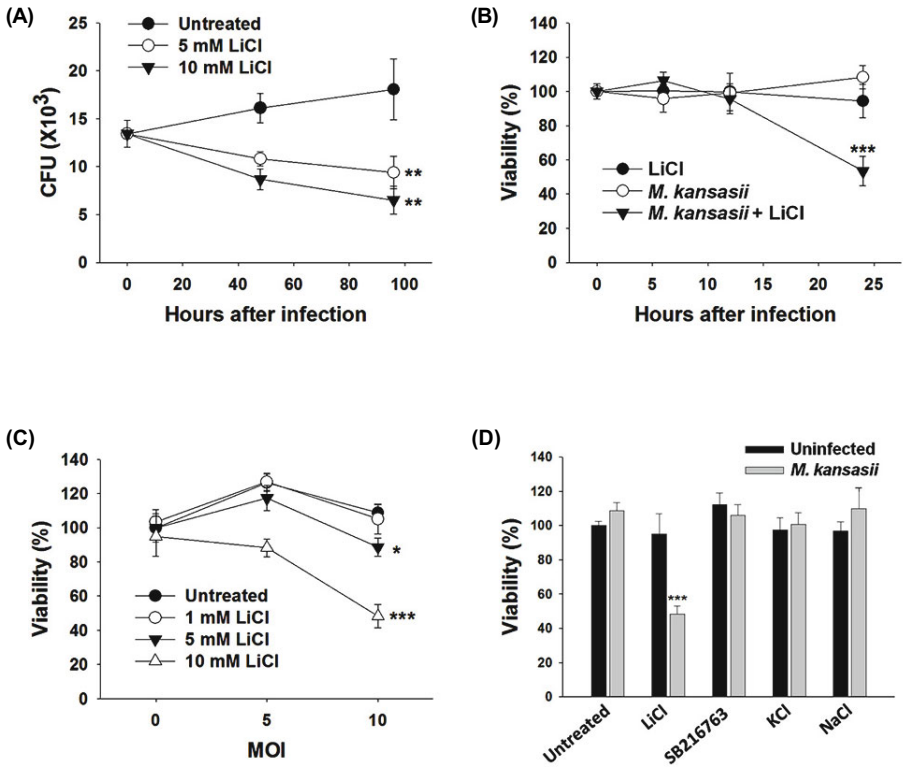

Fig. 1. Effect of lithium on the viability of intracellular *M. kansasii* and infected macrophages. (A) BMDMs were infected with Mk at an MOI of 5 for 4 h, washed, and incubated in the presence or absence of LiCl. Cells were lysed on day 0, 2, or 4 of infection and viable bacteria were quantified using a CFU assay. Results represent the means ± SD of triplicate determinations in three independent experiments. ** $P<0.01$ between Mk-infected cells and Mk-infected cells treated with LiCl. (B–D) BMDMs were incubated in the presence or absence of LiCl (10 mM) for 90 min, followed by infection with or without Mk at an MOI of 10 for the times indicated (B). BMDMs were treated without or with LiCl at the indicated doses for 90 min and infected with Mk at MOI of 0, 5, or 10 for 24 h (C). Cells were incubated in the presence or absence of LiCl (10 mM), SB216763 (10 μM), KCl (10 mM), or NaCl (10 mM) before infection with Mk (MOI = 10) for 24 h (D). Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) from Dojindo Laboratories. Data represent means ± SD for triplicate determinations of three independent experiments. * $P<0.05$, *** $P<0.001$ Mk-infected cells versus Mk-infected cells treated with LiCl.

Statistical analysis

The data represent the means ± standard deviation (SD) from at least three independent experiments. Statistical comparisons were performed using Student's *t*-test. A *P*-value < 0.05 was considered significant.

Results

Lithium reduces the viability of intracellular MK and enhances cell death in infected macrophages

To determine the effect of lithium on the viability of intracellular NTM, bacteria were counted after 2, 3, or 4 days in BMDMs infected with Mk for 4 h and then incubated with LiCl. Under normal conditions, Mk persisted but replicated slowly within macrophages (Fig. 1A). Lithium treatment of Mk-challenged macrophages significantly reduced bacterial viability in a time- and dose-dependent manner. Next, we determined whether lithium affected the viability of Mk-infected macrophages. In BMDMs, Mk alone at an MOI of 10 did not induce significant cell death within 24 h post-infection, although a high MOI (≥ 25) of Mk caused marked macrophage death (data not shown). LiCl dramatically augmented the death of Mk-infected macrophages despite its lack of a significant cytotoxic effect on uninfected macrophages after 24 h (Fig. 1B). Additional evidence of dose-dependency was obtained using macrophages incubated with LiCl at various concentrations followed by infection with Mk at an MOI of 5 or 10 (Fig. 1C). In addition, lithium also reduced the intracellular viability of *M. abscessus*, the other member of NTM, in BMDMs, and significantly enhanced the death of macrophages infected with *M. abscessus* (Supplementary data Fig. S1).

LiCl directly inhibits GSK-3 activity, and the cytotoxic effect of TNF is enhanced in GSK-3β-deficient cells (Hoeflich *et al.*, 2000). To evaluate whether the effect of LiCl on infected macrophages was due to inhibition of GSK-3β activation, we evaluated the effect of a specific inhibitor of GSK-3β, SB216763 (Martin *et al.*, 2005), on Mk-infected macrophage death. Unexpectedly, SB216763 did not affect the viability of macrophages infected with Mk, indicating that it did not mimic the effect of LiCl on Mk-infected macrophages death (Fig. 1D).

To exclude the influence of the chloride ion, the effect of LiCl was compared to those of equivalent concentrations of KCl or NaCl. As shown in Fig. 1D, exposure of BMDMs to KCl or NaCl at concentrations identical to those of LiCl did not change Mk-induced cell death, suggesting the specificity of lithium on Mk sensitization.

LiCl-enhanced cell death occurs by apoptosis

To confirm that the LiCl-mediated enhanced cell death was due to apoptosis, BMDMs were pretreated with LiCl and infected with Mk. Then an apoptosis assay based on DNA fragmentation, a hallmark of apoptotic cell death, was performed. Lithium dose-dependent death of macrophages challenged with Mk was accompanied by nucleosomal fragmentation, indicating that Mk-infected macrophages were susceptible to lithium-induced apoptosis (Fig. 2A). In addition, caspase activation was determined by immunoblotting. Significant activation of caspase-3 and -9 was detected in

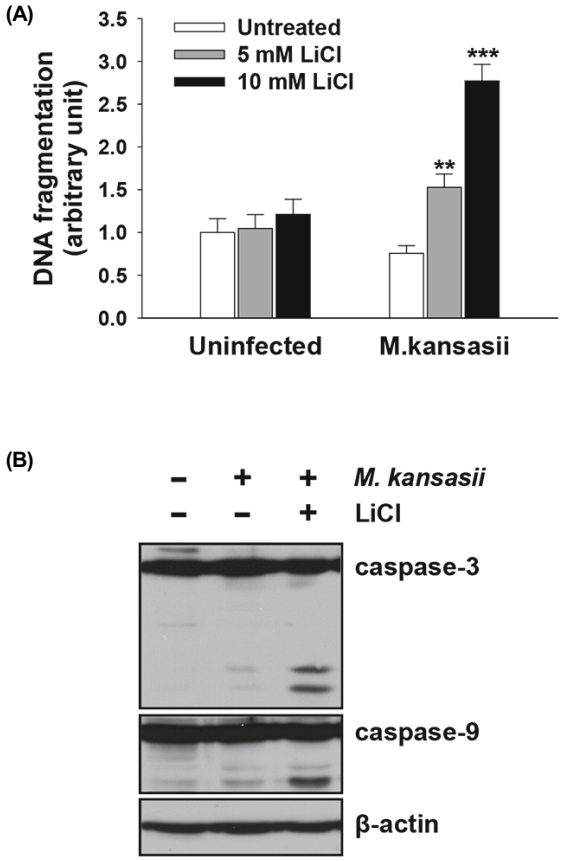

Fig. 2. Lithium increases nucleosomal fragmentation and caspase activation in *M. kansasii*-challenged BMDMs. (A) BMDMs were incubated without or with LiCl at the indicated doses for 90 min and then left uninfected or infected with Mk (MOI=10) for 24 h. Cells were washed and lysed, and their apoptosis was evaluated using the Cell Death Detection ELISA kit (Roche Applied Science). Data shown are means ± SD of triplicates of three independent determinations. ** $P<0.01$, *** $P<0.001$ Mk-infected cells *versus* Mk-infected cells treated with LiCl. (B) BMDMs were pretreated without or with LiCl (10 mM) for 90 min and infected with Mk at an MOI of 10 for 24 h. Total cell extracts were separated by SDS-PAGE and immunoblotted using antibodies specific for caspase-3 and -9. β-Actin was used as a loading control.

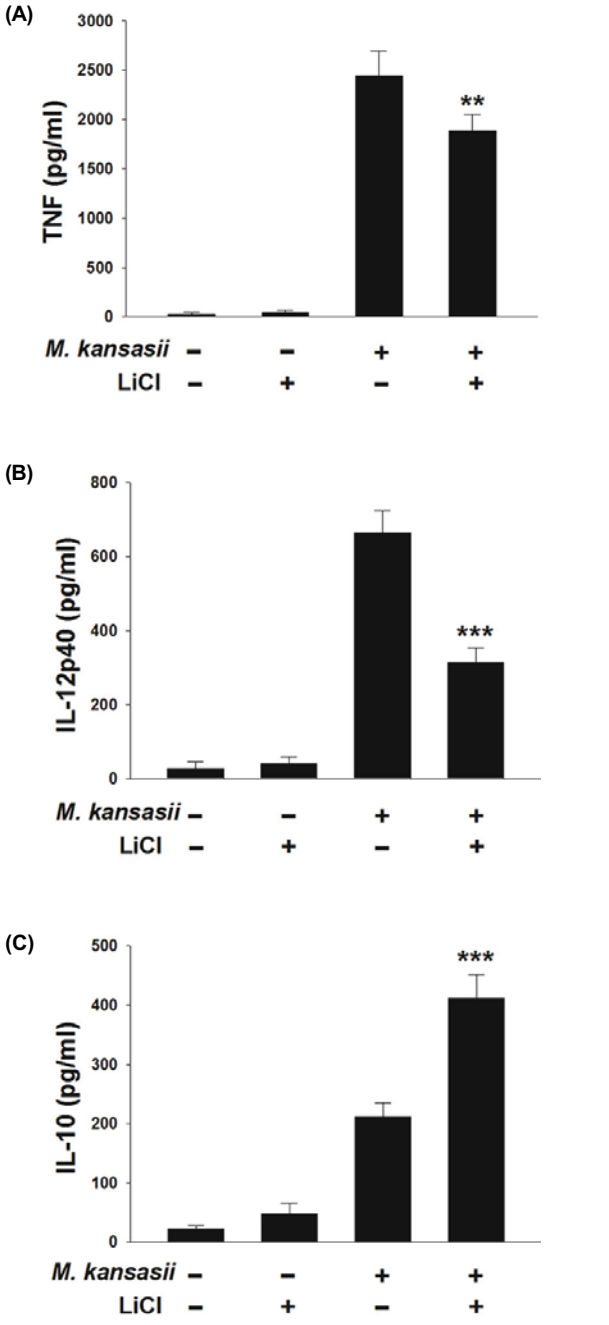

Fig. 3. LiCl modulates TNF (A), IL-12 (B), and IL-10 (C) production by Mk infection. BMDM were untreated or treated with LiCl (10 mM) for 90 min and infected with Mk at an MOI of 5 for 24 h. Supernatants were then collected and the levels of TNF, IL-12p40, and IL-10 were determined by ELISA. The values shown are the means ± SD from three independent experiments. ** $P<0.01$, *** $P<0.001$ Mk-infected cells versus Mk-infected cells treated with LiCl.

macrophages treated with LiCl plus Mk, but not in macrophages treated with LiCl or Mk alone (Fig. 2B), suggesting that the lithium-mediated augmentation of apoptosis in Mk-infected macrophages is caspase-dependent.

Lithium-enhanced apoptosis is not due to TNF

Lithium has been shown to significantly enhance TNF-induced apoptosis in tumor cells (Beyaert 1989; Hoeflich *et al.*, 2000; Schotte *et al.*, 2001) and to differentially regulate LPS-stimulated cytokine production mediated by toll-like receptors in monocytes (Martin *et al.*, 2005). We next examined whether lithium regulates the cytokine production in macrophages after Mk stimulation. Mk induced the significant production of TNF and IL-12p40 in BMDMs (Fig. 3). LiCl pretreatment of BMDMs prior to Mk stimulation substantially reduced the production of IL-12p40 and TNF induced by Mk (Fig. 3A and 3B). However, the production of anti-inflammatory cytokine IL-10 was significantly increased in Mk-infected BMDM in the presence of LiCl (Fig. 3C). This indicates that the lithium-mediated augmentation of apoptosis in Mk-infected macrophages is TNF-independent.

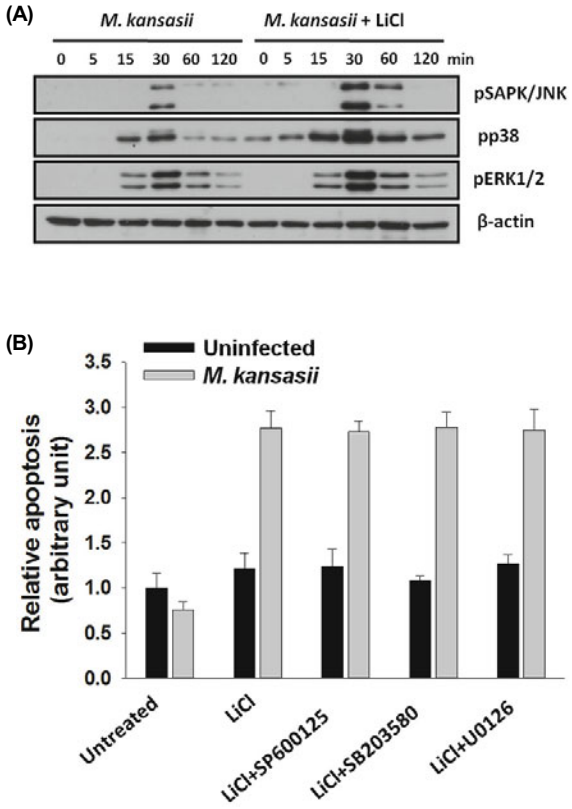

Fig. 4. MAPKs activation during LiCl-induced BMDM apoptosis. (A) BMDMs were incubated in the presence or absence of 10 mM of LiCl for 90 min before infection with Mk at an MOI of 10 for indicated times. Total cell extracts were separated by SDS-PAGE and immunoblotted using anti-phospho-MAPKs antibodies. (B) BMDMs were left either untreated or treated with the MAPK inhibitors SP600125 (20 μM), SB203580 (10 μM), or U0126 (10 μM) for 60 min. After the removal of the inhibitors, cells were incubated with LiCl (10 μM) for 60 min and challenged with or without Mk (MOI = 10) for 24 h. Cells were washed and lysed, and their apoptosis was evaluated using the Cell Death Detection ELISA kit. Data shown are the means ± SD of three experiments.

MAPK activation is independent on lithium-mediated enhancement of apoptosis

MAPK activation is the early event induced by mycobacteria infection. To determine the effect of lithium on the MAPK activation induced by NTM infection, we treated BMDMs with LiCl and infected with Mk for indicated intervals and then examined the activation of MAPK by using immunoblotting. Phosphorylation of all MAPKs tested was markedly increased at 30 min after Mk infection in BMDM and also augmented by lithium (Fig. 4A). For confirmation of which MAPK activations were involved in the enhancement of lithium-mediated macrophage apoptosis, we examined the effects of a specific inhibitor of SAPK/JNK (SP600125), p38 (SB203580), and ERK1/2 (U0126) on lithium-induced macrophage apoptosis. In contrast to our expectation, increased apoptotic responses were not attenuated by MAPKs inhibitors (Fig. 4B), indicating that early MAPK activation is an independent event for the apoptotic effects of lithium on infected macrophages.

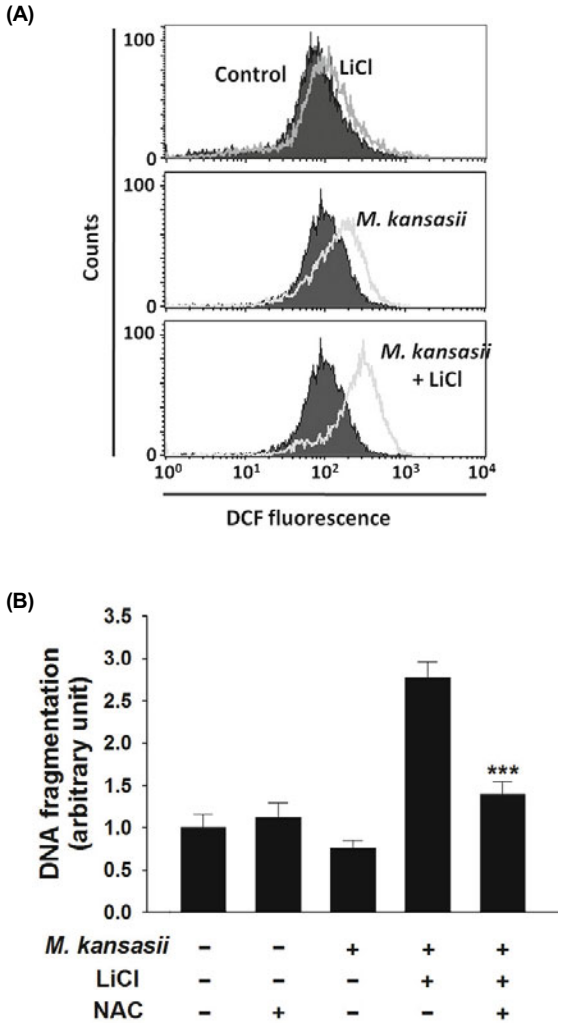

Fig. 5. Effect of LiCl treatment on ROS production by infected BMDMs. (A) BMDMs were either untreated or treated with LiCl (10 mM) for 90 min before infection with Mk at an MOI of 10 for 24 h. ROS levels were measured by FACS analysis after staining with the DCFH-DA fluorescent probe. The shift to the right of the curve due to increased fluorescence indicates an increase in intracellular ROS levels. Results are representative of three independent experiments. Filled, control; unfilled, LiCl, Mk, or LiCl plus Mk. (B) BMDMs were incubated with or without LiCl (10 mM) and NAC (10 mM) for 90 min and then challenged with Mk (MOI = 10) for 24 h. Cells were lysed and apoptosis was evaluated using a cell death ELISA kit. Data represent the means ± SD of triplicate assays and are representative of three independent experiments. *** $P<0.001$ compared to Mk-infected cells treated with LiCl without NAC.

ROS are required for lithium-enhanced apoptosis

Mycobacterial infection leads to increased ROS production in murine BMDMs (Yang *et al.*, 2009). ROS production may lead to apoptosis, which can be inhibited by the antioxidant NAC (Mayer and Novel, 1994). Thus, we evaluated whether intracellular ROS levels increased in response to Mk infection and whether lithium modulated ROS levels. Infection of BMDMs with Mk resulted in elevated intracellular ROS levels (Fig. 5A). Addition of LiCl resulted in a robust burst of ROS production in BMDMs infected with Mk; however, LiCl alone had no such effect (Fig. 5A). Pre-

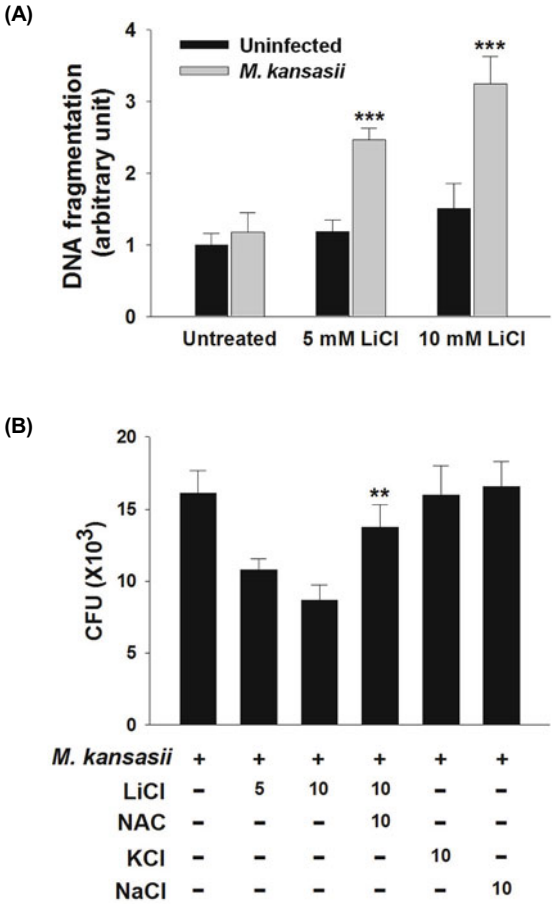

Fig. 6. Correlation between the reduction in intracellular bacterial viability and lithium-mediated enhancement of host cell apoptosis. BMDMs were infected with Mk at an MOI of 5 for 4 h, washed, and further incubated in the presence or absence of KCl (10 mM), NaCl (10 mM), or LiCl (5 or 10 mM) plus or minus NAC (10 mM). After lysis of cells 48 h postinfection, macrophage apoptosis (A) and the viability of intracellular bacteria (B) were evaluated. Apoptosis was determined using a Cell Death Detection ELISA kit, as described earlier. The viability of intracellular bacteria was measured by serial dilutions and colony counting on 7H10 agar plates. Values represent means ± SD of three independent determinations. *** $P<0.001$ both Mk-infected cells versus Mk-infected cells treated with LiCl. ** $P<0.01$ compared to Mk-infected cells treated with LiCl without NAC.

treatment of cells with NAC, an ROS scavenger, prior to stimulation with Mk abrogated the LiCl-mediated enhancement of macrophage apoptosis (Fig. 5B). These data suggest that the enhancement of Mk-induced macrophage death by lithium is accompanied by an increase in oxidative stress, which is a prerequisite for apoptosis.

Lithium-enhanced apoptosis is associated with reduced intracellular Mk viability, which can be restored by inhibition of apoptosis

To determine whether the lithium-mediated enhancement of apoptosis was involved in the control of intracellular Mk viability, parallel apoptosis and CFU assays were performed in BMDMs infected with Mk at an MOI of 5 in the absence or presence of LiCl. As expected, Mk-infected BMDMs were susceptible to lithium-mediated apoptosis in a dose-dependent manner (Fig. 6A). Viable counts indicated that lithium-enhanced apoptosis was accompanied by reduced intracellular numbers of Mk, whereas equivalent concentrations of KCl or NaCl had no such effect (Fig. 6B). Moreover, the lithium-mediated inhibition of intracellular Mk growth was restored by NAC treatment of Mk-infected macrophages. This suggests that the reduction in intracellular bacterial viability was directly related to host cell apoptosis (Fig. 6B).

Discussion

Macrophages infected with mycobacteria undergo death by apoptosis and necrosis. It has been postulated that apoptosis of infected macrophages is associated with killing of intracellular mycobacteria (Molloy *et al.*, 1994; Keane *et al.*, 2000). Virulent *M. tuberculosis* strains cause macrophage necrosis, both to avoid innate host defenses and to escape their host cells (Divangahi *et al.*, 2009). We previously demonstrated that Mk virulence is correlated with its capacity for intracellular growth in macrophages and necrosis-inducing activity, as is the case for *M. tuberculosis* (Sohn *et al.*, 2010). Although the precise mechanism of the antimicrobial activity in infected macrophages undergoing apoptosis is unclear, induction of apoptosis by chemical agents such as adenosine triphosphate or picolinic acid in macrophages infected with mycobacteria is accompanied by a reduction in intracellular bacterial counts (Molloy *et al.*, 1994; Pais and Appelberg, 2000). In the present study, we found that lithium enhanced the apoptosis of Mk-infected macrophages in a caspase- and ROS-dependent manner, which was associated with a substantial reduction in intracellular Mk viability. To the best of our knowledge, this is the first report showing that apoptosis of mycobacteria-infected macrophages is modulated by lithium.

LiCl alone caused no significant cytotoxicity but drastically enhanced the cell death of macrophages infected with Mk or *M. abscessus*. In fact, LiCl at low concentrations had no effect on tumor cell viability in the absence of TNF (Beyaert *et al.*, 1989; Liao *et al.*, 2003). Several studies have demonstrated that lithium, an inhibitor of GSK-3β, enhances TNF-induced cell death in tumor cells (Beyaert *et al.*, 1989; Schotte *et al.*, 2001; Schwabe and Brenner, 2002; Liao *et al.*, 2003). Also, a synthetic GSK-3β-specific inhibitor (SB216763) significantly enhances TNF-related apoptosis-inducing ligand (TRAIL)-mediated cell death in prostate cancer cells (Liao *et al.*, 2003). Genetic deletion of GSK-3β potentiates the cytotoxicity of TNF (Hoeflich *et al.*, 2000), and GSK-3 protects hepatocytes from TNF-induced apoptosis (Schwabe and Brenner, 2002). These results suggest that GSK-3β is associated with protection against TNF-induced cell death. However, in the present study, SB216763 had no effect on the viability of Mk-infected macrophages. Similarly, Schotte *et al.* (2001) reported that GSK-3 is not involved in lithium-induced TNF sensitization of tumor cells. LiCl has multiple competitive targets in addition to GSK-3 (Klein and Melton, 1996; Liao *et al.*, 2003). However, at this stage we cannot completely exclude a role of GSK-3 in lithium-enhanced apoptosis of Mk-infected macrophages.

In vitro infection with *M. tuberculosis* induces macrophage apoptosis in a TNF-dependent manner (Keane *et al.*, 1997). Martin *et al.* (2005) reported that lithium markedly reduces TLR agonist-induced production of the pro-inflammatory cytokines including TNF and increases the production of the anti-inflammatory IL-10 by TLR agonist in monocytes. In this study, we also found that lithium inhibited Mk-induced TNF and IL-12 production and increased Mk-induced IL-10 production in BMDMs. These results suggest that the lithium-mediated enhancement of apoptosis of Mk-infected macrophages was not due to augmentation of TNF production. Our previous study showed that macrophage death caused by Mk is independent of TNF (Sohn *et al.*, 2010).

Lithium is involved in the modulation of the immune response; like some other antidepressant drugs, it has both immune-stimulatory and immune-suppressive functions (Rybakowski, 2000; Lieb, 2004; Martin *et al.*, 2005). Recent studies have suggested that lithium has immunopotentiatory, antimicrobial, and antiviral properties, as well as antidepressant activity (Rybakowski, 2000; Lieb, 2004). Thus, lithium may prevent replication of mycobacteria by stimulating phagocytic cells. The MAPK signaling pathways composed of the ERK1/2, p38, and SAPK/JNK are stimulated during mycobacterial infection and have been implicated in mycobacterial pathogenesis (Yadav *et al.*, 2004). However, in this study, MAPK activations were not involved in the enhancement of macrophage apoptosis by lithium, indicating that macrophage activation do not seem to play a role in the reduction of intracellular Mk viability by lithium.

We observed that LiCl augmented the production of ROS in Mk-infected BMDMs and preincubation with NAC, an ROS scavenger, abrogated the lithium-mediated reduction of intracellular Mk growth and enhancement of apoptosis. These results suggest that ROS are essential for lithium-enhanced apoptosis and that apoptosis serves as a mechanism of reducing bacillary viability. Bhattacharyya *et al.* (2003) reported that challenge of macrophages with *M. avium* resulted in apoptosis through a process in which ROS played a key role. Similarly, Herbst *et al.* (2011) reported that interferon gamma-activated macrophages killed mycobacteria by nitric oxide- induced apoptosis.

In conclusion, our data indicate that lithium modulates the apoptotic response in Mk-infected macrophages. Further investigation of the mechanisms of lithium-enhanced apoptosis may further elucidate the role of lithium in immunity to mycobacteria.

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