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**Mycobacterium tuberculosis** Mce3E Suppresses Host Innate Immune Responses by Targeting ERK1/2 Signaling

Jie Li,* Qi-Yao Chai,* Yong Zhang,* Bing-Xi Li,* Jing Wang,* Xiao-Bo Qiu,† and Cui Hua Liu*†

Crucial to the pathogenesis of the tuberculosis (TB)-causing pathogen *Mycobacterium tuberculosis* is its ability to subvert host immune defenses to promote its intracellular survival. The mammalian cell entry protein 3E (Mce3E), located in the region of difference 15 of the *M. tuberculosis* genome and absent in *Mycobacterium bovis* bacillus Calmette-Guérin, has an essential role in facilitating the internalization of mammalian cells by mycobacteria. However, relatively little is known about the role of Mce3E in modulation of host innate immune responses. In this study, we demonstrate that Mce3E inhibits the activation of the ERK1/2 signaling pathway, leading to the suppression of *Tnf* and *Il6* expression, and the promotion of mycobacterial survival within macrophages. Mce3E interacts and colocalizes with ERK1/2 at the endoplasmic reticulum in a DEF motif (an ERK-docking motif)–dependent manner, relocates ERK1/2 from cytoplasm to the endoplasmic reticulum, and finally reduces the association of ERK1/2 with MEK1 and blocks the nuclear translocation of phospho-ERK1/2. A DEF motif mutant form of Mce3E (F294A) loses its ability to suppress *Tnf* and *Il6* expression and to promote intracellular survival of mycobacteria. Inhibition of the ERK1/2 pathway in macrophages using U0126, a specific inhibitor of the ERK pathway, also leads to the suppressed *Tnf* and *Il6* expression and the enhanced intracellular survival of mycobacteria. Taken together, these results suggest that *M. tuberculosis* Mce3E exploits the ERK1/2 signaling pathway to suppress host innate immune responses, providing a potential Mce3E–ERK1/2 interface–based drug target against *M. tuberculosis*. The *Journal of Immunology*, 2015, 194: 3756–3767.

*Mycobacterium tuberculosis* remains one of the major global health threats, resulting in an annual casualty of ∼2 million people worldwide (1). Although nearly one-third of the human population is infected with *M. tuberculosis*, only ∼10% of the infected individuals develop active disease during their lifetime. The pathogenesis of *M. tuberculosis* is largely because it can escape host immune defense system and thus survive within the hostile environment of human macrophages (2, 3). The prolonged coevolution of *M. tuberculosis* with its hosts has led to multiple survival strategies to interfere with a wide range of host cellular processes, such as production of cytokines, phagolysosome biogenesis, and modulation of macrophage survival (3–6). The majority of the currently available drugs are only partially effective due to the impermeable nature of the mycobacterial cell wall and the propensity of *M. tuberculosis* to develop drug resistance (7). Thus, it is urgent to unravel the molecular details underlying *M. tuberculosis*–macrophage interactions, which could be useful to identify novel targets for anti-tuberculosis (TB) drug development.

Innate immunity constitutes the first line of defense against pathogen infection (8). Two major immune signaling pathways, including NF-κB and MAPK, mediate regulation of innate immune responses through controlling synthesis of various cytokines such as TNF and IL-6, etc. (9–11). Meanwhile, increasing evidence suggests that bacterial pathogens target and manipulate those signaling pathways for their benefits (12). For example, *Yersinia enterocolitica* was indicated to suppress TNF production by inhibiting ERK1/2, p38, and JNK activities (13). Macrophages infected with *Mycobacterium avium* showed decreased MAPK activation compared with the cells infected with nonpathogenic mycobacteria (14). The NF-κB and MAPK signaling pathways can also regulate production of a number of cytokines by the macrophages infected with *M. tuberculosis* (15). But the molecular mechanisms by which specific mycobacterial effectors modulate the host innate immune signaling pathways remain largely unclear.

The genome of *M. tuberculosis* H37Rv harbors four homologous copies of the gene cluster termed *mce operons* (*mce1*–4), which includes two *yrbE* and six *mce* genes (*mceA–F*) (16, 17). Mammalian cell entry (Mce) proteins were initially characterized as invasion-like proteins with putative export signal sequences at the N-terminal end (16, 17). Previous studies suggest that latex beads coated with Mce1A, Mce3A, and Mce3E are internalized by nonphagocytic HeLa cells (18, 19). Additional studies have demonstrated that the *M. tuberculosis* strains with *mce* operon disruption were attenuated in mice (20, 21). Among the four *mce* operons, the *mce3* operon is located in the region of difference 15 of the *M. tuberculosis* genome, which is of special interest because...
it is absent in the vaccine strain of *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) (22). However, relatively little is known about the role of the mce3 operon in modulation of host innate immune responses.

Previous studies have shown that the six Mce proteins encoded by *mce3* operon, especially Mce3A, Mce3D, and Mce3E, are expressed in *M. tuberculosis* during infection in humans and are immunogenic (23). But among all *mce3* operon proteins, only region of difference 1504 (Mce3A) was demonstrated to be able to induce a strong Th1 bias in healthy subjects and a weak Th1 bias in TB patients, whereas other proteins of *mce3* operon induced only moderate to weak Th1 biases in both TB patients and healthy subjects (24), suggesting that other proteins among *mce3* operon might have potential immune-modulating effects. Despite the potential function of Mce proteins on intracellular survival of *M. tuberculosis*, little progress has been made to define the specific functional mechanisms of each Mce protein. In this study, we focus on the Mce3E, aiming at elucidating its potential role in modulation of host innate immune responses. We demonstrate that Mce3E downregulates cytokine expression and promotes survival of mycobacteria in macrophages through inhibiting activation of the ERK1/2 signaling pathway. We further show that Mce3E could be secreted from mycobacteria phosphorylated by macrophages and then translocate into cytosol to localize at the endoplasmic reticulum (ER). Furthermore, Mce3E interacts with ERK1/2 in a DEP motif (an ERK-docking motif)–dependent manner, traps ERK1/2 into ER, and blocks the interaction of ERK1/2 with MEK1 and the nuclear translocation of p-ERK1/2, resulting in inhibition of the ERK1/2 signaling pathway. Our findings not only reveal the specific mechanisms by which Mce3E modulates host innate immune responses during the course of mycobacterial infection, but also provide a potential Mce3E–ERK1/2 interface–based drug target against *M. tuberculosis*.

### Materials and Methods

**Bacterial strains**

*Escherichia coli* DH5α, *E. coli* BL21 (DE3), *Mycobacterium smegmatis* mc²155, and *M. bovis* BCG (Pasteur) were used for genetic manipulation or protein expression. *E. coli* DH5α and *E. coli* BL21 (DE3) were grown in flasks using LB medium. The mycobacterial strains were grown in Middlebrook 7H9 broth (273110; BD Difco) supplemented with 10% oleic acid–albumin–dextrose–catasalase and 0.05% Tween 80 (Sigma–Aldrich), or on Middlebrook 7H10 agar (262710; BD Difco) supplemented with 10% oleic acid–albumin–dextrose–catasalase. The *Mycobacterium* shuttle vector pMV261 (provided by W. Jacobs from Albert Einstein College) was used to express *M. tuberculosis* Mce3E in *M. smegmatis* or BCG. The mycobacterial expression vector with GFP tag pSC300 (Addgene) was used to express GFP–Mce3E in *M. smegmatis* or BCG. Expression of Mce3E in mycobacteria-infected RAW264.7 cells was examined by quantitative PCR analysis.

**Plasmids, Abs, and reagents**

The gene for Mce3E was amplified from genome of *M. bovis* H37Rv. For expression in mammalian cells, the gene was cloned into pcDNA6A, p3XFlag-CMV-14, or pEGFP-C1, respectively. Bacterial expression plasmids were constructed by inserting the genes into pGEX-6p-1, pMV261, or pSC300, respectively. Luciferase assay plasmids for Gal4-Elk, Gal4-mids were constructed by inserting the genes into pGEX-6p-1, pMV261, or pCR2.1. Dual luciferase assay was performed as described by Li et al. (25) using the Promega luciferase reporter system. For the ERK pathway, RAW264.7 cells grown on 12-well plate were cotransfected with 0.6 μg Gal4-Erk, 0.6 μg Gal4-luc, 50 ng pRL-TK and 10 ng RasV12, or 100 ng v-Raf or 100 ng constitutive active MEK1 (MEK1-ED) in the presence or absence of 1 μg Mce3E. To measure JNK activation, RAW264.7 cells were cotransfected with 0.1 μg pFA-Jun, 0.5 μg Gal4-Erk, 0.5 μg Gal4-mids, and 50 ng pRL-TK with or without 1 μg Mce3E plasmid. For the NF-kB pathway, RAW264.7 cells were cotransfected with 1 μg pNF-kB-Luc and 50 ng pRL-TK in the presence or absence of 1 μg Mce3E plasmid. Twenty-four hours later, cells were treated with 100 ng/ml LPS (Sigma–Aldrich) for 5 h to stimulate NF-kB activation.

**Luciferase assay**

Dual luciferase assay was performed as described by Li et al. (25) using the Promega luciferase reporter system. For the ERK pathway, RAW264.7 cells grown on 12-well plate were cotransfected with 0.6 μg Gal4-Erk, 0.6 μg Gal4-luc, 50 ng pRL-TK and 10 ng RasV12, or 100 ng v-Raf or 100 ng constitutive active MEK1 (MEK1-ED) in the presence or absence of 1 μg Mce3E. To measure JNK activation, RAW264.7 cells were cotransfected with 0.1 μg pFA-Jun, 0.5 μg Gal4-Erk, 0.5 μg Gal4-mids, and 50 ng pRL-TK with or without 1 μg Mce3E plasmid. For the NF-kB pathway, RAW264.7 cells were cotransfected with 1 μg pNF-kB-Luc and 50 ng pRL-TK in the presence or absence of 1 μg Mce3E plasmid. Twenty-four hours later, cells were treated with 100 ng/ml LPS (Sigma–Aldrich) for 5 h to stimulate NF-kB activation.

**Protein purification and in vitro binding assay**

*E. coli* BL21 (DE3) strain was used as the host for expression of GST, GST-Mce3E, GST-Mce3D, and GST-Mce3A (P294A), MBP, MBP-Erk2, His-Erk2, and His-p38. Protein expression was induced at 30°C or 16°C with 0.1–0.2 mM isopropyl-β-D-thiogalactopyranoside after OD600 reached 0.6–0.8. GST-tagged proteins, MBP fusion proteins, and His fusion proteins were purified by affinity chromatography (amino resin for MBP fusion proteins; glutathione Sepharose 4B for GST-tagged proteins; Ni-NTA resin for His fusion proteins), followed by size exclusion chromatography on a Superdex 200 column. For pulldown assay, bait proteins were immobilized onto amylase beads (MBP and MBP-Erk2) or glutathione Sepharose 4B (GST, GST-Mce3E and GST-Mce3D [P294A]), followed by incubation with corresponding prey proteins in a buffer containing 20 mM HEPES, 200 mM NaCl, and 1% Nonidet P40 (pH 7.4) supplemented with complete protease inhibitors at 4°C for 2 h. After incubation, the beads were extensively washed with the binding buffer, and the bound proteins were analyzed by immunoblot analysis.

**Cell staining and confocal microscopy**

RAW264.7 cells were seeded on glass coverslips and transfected with Lipofectamine 2000. Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, blocked with 1% milk in PBS for 30
min, and labeled with primary and secondary Abs. Confocal images were taken with a Leica SP8 confocal system.

Quantification of infected RAW264.7 cells with nuclear localization of phospho-ERK

At 24 h after transfection of Flag-ERK2, RAW264.7 cells were infected for 4 h with pSC300-BCG, pSC300-Mce3E-BCG, or pSC300-Mce3E (F294A)-BCG at multiplicity of infection (MOI) of 100 and then subjected to cell staining, as described above. With regard to quantification of the number of infected RAW264.7 cells with nuclear localization of p-ERK, only RAW264.7 cells that were infected with GFP-labeled BCG were counted. At least 100 infected cells were scored blind in each experiment, and all experiments were repeated three times.

Infection of macrophages, CFU counting, quantitative PCR analysis, and ELISA

RAW264.7 cells were seeded at a density of $5 \times 10^5 - 1 \times 10^6$ cells/well in 6-well plates and cultured for 12 h before infection. Frozen mycobacterial strains were thawed and centrifuged, and the pellet was resuspended in DMEM supplemented with 0.05% Tween 80 by vortexing. Then the cells were infected with mycobacterial strains for 2 h at a MOI of 10. After incubation for 2 h, the cells were washed three times with PBS to remove any unphagocytosed bacteria, followed by culturing in fresh medium containing 20 µg/ml gentamicin. At various time points postinfection, cell culture media were obtained for ELISA; cells were washed with PBS three times and harvested for CFU measurement or quantitative PCR assay. For CFU determination, macrophages were solubilized with 0.025% (v/v) SDS. Then intracellular survival was determined by plating serially diluted cultures on 7H10 plates, and the colonies were enumerated after 3 d for M. smegmatis and 3 wk for BCG. For quantitative PCR assay, total RNA was extracted from the collected cells and reverse transcribed into cDNA by using commercially available kits according to the manufacturers’ instructions. The obtained cDNA was then subjected to quantitative real-time PCR analysis (quantitative PCR) with KAPA SYBR FAST qPCR Kit (KAPA Biosystems) on ABI 7300 system (Applied Biosystems). GAPDH mRNA was used as a reference housekeeping gene for normalization. For ELISA, the cell-free supernatants were harvested by centrifugation at 13,000 rpm for 1 min to remove suspension cells and mycobacteria. Levels of TNF protein and IL-6 protein in the supernatants were quantified by RayBio ELISA kits (Mouse TNF ELISA Kit: ELM-TNFα-001; Mouse IL-6 ELISA Kit: ELM-IL6-001; Human TNF ELISA Kit: ELH-TNFα-001; Human IL-6 ELISA Kit: ELH-IL6-001), according to the manufacturer’s instructions. Each sample was done in triplicates.

**FIGURE 1.** Inhibition of the ERK1/2 pathway by M. tuberculosis Mce3E. (A–C) Luciferase assays of RasV12-, V-Raf–, and MEK1-ED–induced ERK1/2 activation in the absence or presence of Mce3E. Mce3E blocks ERK1/2 activation in RAW264.7 macrophage cells stimulated by RasV12 (A), V-Raf (constitutive active Raf) (B), or MEK1-ED (constitutive active MEK1) (C). (D) Western blotting analysis showed the secretion of Mce3E into the cytosol of RAW264.7 macrophage cells infected with BCG-expressing M. tuberculosis Mce3E. The membrane protein Rv3134 was used as a negative control for secretion. α-Tubulin and poly(ADP-ribose) polymerase were used as markers of the cytosolic and nuclear fractions, respectively. (E and F) Expression of M. tuberculosis Mce3E in M. smegmatis (M. smeg) (E) or BCG (F) reduces phosphorylation of ERK1/2 in RAW264.7 macrophage cells infected for 0–24 h with indicated mycobacterial strains as analyzed by immunoblot analysis. (G) Phosphospecific immunoblot analysis of inhibition of MEK1-ED–induced ERK1/2 activation by Mce3E in HEK293T cells. (H) Phosphospecific immunoblot analysis of RasV12-induced MEK1 or ERK1/2 activation in the presence of Mce3E. Data are representative of at least three independent experiments (mean and SEM in A–C). **p < 0.01 (two-tailed unpaired t test).
Isolation of PBMCs and differentiation of monocytes into macrophages

Human PBMCs were derived from whole blood by Ficoll-Hypaque density gradient centrifugation and cultured in RPMI 1640 supplemented with 10% FBS, 4 mM l-glutamine, and 1% penicillin-streptomycin for 2 h at 37°C with 5% CO₂ to allow the adherence of monocytes. After 2 h of adherence, PBMCs were washed five times with warmed PBS to remove those non-adherent cells, including lymphocytes, and then cultured with medium exchanged every 3 d until the cells differentiated into macrophages. PBMC-derived macrophages were infected with mycobacterial strains and subjected to CFU counting, quantitative PCR analysis, and ELISA, as described above.

Cellular fractionation of infected macrophages

RAW264.7 cells infected with pSC300-Mce3E-BCG at MOI of 100 were subjected to hypotonic lysis buffer containing 10 mM HEPES (pH7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.34 M sucrose, 10% glycerol, protease inhibitors, and 0.1% Triton X-100. The lysate mixture was then centrifuged at 1300 × g for 10 min to pellet the macrophage nuclei and BCG. The supernatant was then collected as the cytosolic fraction of infected macrophages for immunoblot analysis (26).

Statistical analysis

Two-tailed unpaired t test was used for statistical analysis. Each p value < 0.05 or < 0.01 was considered statistically significant.

Results

Inhibition of the ERK pathway by M. tuberculosis Mce3E

Given the important contribution of the NF-κB and MAPK signaling pathways in activating host innate immune responses, microbes have evolved a variety of strategies to inhibit activation of those signaling pathways (27–30). To define the potential role of M. tuberculosis Mce3E in modulating host innate immunity as well as to identify the specific signaling pathways it targets, we examined the effects of M. tuberculosis Mce3E on activation of the NF-κB and MAPK signaling pathways. Transient expression of M. tuberculosis Mce3E in RAW264.7 macrophage cells efficiently blocked RasV12-induced ERK1/2 activation (Fig. 1A), and had little, if any, inhibitory effect on the RacL61-induced JNK/p38 activation, while promoting LPS-stimulated NF-κB activation (data not shown). We thus further sought to define the specific step(s) in the ERK1/2 pathway suppressed by M. tuberculosis Mce3E. As shown in Fig. 1B and 1C, M. tuberculosis Mce3E efficiently blocked v-Raf (constitutive active Raf)– and MEK1-ED (constitutive active MEK1)–stimulated ERK1/2 activation in RAW264.7 macrophage cells. Immunoblot analysis was used to confirm the secretion of Mce3E into the cytosolic fraction of macrophages infected with pSC300-Mce3E-BCG. The Rv3134 protein, which was identified in the cell membrane fraction of M. tuberculosis H37Rv (31), was used as a negative control for secretion (Fig. 1D). To further examine whether Mce3E suppressed ERK1/2 signaling in macrophages during the course of mycobacterial infection, we expressed M. tuberculosis Mce3E in M. smegmatis (Mce3E-M. smegmatis) or BCG (Mce3E-BCG) for infection of RAW264.7 cells. We found that expression of M. tuberculosis Mce3E in both M. smegmatis and BCG (as assessed by quantitative PCR analysis; data not shown) resulted in reduced ERK phosphorylation in RAW264.7 cells during the course of infection (Fig. 1E, 1F). Furthermore, Mce3E abrogated the ERK1/2 phosphorylation induced by MEK1-ED (Fig. 1G) or RasV12 (Fig. 1H), whereas it did not affect RasV12-induced MEK1 phosphorylation in HEK2923 cells. Collectively, these data suggest that M. tuberculosis Mce3E

FIGURE 2. Expression of M. tuberculosis Mce3E in BCG decreases expression of TNF and IL-6, and increases the survival of BCG in RAW264.7 macrophages during the course of infection. (A and B) Expression of M. tuberculosis Mce3E in BCG decreases the mRNA expression of Tnf (A) and Il6 (B) in RAW264.7 cells as examined by quantitative PCR. Macrophage cells were infected for 0–24 h with indicated mycobacterial strains. (C and D) ELISA showed that expression of M. tuberculosis Mce3E in RAW264.7 macrophages decreases the TNF protein (C) and IL-6 protein (D) released by RAW264.7 cells infected as in (A) and (B). (E) Expression of M. tuberculosis Mce3E in BCG increases the survival of BCG in RAW264.7 cells infected as in (A) and (B). Quantitative PCR results are presented relative to expression of the control gene Gapdh (in A and B). Data are representative of at least three independent experiments (mean and SEM in A–E). *p < 0.05, **p < 0.01 (two-tailed unpaired t test).
blocks the ERK signaling downstream or at the level of MEK1
along the Ras-Raf-MEK-ERK cascade.

*M. tuberculosis* Mce3E inhibits cytokine production and
promotes survival of mycobacteria in macrophages

Bacterial infection incites the activation of host immune signaling
pathways and thereby induces the production of various cytokines such
as TNF and IL-6. Such mediators are essential for recruitment and
activation of immune cells to respond to bacterial infection (8). In
contrast, bacterial pathogens frequently target the MAPK pathway to
suppress the production of cytokines to counteract host defenses (29,
32). The altered ERK1/2 pathway activation by *M. tuberculosis*
Mce3E therefore raised the possibility that the expression of certain
cytokines might be altered in macrophages infected with *M. tuber-
culosis* Mce3E-expressing mycobacterial strain. To examine *M. tuberculosis* Mce3E’s ability in modulating cytokine expression
independently of other *M. tuberculosis* effector proteins, we analyzed the expression of a number of cytokines in macrophages infected with
Mce3E-*M. smegmatis* or Mce3E-BCG strains by quantitative PCR
analysis and ELISA. Expression of *M. tuberculosis* Mce3E in either
*M. smegmatis* (data not shown) or BCG (Fig. 2) greatly inhibited the
expression of *Tnf* and *Il6* and promoted bacterial survival in
RAW264.7 macrophage cells during mycobacterial infection. These
results establish that *M. tuberculosis* Mce3E alone can effectively
suppress the expression of certain inflammatory cytokines and pro-
mote survival of mycobacteria in macrophages.

*Mce3E interacts with ERK in a DEF-dependent manner

To elucidate the underlying mechanisms of Mce3E-mediated sup-
pression of host innate immune responses, we attempted to search for
its host targets along the ERK1/2 pathway. Based on the observation
that Mce3E blocks MEK1-ED–mediated activation of the
ERK1/2 pathway, we analyzed whether Mce3E interacted with
MEK1 or ERK1/2. When Mce3E and MEK1 were coexpressed in
HEK293T cells, Mce3E was able to coimmunoprecipitate with en-
dogenous ERK1/2, but not expressed MEK1 (Fig. 3A). Consistently,
Flag-ERK2 could also communoprecipitate with Myc-Mce3E when
coexpressed in HEK293T cells (Fig. 3B). Thus, we proposed that
Mce3E interacts with ERK1/2, but not MEK1. Several effectors from
pathogenic bacteria such as *Legionella* effectors display distinctive
eukaryotic domains, among which are protein kinase or phosphatase
domains (33–35). In our efforts to search for the functional domains/
motifs within Mce3E, we identified a potential DEF motif, an ERK-
docking motif that possesses a consensus sequence of FXFP, in
Mce3E (FPFP) using in silico analysis (http://scansite.mit.edu/) (36).
Because the former Phe residue is most critical among the three
conserved amino acids of the DEF motif, we thus mutated the Mce3E
Phe294 into Ala (F294A), and found that this mutant could barely
bind to ERK in vitro (Fig. 3C). Because existing reports suggest that
the DEF domain also mediates interaction with p38 MAPK (37), we
thus further performed experiments to examine whether this DEF
motif in Mce3E also interacts with p38 MAPK. As is shown in Fig.
3D, Mce3E has little binding affinity to p38α, whereas F294A mu-
tation completely abolishes such weak binding. Consistently, such
mutation almost completely abolished Mce3E-mediated inhibition of
ERK1/2 phosphorylation in HEK293T cells (Fig. 3E) and ERK
signaling activation in RAW264.7 macrophage cells (Fig. 3F). Taken
together, these data demonstrate that Mce3E interacts with ERK in
a DEF motif-dependent manner to inhibit ERK pathway activation.

**Mce3E is localized at the ER apparatus, thus trapping ERK
into the ER apparatus**

We then performed immunofluorescence assays to investigate
whether Mce3E colocalizes with ERK in vivo. Intriguingly, immu-
nofluorescent analysis of exogenously expressed Mce3E fused
to GFP in RAW264.7 cells revealed a staining pattern that spec-
ifically overlapped with the ER marker Calnexin, but not the Golgi
marker Giantin. Subcellular distribution of Mce3E remained the
same pattern when using GFP, Flag, or Myc tags at its C terminus
in several cell lines (data not shown). In addition, the Mce3E F294A

**FIGURE 3.** *M. tuberculosis* Mce3E binds to
ERK1/2 in a DEF motif-dependent manner. (A) Flag-Mce3E coexpressed with endogenous
ERK1/2, but not exogenous expressed Myc-
MEK1 from HEK293T cells. (B) Coimmunopre-
cipitation of *M. tuberculosis* Mce3E and ERK2 in
the lysates of the HEK293T cells cotransfected
with *M. tuberculosis* Mce3E and ERK2. (C) WT
Mce3E, but not its F294A mutant, interacts with
ERK2 as analyzed by MBP pulldown assay. (D)
Mce3E displays DEF motif-based strong binding
affinity to ERK2 and little binding affinity to
p38α. (E) F294A mutation abolishes the Mce3E-
mediated inhibition of ERK1/2 phosphorylation
as indicated by immunoblot assay. (F) Luciferase
assay showed that F294A mutation attenuates
the Mce3E-mediated inhibition of ERK1/2 activation
in RAW264.7 macrophage cells stimulated by
MEK1-ED. Data are representative of at least
three independent experiments (mean and SEM in
F). **p ≤ 0.01 (two-tailed unpaired t test).
mutant shared the same localization pattern with the wild-type protein. Unlike the concentrated localization of Mce3E, Mce3A encoded by the mce3 operon displayed diffused distribution mode (Fig. 4A). Thus, these results indicate that Mce3E specifically resides in the ER apparatus in a DEF motif-independent manner. In resting cells, ERKs are diffusely distributed in the cytoplasm due to their interactions with anchoring proteins, such as their upstream activators MEKs. Upon stimulation, ERKs detach from the anchoring proteins and translocate to their sites of action, mainly at nucleus (38). Given the distinct localization of Mce3E as well as its interaction with ERK, we thus sought to investigate whether Mce3E could regulate the cellular location of ERKs. When cotransfected with Mce3E, but not its F294A mutant, in resting RAW264.7 cells, the majority of ERKs were translocated to the ER apparatus and exhibited almost complete colocalization with Mce3E (Fig. 4B), consistent with our results from coimmunoprecipitation and pulldown assays. In addition, the colocalization of Mce3E with ERK appeared to be specific for ERK, because Mce3E had no impact on the localization of MEK1 (Fig. 4B).

FIGURE 4. M. tuberculosis Mce3E colocalizes with ERK1/2 at the ER apparatus in RAW264.7 cells. (A) M. tuberculosis Mce3E is specifically localized at the ER in a DEF motif-independent manner. RAW264.7 cells were transfected with GFP-Mce3E or its mutant F294A or GFP-Mce3A (green). The ER, Golgi, and cell nuclei were labeled by Calnexin (red), Giantin (red), and DAPI (blue), respectively. (B) M. tuberculosis Mce3E colocalizes with ERK1/2 in RAW264.7 cells. RAW264.7 cells were transfected with the indicated plasmids and followed by serum starvation. Coexpression of Mce3E, but not its mutant form F294A, causes the translocation of ERK1/2 from cytosol to the ER. Cells were stained with anti-ERK1/2 or anti-Myc Abs, followed by labeling with anti-rabbit/mouse Alexa594 or anti-mouse FITC Abs (Molecular Probes). After Ab labeling, cells were counterstained with DAPI for nuclei (blue). Scale bars, 10 μm. Data are representative of at least three independent experiments.

Taken together, Mce3E is located at and traps ERK to the ER apparatus in host cells.

Mce3E blocks interactions between ERK and MEK and interrupts nuclear translocation of p-ERK

Because Mce3E could interact with ERK1/2 and trap ERK1/2 to the ER, we thus hypothesized that the Mce3E-ERK1/2 interactions might compete for the interactions between ERK1/2 and MEK1. We addressed this issue by coimmunoprecipitation assay with various expression levels of Mce3E. The interactions between ERK1/2 and Mce3E were increased upon increasing expression of Mce3E, concurrent with decreased interactions between ERK1/2 and MEK1 (Fig. 5A). Similarly, Mce3E (F294A) could not affect the association between ERK1/2 and MEK1 (Fig. 5B). These data suggest that Mce3E, through sequestering ERK1/2 at the ER, reduces the accessibility of ERK1/2 to MEK1 and consequently blocks the signaling from MEK to ERK. Because the nuclear translocation of p-ERK is stimulated by many stimuli such as EGF and could be modulated by pathogen effectors (39–41), we then
further examined whether Mce3E could affect the nuclear translocation of p-ERK1/2.

We found that Mce3E, but not its F294A mutant, also colocalizes with p-ERK1/2 and thus blocks the nuclear translocation of p-ERK1/2 in RAW264.7 cells stimulated with EGF (Fig. 5C). Consistently, RAW264.7 cells infected with pSC300-Mce3E-BCG showed less p-ERK1/2 localization in nucleus than their counterparts infected with pSC300-BCG or pSC300-Mce3E (F294A)-

**FIGURE 5.** *M. tuberculosis* Mce3E blocks ERK1/2-MEK1 interaction and nuclear translocation of phospho-ERK1/2. (A and B) Mce3E, but not its mutant form F294A, interferes with the interaction between ERK1/2 and MEK1. HEK293T cells were transiently transfected with the indicated plasmids. The Flag-tagged ERK2 was immunoprecipitated, and the ERK2-associated Mce3E and MEK1 were analyzed by immunoblot analysis. (C) Mce3E blocks EGF-stimulated nuclear translocation of p-ERK1/2 in RAW264.7 cells, as shown in immunostaining assays. RAW264.7 cells transfected with GFP-Mce3E (or its mutant form F294A, green) were treated with EGF (100 ng/ml) for 5 min and then stained with anti–p-ERK1/2 Ab and anti-rabbit Alexa 594 Ab (red). After Ab labeling, cells were counterstained with DAPI for nuclei (blue). Scale bars, 10 μm. (D) Expression of *M. tuberculosis* Mce3E in BCG, but not its mutant form F294A, blocks the nuclear translocation of p-ERK1/2 in RAW264.7 macrophage cells during infection. RAW264.7 macrophage cells were infected with indicated mycobacterial strains for 4 h and then stained with anti–p-ERK1/2 Ab and anti-rabbit Alexa 594 Ab (red). After Ab labeling, cells were counterstained with DAPI for nuclei (blue). Scale bars, 10 μm. Data are representative of at least three independent experiments. (E) Fraction (%) of infected RAW264.7 cells with nuclear localization of p-ERK1/2. Data are representative of at least three independent experiments (mean and SEM in E). **p < 0.01 (two-tailed unpaired t test).
BCG at the identical time points postinfection (Fig. 5D, 5E). Together, these observations reveal that Mce3E is exclusively localized at the ER apparatus and might inhibit ERK activation by specifically sequestering cytoplasmic ERK1/2 at the ER apparatus and thereby antagonizing the interactions between ERK1/2 and MEK1. In addition, through colocalization with p-ERK1/2, Mce3E also blocks their nuclear translocation, thus further attenuating ERK signaling.

**ERK interaction is required for Mce3E to downregulate Tnf and Il6 expression and to enhance intracellular survival of mycobacteria**

We further examined whether ERK interaction through Mce3E DEF domain is essential for the Mce3E-mediated suppression of cytokine expression in RAW264.7 cells and primary human monocyte-derived macrophage cells during the course of mycobacterial infection. We found that expression of Mce3E in M. smegmatis or BCG efficiently downregulated the expression level of Tnf and Il6 in both RAW264.7 macrophage cells and primary human monocyte-derived macrophage cells during mycobacterial infection (Supplemental Figs. 1A–D, 2A–D, Figs. 6A–D, 7A–D). Consistently, expression of Mce3E also promoted the intracellular survival of mycobacteria in macrophage cells. In contrast, F294A mutation abolished Mce3E-mediated suppression of proinflammatory cytokine production as well as promotion of intracellular survival of mycobacteria (Supplemental Figs. 1E, 2E, Figs. 6E, 7E). We next further examined whether chemical inhibition of the ERK1/2 signaling pathway could affect the expression of proinflammatory cytokines and bacterial intracellular survival during mycobacterial infection. We pretreated RAW264.7 cells for 2 h with U0126, a specific inhibitor of the ERK pathway, and then subjected them to M. smegmatis or BCG infection.

**Infection of U0126-pretreated RAW264.7 cells elicited lower expression of Tnf and Il6 than did infection of untreated RAW264.7 cells (Supplemental Fig. 3A–D, Fig. 8A–D). In addition, U0126 pretreatment promoted the survival of M. smegmatis or BCG in RAW264.7 cells (Supplemental Fig. 3E, Fig. 8E). Collectively, these data suggest that Mce3E might suppress cytokine production and enhance the intracellular survival of mycobacteria via inhibition of ERK1/2 activation in a DEF motif-dependent manner (Fig. 9).**

**Discussion**

The human intracellular pathogen *M. tuberculosis* can persist in host macrophage cells for long periods, even in a fully functioning immune system. Among the repertoire of strategies that *M. tuberculosis* has evolved to evade, divert, or subvert immune responses, the best documented are those that regulate host innate immune signaling pathways to avoid the elimination of the bacilli within macrophages. Many cytokines regulated by NF-κB and MAPK signaling pathways have been implicated in host–mycobacteria interactions. There are increasing numbers of examples of bacterial pathogens that produce and secrete effector molecules that dampen inflammatory cytokine production. For example, the *Yersinia* effector YopJ, which is a ubiquitin-like cysteine protease, targets and downregulates both the NF-κB and MAPK pathways (42). *Shigella flexneri* effector OspG, which is a protein kinase, targets ubiquitin-conjugated enzymes, thereby affecting phospho-IkBα degradation and subsequent NF-κB activation (43). OspF, another *Shigella* type III effector, was also shown to inactivate MAPks, including ERK1/2, JNK, and p38, by irreversibly removing phosphate groups from the phosphothreonine, but not from the phosphotyrosine residue in the activation loop of MAPks (25). But up to now, few mycobacterial effectors as well as their
host-modulatory mechanisms have been revealed. In this study, we demonstrate that Mce3E, a secreted effector protein from \textit{M. tuberculosis}, can inhibit ERK1/2 pathway activation, concurrent with decreased expression of a specific pool of inflammatory cytokines, including \textit{Tnf} and \textit{Il6}, which in turn leads to inhibition of the development of efficient immune responses against the mycobacteria.

Previous studies involving Mce3E and other Mce proteins were mainly focused on their potential role in facilitating the uptake of mycobacteria by macrophages, although relatively little is known about how Mce3E and other Mce proteins function after entering of the pathogen into host macrophage cells. The fact that inactivation of \textit{mce} operons could attenuate \textit{M. tuberculosis} virulence in the murine model provides a convincing evidence that Mce proteins are essential for intracellular survival of mycobacteria; we thus in this study sought to investigate the potential role of Mce3E in the modulation of host innate immune responses during mycobacterial infection of macrophage cells. Mce3E was presumably defined as a lipoprotein and anchored in the outer membrane of \textit{M. tuberculosis} (16). Previous studies suggest that the Mce3 are localized extracellularly, and, based on their topology, they are most likely involved either in transport activity or in functions involving interactions with the environment or the host (17, 19). But up to now, it is still unclear whether Mce3E could be secreted into the cytosol of host cells during mycobacterial infection. In this work, based on our study, we propose that Mce3E is likely to be secreted extracellularly, and, based on their topology, they are most likely involved in vesicular trafficking, and innate immune responses (48). Several effector proteins from pathogenic bacteria were shown to mimick host proteins such as phosphatases or kinases to evade host immune defenses (33, 35, 49–51). Because Mce3E has a DEF motif, which in turn leads to inhibition of the development of efficient immune responses against the mycobacteria.

Microbial pathogens have evolved mechanisms to subvert ER functions, thereby influencing the host immune response. The ER is one of the organelles of the host cell that is targeted by intracellular pathogens. Certain bacterial toxins also hijack the ER for entry and intracellular survival (35). In addition, in the Ras/Raf/MEK/ERK signaling cascade, several mediators within this pathway are exquisitely regulated by subcellular compartmentalization (44, 45). For example, \textit{\beta}-arrestin could function as a scaffold protein to target the MAPK protein complex to early endosome (46). It was reported that Raf kinase can be recruited to the Golgi by Raf kinase trapping to Golgi, and that such spatial regulation inhibits Raf-1 activation and reduces the association of Raf-1 with Ras and MEK, thus blocking the ERK pathway (47). In this study, we unveil another aspect of the ER function that mycobacteria hijack to maintain infection. Our study reveals that \textit{M. tuberculosis} Mce3E modulates the ERK signaling pathway through spatial regulation of subcellular localization of ERK/p-ERK. Because hydrophobicity analysis indicated Mce3E contains no transmembrane domains, we thus hypothesized that Mce3E localizes at ER apparatus by interacting with certain ER resident protein. Further identification of such protein(s) is currently underway in the laboratory.

Phosphorylation plays important roles in regulating numerous eukaryotic cellular processes, including cell cycle progression, vesicular trafficking, and innate immune responses (48). Several effector proteins from pathogenic bacteria were shown to mimick host proteins such as phosphatases or kinases to evade host immune defenses (33, 35, 49–51). Because Mce3E has a DEF motif and could interact with ERK1/2 directly, we initially examined whether Mce3E could be able to dephosphorylate p-ERK directly, but we were not able to demonstrate the phosphatase activity of Mce3E toward p-ERK in vitro. We also conducted yeast two-
hybrid assay to identify potential Mce3E-interacting phosphatase(s) that might be brought to p-ERK1/2 to function more efficiently, but the only Mce3E-interacting phosphatase we identified was Ptpn23, and Mce3E-mediated inhibition of ERK1/2 activation was not affected in Ptpn23 knocked-down cells (data not shown). We thus turned to other explanations for the mechanisms underlying

FIGURE 8. Inhibition of ERK1/2 pathway activation causes downregulation of the expression of Tnf and Il6 and promotion of the survival of BCG in RAW264.7 macrophage cells during the course of infection. (A and B) Inhibition of ERK1/2 activation by U0126 causes decreased expression of Tnf (A) and Il6 (B) in BCG-infected RAW264.7 macrophage cells as examined by quantitative PCR. Macrophage cells were infected for 0–24 h with indicated mycobacterial strains. (C and D) ELISA showed that inhibition of ERK1/2 activation by U0126 decreases the TNF protein (C) and IL-6 protein (D) released by RAW264.7 cells infected as in (A and B). (E) Inhibition of ERK1/2 activation by U0126 promotes survival of BCG in RAW264.7 cells infected as in (A) and (B). Quantitative PCR results are presented relative to expression of the control gene Gapdh (in A and B). Data are representative of at least three independent experiments (mean and SEM in A–E). *p < 0.05, **p < 0.01 (two-tailed unpaired t test).

FIGURE 9. Proposed model for the mechanism of M. tuberculosis Mce3E-mediated host innate immune suppression. Mce3E interacts with and colocalizes with ERK1/2 at the ER apparatus in a DEF motif-dependent manner. Through association with ERK1/2, Mce3E changes the subcellular localization of ERK1/2 from cytoplasm to the ER apparatus, reduces the association of ERK1/2 with MEK1, and blocks the nuclear translocation of phospho-ERK1/2, leading to inhibition of ERK1/2 signaling pathway activation, suppression of Tnf and Il6 expression, and promotion of mycobacterial survival within macrophages.
Mce3E-mediated downregulation of ERK1/2 phosphorylation in host cells. Because Mce3E not only locates at the ER, but also directly and specifically interacts with host ERK1/2 and p-ERK1/2 through a DEF motif, we thus hypothesized that Mce3E might antagonize the interactions between ERK1/2 and MEK1. In addition, through colocalization with p-ERK1/2, Mce3E could also block the nuclear translocation p-ERK1/2, thus further attenuating ERK signaling.

We found that DEF motif-based Mce3E–ERK interaction is required for Mce3E to inhibit ERK1/2 signaling activation, downregulate cytokine expression, and enhance intracellular survival of mycobacteria. Notably, the novel DEF motif identified in M. tuberculosis Mce3E shared conserved residues with typical DEF motifs of molecules from eukaryotic cells. Moreover, consistent to the previous finding that the DEF motif promotes targeting by ERK2 and, to a lesser extent, p38α, but not p38β2 (37), we found that Mce3E displayed DEF motif-based strong binding affinity to ERK2 and little binding affinity to p38α. Such discovery might help to explain the slight, if any, inhibitory effect on infections such as drug regimens, great care could be necessary in targeting the ERK1/2 signaling pathway in host as it may lead to unwanted exacerbation of, or increased susceptibility to, certain bacterial infections such as M. tuberculosis infection. Instead, the DEF motif of M. tuberculosis Mce3E might lead to further identification of similar DEF motifs in mycobacteria and perhaps other pathogenic bacteria. Our results suggest that, although strategies to reduce damaging inflammation and facilitate tissue repair could therefore be helpful in augmenting standard TB treatment, great care could be necessary in targeting the ERK1/2 signaling pathway in host as it may lead to unwanted exacerbation of, or increased susceptibility to, certain bacterial infections such as M. tuberculosis infection. Indeed, the DEF motif of M. tuberculosis Mce3E might provide more efficient and selective target for the development of novel anti-TB therapies.

In summary, this study reveals a previously unknown, yet important, function of M. tuberculosis Mce3E in exploiting the host ERK1/2 signaling pathway to evade host immune defense against mycobacterial infection. We demonstrate that Mce3E localizes at ER, interacts with ERK in a DEF motif-dependent manner, and such interaction traps ERK into the ER and compromises the association between ERK and MEK as well as the nuclear translocation of p-ERK, thus abolishing the activation of ERK. These results suggest that M. tuberculosis Mce3E might exploit the ERK1/2 signaling pathway to suppress host innate immune responses and promote intracellular survival of mycobacteria, providing a novel potential Mce3E–ERK1/2 interface–based drug target against TB diseases.

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Disclosures
The authors have no financial conflicts of interest.

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