Early Secreted Antigenic Target of 6 kDa (ESAT-6) Protein of Mycobacterium tuberculosis Induces Interleukin-8 (IL-8) Expression in Lung Epithelial Cells via Protein Kinase Signaling and Reactive Oxygen Species*

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Background: The role of early secreted antigenic target of 6-kDa in tuberculosis is unclear.

Results: Early secreted antigenic target of 6 kDa induces interleukin-8 in lung epithelial cells via reactive oxygen species and protein kinases and promotes granuloma formation.

Conclusion: Early secreted antigenic target of 6 kDa induces interleukin-8 to promote granuloma formation.

Significance: Induction of interleukin-8 expression by early secreted antigenic target of 6 kDa is a potential mechanism for tuberculosis pathogenesis.

Early secreted antigenic target of 6 kDa (ESAT-6) of Mycobacterium tuberculosis is critical for the virulence and pathogenicity of M. tuberculosis. IL-8, a major chemotactic cytokine for neutrophils and T lymphocytes, plays important roles in the development of lung injury. To further understand the role of ESAT-6 in lung pathology associated with tuberculosis development, we studied the effects of ESAT-6 on the regulation of IL-8 expression in lung epithelial cells. ESAT-6 induced IL-8 expression by increasing IL-8 gene transcription and mRNA stability. ESAT-6 induction of IL-8 promoter activity was dependent on nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) binding and sensitive to pharmacological inhibition of PKC and ERK and p38 MAPK pathways. ESAT-6 activated ERK and p38 MAPK phosphorylation and rapidly induced reactive oxygen species (ROS) production. Dimethylthiourea but not mannitol inhibited IL-8 induction by ESAT-6, further supporting the involvement of ROS in the induction of IL-8 expression. Exposure of mice to ESAT-6 induced localized inflammatory cell aggregate formation with characteristics of early granuloma concomitant with increased keratinocyte chemoattractant CXCL1 staining in bronchiolar and alveolar type II epithelial cells and alveolar macrophages. Our studies have identified a signal transduction pathway involving ROS, PKC, ERK, and p38 MAPKs and NF-κB and AP-1 in the ESAT-6-induced IL-8 expression in lung epithelial cells. This has important implications for the understanding of lung innate immune responses to tuberculosis and the pathogenesis of lung injury in tuberculosis.

Mycobacterium tuberculosis is the causative agent for tuberculosis, and the lung is a primary target for the infection, resulting in respiratory symptoms and chronic lung inflammation. An estimated 2 billion people worldwide are infected with M. tuberculosis, and in 2009 alone, of the 8.8 million people that became ill with tuberculosis, 1.4 million perished. ESAT-6 (early secreted antigenic target of 6 kDa), a protein secreted by M. tuberculosis, is a T cell antigen and a promising vaccine candidate (1, 2). However, it has also been implicated in the virulence and pathogenicity of M. tuberculosis. Based on its ability to bind to laminin and cause lysis of lung epithelial cells, ESAT-6 has been suggested to play roles in the dissemination of M. tuberculosis (3). In addition, ESAT-6 contributes to granuloma formation (4), a hallmark of tuberculosis pathology, through induction of epithelial cell matrix MMP-9 (metalloproteinase-9) in a zebrafish model of tuberculosis. ESAT-6 also regulates cytokine production by immune cells, such as induction of IL-1β secretion by macrophages (5) and inhibition of IFN-γ production by T-cells (6), indicating that it may have important roles in the innate and adaptive immune responses to M. tuberculosis infection.

The respiratory epithelium comprises ~70 m² in area and is vital for the maintenance of alveolar integrity, innate immune defense, and inflammation control through the production of surfactant and cytokines (7–9). IL-8, a member of the CXC chemokine family, serves as a chemoattractant for neutrophils, T-cells, and monocytes and controls trafficking of these cells to the sites of infection (10). Lung epithelial cells produce elevated levels of IL-8 following infection by a variety of respiratory viruses and bacteria (10). High IL-8 levels in the bronchoalveolar lavage fluid were associated with pulmonary tuberculosis (11) and high IL-8 levels in plasma correlated with increased mortality in M. tuberculosis infection (12). Pulmonary epithelial cells were identified as a major source of IL-8 production in response to M. tuberculosis infection (13). These data suggest that elevated IL-8 levels may be responsible for injury to lung architecture commonly seen in pulmonary tuberculosis.

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patients. Infection of A549 lung epithelial cells by *M. tuberculosis* induces IL-8 production (13) that is dependent on reactive oxygen species and mitogen-activated protein kinase activation (14).

Enhanced neutrophil trafficking to sites of infection triggered by elevated IL-8 levels may be involved in the clearance of *M. tuberculosis*, but excess neutrophils may cause tissue damage by release of oxidants and proteases (15) and be detrimental to host protective immunity (16). If mycobacteria are not cleared, neutrophils may actually traffic the bacteria to other sites in the body, aiding in their dissemination (15). Oxidants generated by NADPH oxidase-dependent mechanisms are implicated in the clearance of mycobacteria (17). Considering the importance of IL-8 in innate immune responses to *M. tuberculosis* infection and its role in the development of lung injury, it is important to understand the mechanisms regulating IL-8 expression by *M. tuberculosis*. Although *M. tuberculosis* stimulates lung epithelial cells to produce IL-8 (13, 14), bacterial components responsible for the induction and the underlying mechanisms for IL-8 stimulation are not known. We hypothesized that ESAT-6 is an important modulator of IL-8 expression in lung epithelial cells. In this study, we found that ESAT-6 induced IL-8 levels in lung epithelial cells by increasing gene transcription and IL-8 mRNA stability. ESAT-6 induction of IL-8 expression was sensitive to pharmacological inhibition of protein kinase C and ERK and p38 mitogen-activated protein kinase (MAPK) signaling pathways. ESAT-6 induction of IL-8 expression was associated with the production of reactive oxygen species and inhibited by the hydroxyl radical scavenger dimethylthiourea. Administration of ESAT-6 into lungs of mice produced localized inflammatory cell aggregates concomitant with increased KC staining by lung epithelial cells and macrophages.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—NCI-H441 cells (HTB-174, ATCC), a human lung adenocarcinoma cell line with characteristics of bronchiolar (Clara) epithelial cells, and A549 cells (CCL-185, ATCC), a human lung adenocarcinoma cell line with certain characteristics of alveolar type II cells, were grown on plastic tissue culture dishes in RPMI 1640 and F12K medium, respectively, supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) in a humidified atmosphere of 95% room air and 5% CO₂. Semi-confluent cells were placed in serum-free medium overnight (16–17 h) prior to treatment with ESAT-6.

**Cell Viability**—Cell viability was determined using the CellTiter96AQuenched non-radioactive cell proliferation assay (Promega, Madison, WI). The colorimetric assay measures the reduction of the tetrazolium compound (3-(4,5-di-methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt), which is an indicator of the number of viable cells in culture. Cell death was determined by annexin V staining for apoptotic cells and propidium iodide staining for end stage apoptotic or necrotic cells. Cells were stained with FITC-labeled annexin V and propidium iodide using a kit (BD Biosciences) following the manufacturer’s instructions. The apoptosis and viability of the cells were examined by flow cytometry analysis with FACSCalibur flow cytometer (BD Biosciences), using FlowJo software.

**Materials**—Recombinant ESAT-6 and CFP10 expressed in *Escherichia coli* were purified as described previously (18) and found to contain low LPS (39 pg/mg protein) by a limulus amebocyte assay and to be free of protein aggregates by fast liquid chromatography gel filtration (19). ESAT-6 preparations were essentially free of peptidoglycan by GC-MS/MS analysis. Purified ESAT-6 was prepared in Hanks’ balanced salt solution (HBSS) at 2 mg/ml and stored at −76 °C. Lipofectamine 2000 was from Invitrogen. Protein kinase C inhibitors bisindolylmaleimide, Go6976, and Go6883 and mitogen-activated protein kinase inhibitors PD98059, SB203580, and SP600125 were from Calbiochem or LC Laboratories (Woburn, MA). Luciferase reporter plasmids containing −546/+44 and −133/+44 bp of the IL-8 gene were kindly provided by Dr. Naofumi Mukaida (Cancer Research Institute, Kanazawa University, Kanazawa, Japan). The IL-8 promoter fragments were subcloned into the promoterless pGL3luc(basic) vector (Promega). Antibodies against phosphorylated and total ERK, p38, and JNK mitogen-activated protein kinases and goat anti-rabbit alkaline phosphatase-conjugated secondary antibody were from Cell Signaling (Beverly, MA). Actin antibodies were from Santa Cruz Biotechnology, Inc.

**Plasmids and Transient Transfection**—Plasmids were amplified in *E. coli* Top10 strain (Invitrogen) and purified by anion exchange chromatography (Qiagen, Valencia, CA). Plasmids were transfected into cells along with pcDNA3.1 (Invitrogen), a β-galactosidase expression plasmid, by liposome-mediated DNA transfer using Lipofectamine 2000 according to the manufacturer’s instructions. Luciferase and β-galactosidase activities of cell lysates were determined by chemiluminescent assays (Promega (Madison, WI) and Tropix (Bedford, MA)). Luciferase activities of cell lysates were normalized to cotransfected β-galactosidase activity or protein content to correct for variations in transfection efficiency.

**Site-directed Mutagenesis**—Transcription factor binding sites in the IL-8 promoter were altered by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutated promoter fragments were sequenced to confirm the presence of mutations.

**RNA Isolation and Northern Blotting**—RNA was isolated using TRI- Reagent (Molecular Research Center Inc., Cincinnati, OH) and subjected to Northern blotting analysis according to a method described previously (20). IL-8 mRNA bands were detected with a PhosphorImager, and IL-8 and 18 S rRNA RNA bands were quantified using Quantity One Image Acquisition and Analysis Software (Bio-Rad). IL-8 mRNA levels were normalized to 18 S rRNA levels to correct for variations in the loading and transfer of RNA.

**Enzyme-linked Immunosorbent Assay (ELISA)**—The levels of IL-8 in cell medium were determined by ELISA, using a matched antibody pair according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN).
Fluorescence Microscopy—Cells were grown on Permanox coverslips and then maintained in serum-free medium overnight (16–17 h). Medium was replaced with fresh serum-free medium, and cells were incubated in the presence of 5 or 10 μM dihydroethidium for 1 h in the dark. Afterward, cells were rinsed twice with serum-free medium and incubated with ESAT-6 (5 μg/ml) for various times. Cells were rinsed twice with cold phosphate-buffered saline, and coverslips were air-dried briefly and mounted with Vectashield. Images were captured with a Nikon Eclipse TE2000-5 inverted fluorescent microscope equipped with an UltraVIEW LCI scanning confocal system (PerkinElmer Life Sciences) using 488-nm excitation and 568-nm emission filters. Imaging Suite TM version 5.0 acquisition and processing software was used to acquire the images.

Isolation of Nuclei and Nuclear Run-on Assay—The methods for the isolation of nuclei and run-on transcription assay were as described previously (20, 21). Equal amounts of [32P]-labeled total RNA were hybridized to nitrocellulose membranes containing immobilized linearized plasmids. After washing, radioactivity bound to membranes was quantified by PhosphoImager scanning.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—The methods for nuclear extract isolation (22) and electrophoretic mobility shift assay (23) were as described previously. The protein concentration of nuclear extracts was determined by the Bradford assay. Double-stranded oligonucleotides were end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. The sense strand sequences of IL-8 promoter oligonucleotides (binding sites underlined) were as follows: AP-1, 5'-AGTGTGATGACTCAGGTTTG-3' (−133/−114 bp); NF-κB, 5'-AACCTGGAATTTTCCCTGTA-3' (−84/−65 bp). Protein-DNA complexes were formed by incubating 0.5–1 ng (100,000 cpm) of the labeled oligonucleotide with 5 μg of nuclear protein in 20 μl of binding buffer (13 mM Hepes, pH 7.9, containing 13% glycerol, 80 mM KCl, 5 mM MgCl2, 1 mM DTT, 1 mM EDTA) and 1 μg of poly(dI-dC) as nonspecific competitor DNA at 30 °C for 20 min. The protein-DNA complexes were resolved by non-denaturing polyacrylamide gel electrophoresis and visualized by autoradiography or PhosphorImager scanning.

Western Immunoblotting—Proteins (10–15 μg) were separated by SDS-PAGE on 10% BisTris gels using 3-morpholinopropanesulfonic acid as running buffer and electroblotted to PVDF membranes using an XCell II Mini-Cell apparatus (Novex, San Diego, CA). After blocking with 5% nonfat dry milk in TBS-T buffer, the membranes were incubated with primary antibodies or antiserum at 1:1000 dilution at 4 °C overnight, followed by goat anti-rabbit alkaline phosphatase-conjugated secondary antibody at 1:2000 dilution for 1 h at room temperature. Protein bands were visualized by the enhanced chemiluminescence detection method (GE Healthcare) and quantified using Quantity One Image acquisition and analysis software (Bio-Rad).

Animals—Animal studies were approved by our institutional animal care and use committee. Female C57BL6 mice (6–8 weeks) (Taconic Farms) (n = 5) were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (8.5 mg/kg) and administered 40 μl of HBSS, recombinant ESAT-6, or CFP10 in HBSS via intranasal inhalation. Mice were sacrificed 3 days after administration of ESAT-6 or CFP-10, and lungs were instilled with 5% formaldehyde in phosphate-buffered saline under constant pressure of 20 cm H2O and stored in the fixative for 24 h. Formaldehyde-fixed lungs were embedded in paraffin, and 5-μm sections were cut for immunohistochemical analysis.

Immunohistochemistry—Immunohistochemical detection was performed with a kit (UltraVision Plus Detection System, Thermo Scientific) that uses horseradish peroxidase (HRP) and 3-amo-no-9-ethylcarbazole as a chromogenic substrate. Lung sections were serially incubated with KC polyclonal antibody (0.5 μg/ml) (1:100) (Biovision) or non-immune rabbit IgG, biotin-conjugated goat secondary antibody, and HRP-labeled streptavidin. Afterward, sections were stained with 3-amino-9-ethylcarbazole and counterstained with hematoxylin. Lung sections were also subjected to hematoxylin and eosin staining according to standard procedures.

Statistical Analyses—Data are shown as means ± S.D. or S.E. In experiments in which IL-8 promoter activity/mRNA/protein levels in untreated cells were arbitrarily considered as 100, statistical significance was analyzed by the one-sample t test. One-tailed p values of <0.05 were considered significant. Effects of ESAT-6 and CFP-10 on mouse weight were analyzed by the paired t test, and two-tailed p values of <0.05 were considered significant.

RESULTS

ESAT-6 Induces IL-8 mRNA and Protein Levels in Lung Epithelial Cells—ESAT-6 and its molecular partner, CFP10 (culture filtrate protein of 10 kDa), are encoded by genes located in region of difference 1 (RD1), which is deleted in the attenuated vaccine strain of Mycobacterium bovis bacillus Calmette-Guerin and secreted by the ESX-1 protein secretion system of M. tuberculosis (24) (25). Secretion of ESAT-6 together with CFP10 is associated with the virulence and pathogenicity of M. tuberculosis, and production of IL-8 by lung epithelial cells can contribute to the inflammation that is characteristic of tuberculosis and other lung diseases. Therefore, we studied the effects of ESAT-6 on IL-8 production by H441 lung epithelial cells. H441 lung epithelial cells possess certain morphologic and ultrastructural characteristics of bronchiolar (Clara) epithelial cells and express surfactant proteins A, B, and D, markers of type II and bronchiolar epithelial cells, making them suitable as a model for cells lining the distal lung epithelium.

ESAT-6 but not CFP10 at concentrations ranging from 1 to 5 μg/ml induced significant amounts of IL-8 mRNA compared with untreated cells (Fig. 1). Because ESAT-6 and CFP10 were purified from recombinant E. coli lysates and could contain LPS, we repeated experiments in the presence of polymyxin B, an inhibitor of LPS. Polymyxin B did not alter ESAT-6 stimulation of IL-8 mRNA expression, indicating that this effect is not due to LPS contamination. In separate experiments, polymyxin B (10 μg/ml) blocked LPS (1 μg/ml) induction of TNF-α production by human monocytes (data not shown). Because peptidoglycan is known to induce IL-8 expression, we wanted to know if recombinant ESAT-6 preparations contain peptidogly-
can. GC-MS/MS analysis of muramic acid (a marker of peptidoglycan) levels (HBSS = 1.7 ng/0.5 ml; ESAT-6 = 1.96 ± 0.9 ng/5 μg of ESAT-6 in 0.5 ml of HBSS, n = 3) indicated that recombinant ESAT-6 is essentially free of peptidoglycan. We also found that ESAT-6 induced IL-8 mRNA and IL-8 protein levels in a dose- and time-dependent manner (Fig. 1, C–H). The inductive effect of ESAT-6 was apparent at 1 h of treatment and increased over time, as reflected in the increased IL-8 levels in the cell culture medium. Quantitative RT-PCR analysis in agreement with Northern blotting analysis indicated that ESAT-6 increased IL-8 mRNA levels in a time-dependent manner with significant inductive effects at 1 h of treatment (2.4 – 6-fold compared with control). Similarly ESAT-6 induced IL-8 expression in A549 lung epithelial cells that have certain characteristics of alveolar type II epithelial cells (data not shown).

Collectively, these data indicated that ESAT-6 is a strong inducer of IL-8 expression in lung epithelial cells.

Because ESAT-6 is implicated in the lysis of lung epithelial cells (3, 26) and apoptosis of macrophages (27), we examined the effect of recombinant ESAT-6 on apoptosis and cell death by annexin V and propidium iodide staining. ESAT-6 at a concentration of 5 μg/ml and after 6 h of exposure did not induce cell apoptosis (control = 2.5% ± 0.28, ESAT-6 = 2.75% ± 1.1, n = 3–4) but increased necrotic cell death by ~7% (control = 5.75 ± 0.62%, ESAT-6 = 13.0% ± 1.78, n = 3–4, p < 0.01), suggesting that the observed inductive effects of ESAT-6 on IL-8 expression are specific and not due to toxicity.

ESAT-6 Induces IL-8 Expression by Increasing Gene Transcription and mRNA Stability—We next determined whether ESAT-6 increases IL-8 mRNA levels through increased IL-8
Regulation of IL-8 Expression by M. tuberculosis ESAT-6

FIGURE 2. ESAT-6 increases IL-8 gene transcription and IL-8 mRNA stability. A, H441 cells were treated with or without ESAT-6 (5 ng/ml) for the indicated times, and the IL-8 gene transcription rate was determined by a nuclear run-on assay using isolated nuclei. Actin gene transcription rate was measured as a sample loading control. Data shown are representatives of two independent experiments. B, H441 cells transiently transfected with an IL-8-luciferase promoter plasmid (−546/+44 bp) were treated with or without ESAT-6 (5 ng/ml) for 6 h, and luciferase activity was measured and normalized to total cell protein. Data shown are means ± S.E. (error bars) (n = 4), *p < 0.05 compared with control cells. C and D, H441 cells were treated with or without ESAT-6 (5 ng/ml) for 6 h to induce IL-8 mRNA, and then incubation continued in the presence of actinomycin D (5 μg/ml) to block new transcription. Cells were collected at different times for isolation of RNA, and IL-8 and GAPDH mRNA levels were determined by Northern blotting. Data for GAPDH mRNA levels are not shown. Data shown are means ± S.E. (n = 3).

gene transcription and/or mRNA stability. Using a transcription run-on assay in isolated nuclei, we found that ESAT-6 increased IL-8 gene transcription in a time-dependent manner with significant effect after 6 h (control = 1; ESAT-6 at 3 h = 2.5 ± 0.7; ESAT-6 at 6 h = 6.0 ± 4.0, n = 2) (Fig. 2A). In agreement with these data, ESAT-6 increased IL-8 promoter activity in transient transfection experiments (Fig. 2B). Because mRNA stability plays a significant role in the control of IL-8 mRNA induction by cytokines and other agents (28), we determined if ESAT-6 also increased IL-8 mRNA half-life in H441 cells after blockade of new transcription with actinomycin D. Our experiments to determine IL-8 mRNA half-life by [3H]uridine pulse-chase were not successful. IL-8 mRNA half-life increased from <1 h in control cells to >3 h in ESAT-6-treated cells (Fig. 2, C and D), indicating that enhanced mRNA stability contributes to ESAT-6-mediated increase of IL-8 mRNA levels. GAPDH mRNA stability (half-life in control and ESAT-6-treated cells = 2.5–3 h) was not affected by ESAT-6 treatment.

ESAT-6 Induction of IL-8 Promoter Activity Is Dependent on the Binding of AP-1 and NF-kB—A minimal IL-8 promoter region consisting of −133/+44 bp is necessary and sufficient for the basal and TNF-α induction of IL-8 promoter activity (29). The minimal promoter region contains binding sites for NFIL-6, NF-kB, and AP-1 transcription factors that act independently and synergistically to activate IL-8 promoter in a cell-specific manner in response to stimulatory agents (29). Of these transcription factors, NF-kB and AP-1 play critical roles in the induction of IL-8 promoter activity. We therefore analyzed the effects of ESAT-6 on the binding of NF-kB and AP-1 to IL-8 promoter by an electrophoretic mobility shift assay (Fig. 3A). H441 cells expressed constitutive levels of AP-1 and NF-kB DNA binding activities, and ESAT-6 treatment markedly increased AP-1 DNA binding activity but had only modest effects on NF-kB binding. To assess the roles of AP-1 and NF-kB, we mutated AP-1 and NF-kB binding sites in IL-8 promoter (−546/+44 bp). ESAT-6 increased activities of −546/+44 and −133/+44 bp IL-8 promoter constructs by a similar degree (−2-fold compared with control). Mutations of AP-1 or NF-kB binding sites, individually or in combination, significantly decreased basal and ESAT-6-induced IL-8 promoter activity (Fig. 3B). Mutation of the NF-kB site alone and in combination with AP-1 drastically reduced basal promoter activity and rendered the promoter insensitive to ESAT-6 stimulation. Although mutation of the AP-1 site alone decreased basal promoter activity by greater than 75%, the promoter was sensitive to ESAT-6 induction, albeit to a significantly lesser degree than the wild type promoter. Together, these data indicated that both AP-1 and NF-kB are essential for ESAT-6 induction of the IL-8 promoter.

Protein Kinase C and Mitogen-activated Protein Kinase Signaling Pathways Are Important for ESAT-6 Induction of IL-8 Expression—Protein kinase C (PKC) enzymes are intracellular serine-threonine protein kinases activated by multiple proin-
flamatory stimuli, such as TNF-α, lipopolysaccharide, and oxidants (30). Activation of PKC appears to be one of the key contributing factors for the development of lung injury (31). We hypothesized that ESAT-6 activation of PKC could be involved in the induction of IL-8 expression and tested this with pharmacological inhibitors of PKC. Bisindolylmaleimide and Go6983 inhibited IL-8 mRNA and protein levels by greater than 80% (Fig. 4). The effect of Go6976 on ESAT-6-induced changes was less than that of the other two PKC inhibitors, with more pronounced inhibition of levels of IL-8 mRNA than IL-8 protein (Fig. 4). These data indicated that PKC activation is an important step for the ESAT-6 induction of IL-8 expression.

MAPK signaling cascade couples various cell surface stimuli to cellular responses and are regulated by protein kinase C (32), and ESAT-6 activates p38 MAPK in human lymphocytes (6). We determined if ESAT-6 induction of IL-8 expression was associated with activation of MAPKs and if pharmacological inhibitors of MAPKs affected it. In separate experiments, we evaluated the specificity of MAPK inhibitors at concentrations used in this study by determining their effects on phorbol myristate acetate (10 nm) activation of MAPKs and found that they displayed specificity of inhibition, except that ERK inhibitor PD98059 also inhibited JNK activation (data not shown). PD98059 is known to inhibit JNK MAPK (33). Western blotting analysis showed that ESAT-6 increased ERK and p38 phosphorylation, but the effect on JNK phosphorylation was modest (Fig. 5A). Similarly, inhibitors of ERK and p38 but not JNK MAPK significantly inhibited ESAT-6 induction of IL-8 mRNA and protein levels (Figs. 5, B–D). At a concentration as high as 50 μM, SP600125, a JNK MAPK inhibitor, failed to suppress ESAT-6 induction of IL-8 mRNA levels (data not shown). On the contrary, inhibition of JNK MAPK enhanced IL-8 protein but not mRNA levels (Fig. 5D). These findings indicated that ERK and p38 but not JNK MAPK signal transduction pathways are important for ESAT-6-mediated induction of IL-8 mRNA levels; however, the JNK pathway could contribute to the enhancement of IL-8 protein levels.

**ERK and p38 MAPK Inhibitors Inhibit AP-1 DNA Binding Activity**—We found that pharmacological inhibitors of ERK and p38 but not JNK MAPK suppressed ESAT-6 induction of IL-8 expression. We also found that ESAT-6 increased AP-1 DNA binding activity, and the AP-1 site is important for basal and ESAT-6 induction of IL-8 promoter activity. To determine if ESAT-6 activation of ERK and p38 MAPK pathways control induction of AP-1 activity, we tested the effects of pharmacological inhibitors on ESAT-6 induction of AP-1 DNA binding activity. We found that ERK inhibitor PD98059 and p38 inhibitor SB203580 reduced basal and ESAT-6-induced AP-1 activity, whereas JNK inhibitor SP600125 modestly inhibited induction of AP-1 activity (Fig. 6). These data indicated that ERK and p38 but not JNK MAPK pathways control the ESAT-6-dependent increase of AP-1 activity to induce IL-8 expression.

**Intracellular Oxidants Mediate ESAT-6 Induction of IL-8 Expression**—The intracellular redox state controls the activation of AP-1 and NF-κB to regulate expression of a wide variety of genes involved in inflammatory responses (34). Because ESAT-6 induction of IL-8 was associated with increases in AP-1 and NF-κB binding activities, and AP-1 and NF-κB are necessary for the induction of IL-8 promoter activity, we hypothesized that intracellular oxidants generated in response to ESAT-6 exposure may be involved in the induction of IL-8 expression. We tested the effects of hydroxyl radical scavengers on ESAT-6 induction of IL-8 expression and found that dimethylthiourea inhibited IL-8 expression, but mannitol did not (Fig. 7). Due to cell impermeability, mannitol can only scavenge extracellular hydroxyl radicals, whereas dimethylthiourea can also neutralize intracellular hydroxyl radicals. To obtain further evidence for the generation of oxidants in cells exposed to ESAT-6, we performed direct imaging of reactive oxygen species (ROS) production by confocal microscopy, using dihydroethidium (35), which specifically reacts with superoxide anion to form the red fluorescent product, 2-hydroethidium, that intercalates with DNA. Exposure of cells to ESAT-6 rapidly induced superoxide generation as visualized by red fluorescence, whereas control cells showed very low or undetectable fluorescence (Fig. 8).

**ESAT-6 Induces Acute Lung Inflammation and Early Granuloma Formation in Mice**—Our *in vitro* cell culture experiments demonstrated that ESAT-6 is a potent inducer of IL-8 expression. Because IL-8 is a chemoattractant for neutrophils, T-cells, and monocytes, we determined the effects of ESAT-6 on lung inflammatory responses in mice. There appears to be no information on the physiologic levels of ESAT-6. We used ESAT-6 in the range of 2.5–40 μg/mouse based on our *in vitro* cell culture data. Recombinant ESAT-6 or CFP-10 in HBSS or HBSS alone was administered into mouse lungs via the intranasal route, and lung histology and KC expression were determined by hematoxylin and eosin staining and immunostaining, respectively, after 3 days. Results indicated that ESAT-6, but
not CFP-10 at a dose of 40 μg/mouse caused acute lung inflammation, as indicated by the presence of localized inflammatory cell aggregates (Fig. 9A). ESAT-6 at lower doses (2.5 and 10 μg) caused considerably less inflammation, as indicated by the presence of fewer inflammatory cell aggregates in the lung (data not shown). Inflammatory cell aggregates in lungs were found in all mice (n = 5) exposed to ESAT-6 but not CFP-10. Immunostaining demonstrated that ESAT-6, but not CFP-10, significantly increased KC staining in alveolar type II and bronchiolar epithelial cells and macrophages (Fig. 9B), indicating increased KC expression in vivo by these cells. Inflammatory cell aggregates appeared to contain macrophages and lymphocytes with the appearance of epithelioid cells, suggesting the identity of cell aggregates as early stage granuloma. We found that mice that received 40 μg of ESAT-6, but not CFP-10, suffered weight loss by ~11% after 3 days (Table 1).

DISCUSSION

ESAT-6 is associated with the virulence and pathogenicity of M. tuberculosis (36–38). Recently, ESAT-6 was demonstrated to activate the inflammasome (5) and promote apoptosis and lysis (3) of lung epithelial cells. The lung epithelium contributes to the initiation and amplification of innate and inflammatory responses to various insults (8, 39, 40). IL-8 plays a key role in mediating inflammatory responses and is implicated in the pathogenesis of acute and chronic lung diseases (10). In this study, we found that ESAT-6 induced IL-8 mRNA and protein levels in A549 and H441 lung epithelial cells in a time- and concentration-dependent manner. Interestingly, CFP-10 that is secreted with ESAT-6 and functions as its molecular partner (41) had no effect on IL-8 expression. The inductive effect of ESAT-6 was not sensitive to polymyxin B, indicating that the effects are indeed due to ESAT-6 per se and not due to trace amounts of lipopolysaccharide that may be present in recombinant ESAT-6 preparations. ESAT-6 induction of IL-8 expression cannot be due to peptidoglycan contamination because ESAT-6 preparations were essentially free of peptidoglycan.
ESAT-6 increased the IL-8 gene transcription rate and IL-8 mRNA stability, indicating that both transcriptional and mRNA stability mechanisms control IL-8 induction. The importance of transcriptional mechanisms is further supported by the stimulatory effect of ESAT-6 on IL-8 promoter activity. Electrophoretic mobility shift experiments demonstrated that H441 cells express constitutive binding activities for AP-1 and NF-κB with the NF-κB binding activity present at a lower level than that of AP-1. ESAT-6 treatment increased AP-1 DNA binding activity, but its stimulatory effect on NF-κB binding activity was modest. Consistent with the functional roles for AP-1 and NF-κB in IL-8 induction, mutations of AP-1 and NF-κB reduced basal and ESAT-6-induced IL-8 promoter activity. These data also indicated that AP-1 and NF-κB function in a combinatorial/cooperative manner to induce IL-8 promoter activity in response to ESAT-6 stimulation. A relatively short sequence of −133/+41 bp of the 5′-flanking region is necessary and sufficient for the basal activity and stimulus-specific induction of IL-8 promoter activity (29). Transcription factors AP-1, NF-κB, and NFIL-6 function independently and synergistically to activate IL-8 promoter activity in a stimulus- and cell type-specific manner (29). Although NF-κB has been suggested to be essential for IL-8 induction, there are exceptions, as in the case of H2O2 induction of IL-8 in A549 cells (42). Our data demonstrating a modest increase in NF-κB binding activity in ESAT-6-treated cells suggested that IL-8 induction could occur independently of NF-κB. However, NF-κB is essential for basal promoter activity. AP-1 and NFIL-6 are known to synergize with NF-κB for maximal induction of IL-8 (29).

We also found that ESAT-6 treatment increased the half-life of IL-8 mRNA, indicating that enhanced mRNA stability contributes to the induction of IL-8 levels. Control of IL-8 mRNA degradation is an important mechanism for the regulation of
IL-8 levels, and the p38 MAPK pathway and AU-rich elements located in the 3′-untranslated region have been found to be involved in the control of IL-8 mRNA stability (43). Activation of p38 MAPK by ESAT-6 could suggest a role for the p38 MAPK signaling pathway in the stabilization of IL-8 mRNA to increase IL-8 expression. A variety of agents, such as nitric oxide (44, 45), adenovirus (46), and Shiga toxin (47), increase IL-8 mRNA stability to increase IL-8 mRNA levels in epithelial cells, fibroblasts, and monocytic cells.

ESAT-6 associates with lung epithelial cells by binding to laminin (3) and inhibits IL-12 production in macrophages via TLR signaling by binding to TLR2 (48). The identities of ESAT-6-interacting protein(s) on lung epithelial cells and the ensuing signaling events are not known. We investigated the potential roles of signal transduction pathways involved in the ESAT-6 induction of IL-8 expression. We found that PKC inhibitors reduced IL-8 induction, indicating that ESAT-6 activation of PKC is a key step in the induction of IL-8 expression. Sensitivity to inhibition by bisindolylmaleimide, Go6976, and Go6983 indicated that calcium-dependent conventional protein kinase C enzymes are involved. Sensitivity to Go6976 indicated that the novel and calcium-independent PKCδ enzymes might also be involved. Activation of the PKC signaling pathway has been linked to the development of acute and chronic lung diseases, including those that are caused by a variety of environmental and infectious agents (30). Inhalation of occupational dusts, such as asbestos, silica, and allergens present in home and occupational environments, activate PKC signaling that probably contributes to the development of lung diseases (30). Activation or down-regulation of PKC has been implicated in the host inflammatory responses to M. tuberculosis infection (49) or survival of mycobacteria (50), respectively. Mycobacterial cord factor trehalose 6-monomycolate activated protein kinase C enzymes are involved. Sensitivity to Go6976 indicated that the novel and calcium-independent PKCδ might also be involved.

Our data demonstrated that ESAT-6 activated ERK and p38 and, to a lesser degree, JNK phosphorylation, and pharmacological inhibitors of ERK and p38 but not JNK MAPKs inhibited IL-8 induction, indicating that ERK and p38 MAPK activations are necessary for IL-8 induction. Although additional studies are required to map the exact sequence of signaling pathways, it is likely that PKC activation precedes MAPK activation. Previous studies have shown that activation of PKC by agonists and other agents mediates activation of MAPK. Induction of MUC5Ac by phorbol myristate acetate, a PKC agonist, was mediated via activation of MEK and ERK MAPK signaling pathways (53). Activation of p38 MAPK in epithelial cells is mediated via Src and PKC signaling pathways (54). Lipopolysaccharide induction of c-Jun and c-Fos in tracheal smooth muscle cells was mediated by PKC via activation of ERK, p38, and JNK MAPKs (55). The identities of PKC enzymes involved in the ESAT-6 induction of IL-8 expression remains to be investigated.

We found that ESAT-6-induced IL-8 expression requires ERK and p38 activation and is linked to an increase of AP-1 DNA binding activity. Although the JNK MAPK pathway is a known activator of AP-1, ERK and p38 MAPK pathways also regulate AP-1 activation via control of expression and activations of c-Jun and c-Fos (56–58). The involvement of a particular MAPK signaling pathway(s) in IL-8 induction appears to be dependent on the cell type and the stimulus. Apart from JNK, ERK, and p38 MAPK pathways also play major roles in the regulation of IL-8 expression. Cadmium induction of IL-8 expression in airway epithelial cells is mediated by an NF-κB-independent but ERK MAPK-dependent pathway (59). JNK and ERK MAPK were found to control IL-8 induction by YKL-40, a chitinase-like glycoprotein, in bronchial epithelial cells (60). In U937 monocytic cells, Helicobacter pylori induces IL-8 expression primarily via the p38 MAPK pathway and NF-κB activation (61). Our studies indicated that ERK and p38 but not JNK MAPK pathways are required for an increase in AP-1 DNA binding to the IL-8 promoter. Mechanisms underlying ERK and p38 MAPK-mediated increase of AP-1 DNA binding activity remain to be investigated. H441 cells express c-Jun, c-Fos, Jun B, Jun D, Fru 1, and Fru 2 (23, 62), indicating that the AP-1 complex on the IL-8 promoter could be composed of combinations of these proteins. Inhibition of ESAT-6 increase of AP-1 DNA binding activity by PD 98059 and SB 203580 could be due to inhibition of expression and/or activities of one or more components of the AP-1 complex. The identities of AP-1 components targeted by PD 98059 and SB 203580 are not known. In mouse macrophages, H. pylori induces phosphorylation of c-Fos and formation of the AP-1 complex via ERK MAPK activation to induce expression of c-Myc (63). We found that inhibition of the JNK MAPK pathway did not suppress ESAT-6 induction of IL-8 levels. On the contrary, JNK MAPK inhibition enhanced ESAT-6 induction of IL-8 protein but not mRNA levels, suggesting that the effects are exerted at the posttranslational level. Inhibition of the JNK MAPK pathway induces IL-8 levels via enhanced stabilization of IL-8 mRNA in cystic fibrosis lung epithelial cells expressing high levels of tristetrapolin (64).

We found that ESAT-6 rapidly induced superoxide anion (O2∙−) production in cells, as demonstrated by dihydroethidium fluorescence. The associations between PKC and MAPK activation and ROS production indicate an intracellular signaling pathway that mediates ESAT-6 induction of IL-8 expression, although the exact sequence of the signaling events remains to be determined. ROS are composed of partially reduced metabolites of oxygen, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, and are produced intracellularly as byproducts of normal aerobic metabolism by the mitochondrial electron transport chain (65). Elevated levels of ROS can also be produced as a consequence of exposure to environmental agents, or under pathological conditions, by the actions of enzymes, such as NADPH oxidase, 5-lipooxygenase, xanthine oxidase, and nitric-oxide synthases (66). ROS serve as important second messengers to control a wide range of physiological and pathological processes (66). Elevated ROS activate PKC and MAPK signaling pathways to target NF-κB (67) and AP-1 (68) transcription factor pathways to ultimately alter target gene expression. The major source for ROS and its role in the...
initiation of signal transduction to ultimately induce IL-8 expression in lung epithelial cells exposed to ESAT-6 remains to be determined.

The roles of intracellular ROS in mediating inflammatory responses to mycobacterial infection are not well understood. Recent studies have demonstrated that M. tuberculosis elicits inflammatory responses in monocytes/macrophages (69) and lung epithelial cells (70) via Toll-like receptor 2 and ROS-mediated activation of MAPK signaling pathways. Specifically, tuberculin purified protein derivative was found to elicit inflammatory responses in monocytes/macrophages via the TLR2-ROS-MAPK signaling pathway (69). Our studies have demonstrated that ESAT-6 is another mycobacterial protein that causes elevated intracellular ROS to induce IL-8 levels. The ability of ESAT-6 to induce IL-8 levels suggests that it could promote initiation of granuloma formation via IL-8-mediated recruitment of T cells, neutrophils, and monocytes. Furthermore, elevated IL-8 can induce release of matrix metalloproteinase-9 from neutrophils (71) for the recruitment of macrophages to contribute to nascent granuloma maturation. ESAT-6 induces epithelial cell matrix MMP-9 to recruit macrophages to promote granuloma formation in a zebrafish model of Mycobacterium marinum infection (4). Administration of neutralizing anti-IL-8 antibody suppressed the development of tuberculin skin reaction by reducing the number of neutrophils and lymphocytes at the injection site in rabbits, indicating that IL-8 is important for granuloma formation (72). ESAT-6 could also synergize cytokine-induced IL-8 production at later stages of infection to contribute to lung injury commonly seen in pulmonary tuberculosis.

ESAT-6 promoted the formation of inflammatory cell aggregates in lungs of mice concomitant with increased KC staining in macrophages and bronchiolar and alveolar type II epithelial cells. Cells in the aggregates had the appearance of epithelioid cells, indicating the identity of cell aggregates as early stage granuloma. Results of animal experiments, namely induction of KC in bronchiolar and alveolar type II epithelial cells and infiltration of inflammatory cells into lungs of mice exposed to ESAT-6, are consistent with the inductive effects of ESAT-6 on IL-8 levels in lung cells in vitro. Consistent with the lack of effects of CFP-10 on IL-8 levels in lung cells in vitro, CFP-10 did not significantly induce KC immunostaining or promote inflammatory cell infiltration into lungs of mice. Together, our in vitro cell culture and in vivo mouse studies indicated that ESAT-6 interacts with lung alveolar type II and bronchiolar epithelial cells to induce IL-8 production, which in turn promotes trafficking of macrophages and other cells to initiate granuloma formation in the lung. Results also demonstrated that ESAT-6 increased KC staining in macrophages, suggesting that increased KC expression by alveolar macrophages could also contribute to trafficking of macrophages and lymphocytes to initiate granuloma formation in lung. Our studies have identified ESAT-6-mediated IL-8 increase in alveolar type II and bronchiolar epithelial cells as a potential pathway for granuloma formation in M. tuberculosis infection, adding to the importance of epithelial cells in the pathogenesis of tuberculosis. Previously, ESAT-6 induction of MMP-9 in epithelial cells was shown to promote granuloma formation in zebrafish (4). In conclusion, our studies have demonstrated that ESAT-6 induces IL-8 expression in H441 lung epithelial cells by increasing gene transcription and mRNA stability. IL-8 induction was dependent on NF-κB and AP-1 binding and sensitive to PKC and ERK and p38 MAPK inhibitors. ESAT-6 rapidly induced ROS production in cells, and IL-8 induction was inhibited by the hydroxyl radical scavenger dimethylthiourea. Our results have identified a signaling mechanism involving intracellular ROS, PKC, ERK and p38 MAPK, and AP-1/NF-κB pathways for ESAT-6 induction of IL-8; however, the exact sequence of signaling events remains to be elucidated. In agreement with results of our in vitro cell culture studies, ESAT-6 increased KC, a mouse homolog of IL-8, in lung epithelial cells and macrophages concomitant with inflammatory cell aggregate or granuloma formation in mice. ESAT-6 induction of IL-8 could be one of the mechanisms for granuloma formation and lung innate immune responses to tuberculosis and a contributing factor to the pathogenesis of pulmonary tuberculosis.

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