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Activation of the Mitogen-Activated Protein Kinase Signaling Pathway Is Instrumental in Determining the Ability of Mycobacterium avium to Grow in Murine Macrophages

Hubert M. Tse,* Steven I. Josephy,⁎ Edward D. Chan,†‡ Darren Fouts,* and Andrea M. Cooper2*

Of the two common morphotypes of Mycobacterium avium, designated smooth transparent (SmT) or smooth opaque (SmO), the SmO morphotype is avirulent, whereas the SmT morphotype is virulent. The role of the host macrophage in determining these different virulence phenotypes was analyzed using an in vitro model of macrophage infection. Initial studies confirmed previous reports of the increased ability of the SmT bacteria to grow in macrophages; this increased virulence correlated with reduced induction of inflammatory cytokines. Examination of the response of the mitogen-activated protein kinase (MAPK) pathway following infection with either morphotype revealed that all three members of the MAPK pathway were activated. Pharmacologic inhibition of either the extracellular signal-regulated kinase (ERK) or p38MAPK pathways resulted in distinct consequences for the growth of the two morphotypes. In particular, inhibition of the p38MAPK resulted in attenuated growth of the SmT morphotype, which correlated with reduced PGE2 production. Inhibition of cyclooxygenase 2 by indomethacin also inhibited growth of SmT, substantiating the role for PGE2 in promoting the growth of SmT. In contrast, SmO induction of the ERK pathway was increased compared with the SmT morphotype, and inhibition of ERK resulted in decreased TNF-α synthesis and enhanced SmO growth. Pharmacologic inhibitors of the MAPK pathway were present for only the first 4 h of infection and yet had consequences for bacterial growth at 7 days. Therefore, the data suggest that induction of the MAPK pathway during uptake of bacteria is instrumental in determining the eventual fate of the bacteria.

The facultative intracellular pathogen Mycobacterium avium causes opportunistic infections in AIDS patients and in other susceptible hosts such as individuals with emphysema (1–4). M. avium can exhibit a variety of colony morphotypes when grown on agar, with the predominant morphotypes being either smooth transparent (SmT) or smooth opaque (SmO) in nature (5, 6). The SmT morphotype typically has greater virulence, antibiotic resistance, and prevalence of dissemination than the SmO morphotype (7–9). Although there are subtle differences in the nature of the major surface glycopeptidolipid and in certain proteins between the SmT and SmO morphotypes, the basis for the difference in virulence is not currently fully defined (10–13).

The clinical observations regarding virulence of the morphotypes have been supported by studies using the SmT and SmO morphotypes in in vivo infection models of mice (7). The virulent SmT morphotype grows progressively for some time before being limited, but not cleared, by acquired cellular responses. In contrast, the isogenic avirulent SmO morphotype is rapidly cleared from the infected animal (7, 14). The mechanisms underlying the differences in virulence between these two morphotypes have not been defined either at the level of the host response or by the identification of M. avium virulence factors. However, previous studies have identified the cytokine TNF-α as being differentially induced and differentially protective during infection with the two morphotypes (15–18). Induction of other cytokines by the two morphotypes is also different, with the SmT bacteria being less stimulatory (19, 20). This reduced stimulatory activity of the SmT morphotype is a potential virulence factor and may result from modulation of macrophage receptor-mediated stimulation by molecules unique to the SmT morphotype.

The initial interaction between host macrophages and bacteria results in the induction of intracellular signaling pathways that connect receptor-mediated events to transcriptional responses within the nucleus. In eukaryotic cells, an important group of signaling pathways is the mitogen-activated protein kinase (MAPK) signaling cascades. MAPKs are comprised of three principal serine/threonine kinases with multiple subisoforms within each member: p38MAPK with subisoforms α, β, γ, and δ; extracellular signal-regulated kinase (ERK) 1/2 with p44 (ERK1) and p42 subisoforms; and c-Jun NH2-terminal kinase (JNK) or stress-activated protein kinase with two principal subisoforms, p46 JNK1 and p54 JNK2. Previous studies have implicated the MAPKs in bacterial pathogenesis as evidenced by their induction or inhibition by Salmonella typhimurium (21), Yersinia sp. (22–24), and Listeria monocytogenes (25, 26).

We hypothesized that the virulence of the SmT morphotype was linked to events occurring during the induction of macrophage activation and that modulation of that activation would reduce the virulence of the SmT bacteria. To test these hypotheses, we chose...
to examine the activation of the MAPK pathway by the clinical isolate of *M. avium* designated 2151 (serovar 2), which exists as stable SmT and SmO morphotypes (27). These morphotypes exhibit the expected virulence phenotypes in vivo (A. M. Cooper, unpublished observation). We observed that both the SmO and the SmT morphotypes of *M. avium* activated the principal components of the MAPK pathway. However, pharmacological inhibition of the p38\(^{MAPK}\) resulted in reduced virulence of the SmT morphotype, whereas inhibition of the ERK pathway resulted in slightly enhanced growth of the avirulent SmO morphotype.

### Materials and Methods

**Materials**

Female C57BL/6 mice 6–8 wk in age were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used as a source for bone marrow-derived macrophages. Phospho-p38\(^{MAPK}\) (Thr\(^{180}\)/Tyr\(^{182}\)), phospho-JNK (Thr\(^{183}\)/Tyr\(^{185}\)), phospho-Akt (Ser\(^{473}\)), phospho-Akt (Thr\(^{308}\)), and Akt Abs were purchased from Cell Signaling (Beverly, MA). Phospho-ERK (E-4), JNK1 (F-3), ERK1 (K-23), and p38\(^{MAPK}\) (N-20) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IL-10, anti-mouse TNF-\(\alpha\), and rat isotype IgG1 control Abs were purchased from BD Pharmingen (San Diego, CA). SB203580, PD98059, and indomethacin were from Calbiochem (La Jolla, CA).

**Isolation of mouse bone marrow-derived macrophages**

Bone marrow-derived macrophages were cultured as described previously (28) using complete media comprised of DMEM supplemented with 10% heat-inactivated FCS, 10% L-929 cell-conditioned medium, 10 mM HEPES buffer, 2 mM L-glutamine, 2X nonessential amino acids, and 1% penicillin/streptomycin/amphotericin B (complete DMEM; Sigma-Aldrich, St. Louis, MO). Six- and 24-well tissue culture plates were initially seeded with \(4 \times 10^6\) cells/well and \(1 \times 10^6\) cells/well, respectively, and incubated at 37°C in a 5% CO\(_2\) humidified air chamber for 24 h. The following day, the macrophages were supplemented with an additional 4 or 1 ml/well of complete DMEM for the 6- or 24-well plates, respectively. The cultures were incubated for a total of 7–10 days at 37°C in a 5% CO\(_2\) humidified air chamber with a change of fresh complete DMEM every 2 days. Twenty-four hours before infection, the macrophages were washed twice with PBS and then replated with incomplete DMEM (complete DMEM lacking 10% L-929 conditioned medium and antibiotics).

**Growth and isolation of *M. avium* strains**

*M. avium* 2151 was originally isolated from an AIDS patient, and both the SmT and SmO morphotypes were used. The morphotypes were picked from 7H11 agar (Difco, Detroit, MI) plates supplemented with Middlebrook oleic acid dextrose complex and were expanded on similar plates at 37°C for 10–14 days. The cells were harvested by scraping the plates with a sterile cotton swab stick and they were resuspended in 7H9 broth containing 0.04% Tween 80. The cell suspension was dispersed of bacterial clumps by stirring with a flea bar at room temperature for several hours and it was then frozen at –70°C in 2-ml aliquots. The concentration and morphotypes of the frozen stocks were confirmed by performing serial dilutions on 7H11 agar quad plates containing oleic acid dextrose complex and they were then incubated at 37°C for 10–14 days.

**In vitro infection of bone marrow-derived macrophages**

Macrophages in 6- and 24-well plates were infected with a suspension of *M. avium* 2151 SmT or SmO in incomplete DMEM at a multiplicity of infection (MOI) of 2:1. In certain wells, macrophages were first pretreated with 1, 10, or 30 \(\mu\)M SB203580 or PD98059, or 1, 10, or 20 \(\mu\)M indomethacin for 60 min before infection. To serve as a control, the volume of the diluent DMSO contained in 30 \(\mu\)M SB203580 or PD98059 was added to the cell culture. Twenty-four-well macrophage cultures were infected for 4 h at 37°C, washed five times with PBS to remove nonadherent bacteria, and then replanted with 1 ml of incomplete DMEM per well (no inhibitors were added following the initial 4-h infection period). After this initial uptake period (4 h), the number of phagocytosed bacteria was determined by lysing macrophages using osmotic shock and plating the lysate on 7H11 agar at 37°C. The number of bacteria phagocytosed by macrophages over the 4 h of incubation was reproducible and was not altered by the presence of inhibitors (data not shown). The bacterial count made after 4 h of incubation is shown as \(t = 0\) on the graphs. That bacteria were being phagocytosed was confirmed by fluorescent microscopy using the TB Fluorescent Stain kit M (BD Biosciences Microbiology Systems, Sparks, MD; data not shown). Macrophages were subsequently lysed at days 3 and 7 postinfection to quantitate the growth of the bacteria. Due to the low MOI, macrophages were not susceptible to apoptosis following infection, nor did they exhibit the expected virulence phenotypes in vivo (A. M. Cooper, unpublished observation). We observed that both the SmO and the SmT morphotypes of *M. avium* activated the principal components of the MAPK pathway. However, pharmacological inhibition of the p38\(^{MAPK}\) resulted in reduced virulence of the SmT morphotype, whereas inhibition of the ERK pathway resulted in slightly enhanced growth of the avirulent SmO morphotype.

**Preparation of cell lysates**

Bone marrow-derived macrophages prepared as above and cultured in six-well tissue culture plates were infected with *M. avium* 2151 SmT or SmO (MOI of 2:1). At various time points immediately postinoculation, the macrophages were washed with ice-cold PBS and cellular extracts were harvested by using a lysis buffer containing 50 mM Tris-base, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM NaF, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 2 mM NaVO\(_4\), and 1 mM PMSF, all purchased from Sigma-Aldrich. After the addition of 500 \(\mu\)l of lysis buffer per well, the macrophages were incubated on ice for 10 min, scraped, and transferred to a 1.5-ml centrifuge tube. Nuclei were spun down in an Allegra 21R refrigerated microcentrifuge (Beckman Coulter, Palo Alto, CA) at 17,000 \(\times\) g for 10 min at 4°C and the supernatant (whole cell lysate) was collected. The protein concentration of the whole cell lysate was determined by the bichoninic acid protein assay according to the manufacturer’s instructions (Fierce, Rockford, IL).

**Western immunoblotting**

For total p38\(^{MAPK}\), JNK, ERK, and Akt Abs purchased from Cell Signaling. The membranes were incubated for a total of 4 h at room temperature. The membranes were incubated overnight at 4°C with anti-p38\(^{MAPK}\) Ab or anti-phospho-JNK, anti-phospho-ERK, or anti-phospho-Akt in 5% BSA in PBST for 1 h at room temperature. The membranes were washed four times with PBST for 5 min each in PBST and then blocked with either anti-rabbit or anti-mouse IgG HRP-conjugated Abs (Santa Cruz Biotechnology) in PBST for 1 h at room temperature. Chemiluminescence was detected using the ECL plus reagent (Amersham Pharmacia Biotech, Little Chalfont, U.K.) according to the manufacturer’s recommended protocol.

Western immunoblotting for total p38\(^{MAPK}\), ERK, JNK, or Akt was performed on the same nitrocellulose membranes that had been probed with the respective-Ab. After washing with PBS, the membranes were then washed four times with PBST for 5 min and were incubated with either anti-rabbit or anti-mouse IgG HRP-conjugated Abs (Santa Cruz Biotechnology) in PBST for 1 h at room temperature. Chemiluminescence was detected using the ECL plus reagent and was performed according to the manufacturer’s protocol.

**Anti-IL-10 and anti-TNF-\(\alpha\) treatment of infected macrophages**

Bone marrow-derived macrophages were preincubated with 10 \(\mu\)g/ml anti-mouse IL-10 or 5 \(\mu\)g/ml anti-mouse TNF-\(\alpha\) Abs for 30 min before infection with *M. avium* 2151 SmT or SmO as described above. After the 4-h incubation, macrophages were washed three times with PBS and were then replanted with antibiotic-free DMEM without the anti-mouse IL-10 or anti-mouse TNF-\(\alpha\) Abs. The number of viable bacteria was determined at 4 h and 3 and 7 days postinfection by lysing infected macrophages by osmotic shock. The cell lysate containing viable bacteria was diluted and plated onto 7H11 quad plates as described above.

**ELISAs**

TNF-\(\alpha\) and PGE\(_2\) produced in the supernatants from infected macrophages were measured using commercial ELISA kits from R&D Systems (Minneapolis, MN) and Cayman Chemicals (Ann Arbor, MI), respectively. IL-6 and IL-12p40 ELISAs were performed according to a standard protocol from BD Pharmingen. Streptavidin-conjugated HRP (Zymed Laboratories, San Francisco, CA) was used at a final dilution of 1/4000, and tetramethylbenzidine was used according to the manufacturer’s protocol (DAKO, Carpenteria, CA). ELISA plates were analyzed for absorbance at 450 nm.

\(826\) MAPK ACTIVATION IN *M. avium* COMPLEX INFECTION

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Nitrite assay

NO$_2^-$ production was measured by adding 50 µl of Greiss reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 50 µl of supernatant obtained from M. avium-infected macrophage culture as described previously (29). The NO$_2^-$ concentration was determined by comparing the optical density at 550 nm with a standard curve generated from various known concentrations of sodium nitrite dissolved in culture medium.

Statistical analysis

Determination of the difference between mean values for each experimental group were assessed by the Student t test, with p < 0.05 considered significant. All experiments were performed at least three separate times with data obtained in duplicate wells in each experiment.

Results

The SmT morphotype of M. avium can proliferate within macrophages, whereas the SmO morphotype can merely persist

The growth phenotype of the morphotypes derived from M. avium strain 2151 has not previously been described; therefore, bacteria were added to macrophages and internalization and growth were determined. As shown in Fig. 1, both SmT and SmO were equally infective (t = 0). Following 3 days in culture, the SmO bacteria had reduced in number slightly, whereas the SmT bacteria persisted at levels equal to the initial infection. By 7 days of infection, the phenotype was more defined, with the SmT exhibiting a 10-fold increase in number compared with an absence of growth for the SmO bacteria.

Exposure of macrophages to bacteria of the SmO morphotype resulted in pronounced production of the cytokines IL-12p40 and IL-6

To determine whether the SmT and SmO M. avium 2151 morphotypes were inducing different levels of macrophage activation following infection, the production of cytokines and NO was followed over time. As expected, SmO-infected macrophages induced higher levels of IL-6, IL-12p40, and reactive nitrogen intermediates when compared with SmT-infected macrophages (Fig. 2, A–C). However, it is important to note that IL-12p40, IL-6, and TNF-α were all induced by the SmT infection (Fig. 2).

TNF-α was induced to comparable levels by both morphotypes during the first 4 h of infection (Fig. 2D). After the wash to remove extracellular bacteria at 4 h, little to no TNF-α could be detected in response to either morphotype (data not shown). This observation may relate to the reportedly reduced ability of this particular strain to induce secreted TNF-α (16, 18).

Although both morphotypes could induce macrophage activation, the observed differences may reflect altered stimulation of the signal transduction pathways involved in macrophage activation.

p38$^{\text{MAPK}}$, ERK, and JNK are phosphorylated following interaction of macrophages with either SmT or SmO morphotypes

The MAPK signaling pathways have been implicated in mediating the expression of a wide variety of cytokines in cells stimulated with bacterial products (30). To compare the ability of the SmT and SmO morphotypes to induce macrophage MAPKs, activation of p38$^{\text{MAPK}}$, ERK, and JNK was analyzed directly upon infection. Western blot analysis of cell lysates using Abs specific for the activated, (i.e., phosphorylated) forms of the MAPKs proteins revealed that all three MAPKs were activated as early as 15 min following infection (Fig. 3, A–C). Interestingly, the SmO-infected macrophages demonstrated an ~3-fold increase in phosphorylation of ERK in comparison with SmT-infected macrophages at 60 and 120 min postinfection (Fig. 3B). Fig. 3D shows that the increased phosphorylation of the ERK protein seen in the SmO-stimulated cells translates into increased ERK activity. In contrast, the level and kinetics of p38$^{\text{MAPK}}$ and JNK1/2 phosphorylation...
A–C, JNK (C) between the SmT or SmO morphotypes after 15, 30, or 60 min were separated by SDS-PAGE and immunoblotted with phospho-specific Abs to p38 MAPK. All three MAPKs were activated following infection or with (100 ng/ml) LPS treatment with no observable difference in the levels of phospho-p38 MAPK, ERK, and JNK. The increased ERK activation by the SmO morphotype suggested that the bacteria are, unlike the SmO bacteria, resistant to the effects of TNF-α-mediated macrophage activation. Conversely, SmT bacteria may induce TNF-α by impurity due to their ability to down-regulate macrophage activation directly. This possibility is addressed in the following section.

**Induction of the p38MAPK pathway is a requirement for the optimal growth of SmT bacteria**

The observed difference in cytokine induction by the two morphotypes (Fig. 2) suggested that although SmT bacteria are capable of activating the macrophage, they may also induce other molecules capable of moderating macrophage activation. To address whether

![Image](http://www.jimmunol.org/)
In addition, in macrophages all concentrations of SB203580 tested as determined by an MTT-formazan assay (data not shown). Inhibition of p38MAPK, was used. As shown in Fig. 5 (top), a significant decrease in SmT growth was observed at 7 days postinfection as a result of preincubation of the macrophages with SB203580. This occurred despite the fact that SB203580 was washed away following the initial 4-h treatment. The decrease in SmT growth was not due to SB203580 toxicity, as macrophages were viable at all concentrations of SB203580 tested as determined by an MTT-formazan assay (data not shown). In addition, in macrophages treated with SB203580, there was little or no effect on SmO survival (Fig. 5, bottom).

That the SmO bacteria were unable to take advantage of the inhibition of p38MAPK suggests that there are other antibacterial mechanisms active against the SmO bacteria that do not affect the SmT bacteria.

**FIGURE 5. Inhibition of p38MAPK enhances killing of M. avium 2151 SmT.** Macrophages were pretreated with 30 μM SB203580 or with DMSO for 1 h before infection with M. avium 2151 SmT (top panel) or SmO (bottom panel) at MOI of 2:1. Growth of SmT bacteria was reduced, whereas the survival of the SmO bacteria was unchanged. The results represent the mean (±SEM) of five independent experiments performed, each of which was performed in triplicate. *p < 0.05 vs DMSO control.

**TABLE 1. Increase in CFU following anti-TNF-α treatment of SmO- but not SmT-infected bone marrow-derived macrophages**

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>Condition</th>
<th>Viable CFU</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>SmT</td>
<td>IgG</td>
<td>1.7 ± 0.22 × 10⁶</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>Anti-TNF-α</td>
<td>1.5 ± 0.44 × 10⁶</td>
<td>0.87</td>
</tr>
<tr>
<td>SmO</td>
<td>IgG</td>
<td>3.3 ± 0.96 × 10⁴</td>
<td>2.08</td>
</tr>
<tr>
<td></td>
<td>Anti-TNF-α</td>
<td>6.8 ± 2.8 × 10⁴*</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Macrophages were pretreated with anti-TNF-α Abs or the IgG isotype control for 30 min prior to and during the 4-h infection with M. avium 2151 SmT or SmO. Macrophages were then washed and cultured in the absence of Ab for 7 days.

**FIGURE 6. Early neutralization of IL-10 has no effect on the growth of SmT in macrophages.** Macrophages were incubated with 10 μg/ml of IgG or anti-IL-10 for 1 h before and during the first 4 h of infection with the SmT morphotype (MOI of 2:1). No significant differences in bacteria growth were detected in the presence of the anti-IL-10 Ab. Results are representative of the mean (±SEM) of three independent experiments performed in duplicate.

Increases in CFU following anti-TNF-α treatment of SmO- but not SmT-infected bone marrow-derived macrophages

IL-10 is not required for bacteria of the SmT morphotype to grow in macrophages

That inhibition of p38MAPK activation resulted in reduced virulence of the SmT morphotype suggests that this morphotype may actively induce down-regulatory molecules. In particular, the reduced induction of IL-12p40 implicates the known inhibitor of IL-12p40, IL-10. In addition, the p38MAPK pathway has been shown to elicit an immune suppressive macrophage phenotype through the induction of IL-10 (32, 33). To determine whether this cytokine was important in permitting SmT growth, infected macrophages were cultured with anti-IL-10 Ab during the infection process. As expected and as confirmation that the anti-IL-10 was functioning, anti-IL-10 (10 μg/ml) treatment enhanced IL-12p40 synthesis 5-fold in SmT-infected macrophages (data not shown). However, interestingly, the addition of anti-IL-10 Ab to M. avium 2151 SmT-infected macrophages did not change the in vitro growth characteristics of either strain. The inability of anti-IL-10 to affect bacterial growth suggests that although IL-10 is produced and does down-regulate IL-12p40 production by SmT-infected macrophages, its induction is not an important factor in the virulence of SmT.

Macrophages infected with SmT express PGE₂ in a p38MAPK pathway-dependent fashion

Other mediators of macrophage down-regulation are the PGs, specifically PGE₂ and PGI₂. In addition, the p38MAPK-signaling cascade is known to regulate the synthesis of PGs at the arachidonic acid substrate level and also at the level of cyclooxygenase-2 mRNA stability (34, 35). In support of a role for PGE₂ in mediating the increased growth of the SmT, this molecule was detected in the supernatant of infected macrophages as early as the first hour of infection. By 4 h, the level was significantly higher than the level seen for the SmO-infected macrophages (Fig. 7). Further in support of the hypothesis was the observation that increasing concentrations of the p38MAPK inhibitor, SB203580, significantly decreased the induction of PGE₂ by both morphotypes (Fig. 7, upper right panel and lower panel).

**Inhibition of PGE₂ by indomethacin results in decreased ability of the SmT bacteria to grow within infected macrophages**

To confirm the role of PGE₂ synthesis in increased growth of the SmT morphotype, macrophages were treated with the cyclooxygenase inhibitor, indomethacin, before and for the 4-h infection...
with SmT and SmO bacteria. At all concentrations of indomethacin (1, 10, or 20 μM) tested, the growth of the SmT morphotype 7 days postinfection was decreased 3-fold (Fig. 8, top; only 10 μM data are shown for clarity). Interestingly, indomethacin treatment did not have an effect on the survival of the SmO morphotype within bone marrow-derived macrophages (Fig. 8, bottom). As expected, PGE2 levels decreased to basal levels in the presence of indomethacin (data not shown).

SmT- and SmO-induced activation of the PKB/Akt pathway is inhibited in the presence of 30 μM SB203580

Although significant and distinct effects on the growth of each morphotype were seen in the presence of inhibitors of both the ERK and p38MAPK MAPK pathways, the only difference between the induction of MAPK pathways by SmO and SmT bacteria was the increased ERK activation by SmO. Although this may explain the specific effect of the ERK inhibitor on the increased growth of SmO, it does not support a unique role for the p38MAPK pathway in SmT virulence. It is likely that other signaling pathways are induced during phagocytosis of M. avium and that these pathways modulate the consequences of p38MAPK pathway activation. In addition, the pharmacological inhibitor of p38MAPK used in this study is known to inhibit activation of phosphoinositide-dependent protein kinase 1, the upstream protein kinase of the protein kinase B (PKB)/Akt signaling pathway (36). In determining whether this was the case in M. avium-infected macrophages, we found that both SmT- and SmO-infected macrophages expressed phosphorylated-Akt as early as 15 min after infection. Of particular interest was the observation that in the presence of 30 μM SB203580, this induction of the Akt pathway was inhibited (Fig. 9A).

In addition to the inhibitory effects of SB203580 on the PKB/Akt pathway, we also observed enhanced JNK and ERK activation when macrophages were treated even with 1 μM of SB203580. The increase in SmO- and SmT-induced ERK activity at 15 min of infection (Fig. 9C) occurs before the difference between ERK activation in SmO and SmT untreated macrophages is seen (Fig. 3C). Increased phosphorylation of JNK1/2 also resulted from SB203580; however, we were unable to address the role of this pathway in the current study (Fig. 9B).

Discussion

The response of macrophages to invasion by intracellular pathogens depends to an extent upon the signals initiated during phagocytosis. In this study, we have identified some of the pathways induced during uptake of M. avium, and we have linked the induction of these pathways to the ability of the bacteria to grow in macrophages. Of particular interest was the observation that the virulent morphotype of M. avium activates macrophages, but that this activation is reduced by the p38MAPK-dependent production of PGE2. In contrast, the avirulent morphotype induces greater activation of macrophages, and its growth appears to be controlled, to some extent, by the induction of ERK and the production TNF-α. Taken together, the data suggest that the initial induction of the MAPK pathway by the morphotypes is a contributory factor in determining the fate of the M. avium morphotypes in macrophages.

We had hypothesized that very early interactions between the macrophage and the SmT bacteria were responsible for the virulence of this morphotype. We found this to be true in that inhibition of the p38MAPK pathway during the first 4 h of infection had profound effects on the ability of the bacteria to grow over the following 7-day period. The ability of the virulent SmT to downregulate macrophage activation is the most obvious explanation for
its increased virulence. In studying this inhibitory activity of the SmT morphotype, we describe in this study that both IL-10 and PGE₂ were acting on the macrophages to down-regulate IL-12p40 induction and antimycobacterial activity, respectively. Three lines of evidence support a role for PGE₂ in mediating SmT virulence. First, SmT-infected macrophages expressed an increased level of PGE₂ compared with SmO-infected macrophages. Second, pretreatment with the p38MAPK inhibitor, SB203580, prevented PGE₂ synthesis after SmT infection and enhanced macrophage control of SmT. Finally, treatment of SmT-infected macrophages with indomethacin (an independent inhibitor of PGE₂ expression by inhibition of cyclooxygenase-1 and -2 activity) also diminished SmT viability 5-fold. The relevance of this observation to clinical disease is highlighted by a case report showing that monocytes from a 3-year-old girl with disseminated *M. avium* infection demonstrated a defect in bactericidal activity that was restored when the patient was treated with indomethacin (37). In addition, HIV infection may augment *M. avium* infection through a similar mechanism, as the HIV envelope glycoprotein (gp120) can enhance *M. avium* replication within macrophages by induction of PGE₂ (38).

The inhibition of p38MAPK activation by SB203580 resulting in decreased cyclooxygenase-2 activity and thus production of PGE₂ has been previously reported (39). In addition, the SmT-mediated induction of PGE₂ was inhibited by a concentration of SB203580 close to the reported physiological inhibitory concentration of SB203580 for p38MAPK (40), supporting the hypothesis that PGE₂ induction was dependent upon activation of p38MAPK. However, it is possible that the SB203580 was acting directly on the enzymes cyclooxygenase-1 and -2 required for synthesis of PGs, as has been previously shown to occur in platelets (41). This direct activity occurs at a higher concentration of SB203580 (30 μM; Ref. 41), which is the same concentration at which we detected significantly decreased growth of the SmT bacteria. Therefore, it is plausible that although inhibition of PGE₂ can occur at the lower concentration of SB203580, the higher concentration is required to directly inhibit its production. That the direct inhibitor of the cyclooxygenase enzymes, indomethacin, was a more potent inhibitor of SmT virulence further supports the role of these enzymes in SmT virulence.

Although the evidence suggests that p38MAPK-mediated induction of PGE₂ is an important mediator of increased SmT growth, it is possible that the higher levels of SB203580 required to inhibit growth may affect other macrophage signaling pathways. Indeed, there is growing evidence that the use of this pyridinyl imidazole compound at high concentrations results in the inhibition of several signaling and metabolic pathways (36, 41, 42). At the higher concentration of SB203580, we observed inhibition of the PKB/Akt pathway as shown by reduced phosphorylation of phosphoinositide-dependent protein kinase 1 (also reported in Ref. 36). The PKB/Akt pathway regulates programmed cell death, and apoptosis has been implicated as a method by which macrophages limit mycobacterial growth. Interestingly, we were not able to detect any decrease in cell viability as determined by trypan blue exclusion or MTT-formazan assay (data not shown) following *M. avium* infection either in the presence or absence of SB203580. It is likely that the low MOI and the strain of *M. avium* used greatly reduced the tendency of the bacteria to induce apoptosis in the model reported in this study (43–45).

An alternative mechanism by which inhibition of the PKB/Akt pathway by SB203580 results in decreased SmT viability may be via the repressive effects of PKB/Akt on the Raf-MEK-ERK pathway (46, 47). PKB/Akt has been shown to phosphorylate Raf-1 at a conserved serine residue in the catalytic domain and to prevent the phosphorylation of MEK, the upstream protein kinase and activator of ERK (46). That this mechanism may be active in our model is suggested by our observation that ERK and JNK are up-regulated following SB203580 treatment and by the reported inhibitory effect of the p38MAPK on the ERK and JNK pathways (48–50). Interestingly, we also noted increased ERK activity in the SmO-infected macrophages when compared with the SmT-infected macrophages, suggesting that p38MAPK-dependent inhibition of ERK may be another defense mechanism of the SmT morphotype.

The data suggest that the SmO bacteria induce enhanced ERK activation, resulting in TNF-α production and macrophage activation, which in turn contributes to the control of bacterial growth. In contrast, although the virulent SmT bacteria induce ERK, they also induce a p38MAPK-dependent inhibitory pathway that results in the expression of IL-10, PGE₂, and the down-regulation of ERK. The induction of PGE₂ is a contributing factor in the ability of the SmT bacteria to grow.

In conclusion, we have shown that pharmacological modulation of the MAPK pathway for only the first 4 h of the macrophage-mycobacterial interaction results in consequences in bacterial...
growth up to 7 days later. This demonstrates that the very earliest signals initiated during uptake of the bacteria contribute to the environment in which the bacteria exist throughout infection. The response of the two morphotypes to the intracellular environment is likely to be distinct and, thus, the same protective mechanisms induced by the SmT bacteria may fail to enhance growth of the SmO bacteria. However, this difference in bacterial response to macrophage defense mechanisms is outside the scope of the work reported in this study. The more we understand the mechanisms by which mycobacteria subvert the protective immune response, the easier it will be to both vaccinate against disease and to design novel therapeutics. The data presented in this study suggest that the use of indomethacin and other inhibitors of PGE₂ may be useful in treating chronic M. avium infections.

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