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Mitogen-activated protein kinases regulate *Mycobacterium avium*-induced tumor necrosis factor-α release from macrophages

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Abstract

Tumor necrosis factor-α (TNF-α) is one of the key cytokines elicited by host macrophages upon challenge with pathogenic mycobacteria. Infection of human peripheral blood mononuclear cells or the murine macrophage cell line J774A-1 with *Mycobacterium avium* induced activation of the mitogen-activated protein kinases (MAPKs) ERK1/2, p38 and c-Jun N-terminal kinase. U0126, an MEK-specific inhibitor, abrogated *M. avium*-induced TNF-α secretion. Transfection of cells with dominant-negative MEK1 led to the suppression of TNF-α release in *M. avium*-challenged macrophages. *M. avium* activated p38 MAPK and use of the p38 MAPK inhibitor, SB203580, revealed that the p38 signaling pathway negatively regulates activation of ERK1/2 and release of TNF-α. Taken together, these results provide evidence that *M. avium*-induced TNF-α release from macrophages depends on an interplay between the ERK1/2 and the p38 MAPK signaling pathways.

Keywords: Mycobacterial pathogenesis; Cytokine; Signal transduction; p38 mitogen-activated protein kinase; ERK1/2 mitogen-activated protein kinase

1. Introduction

Mycobacterium avium is an intracellular facultative bacterium that proliferates inside host macrophages [1]. It causes severe pulmonary and disseminated disease in immunocompromised hosts, especially in individuals with AIDS. The bacterium poses a particular threat in these individuals in view of the fact that 40–50% of all patients with *M. avium* infection are refractory to conventional antibiotics and antimycobacterial drugs [2]. The success of virulent mycobacteria as pathogens is attributed largely to their ability to survive and multiply within macrophages for extended periods of time. These phagosomes, unlike those containing other bacteria, are arrested in their maturation [3–6], lack lysosomal markers [7] and fail to be acidified [8].

Designing effective agents to combat *M. avium* necessitates a thorough understanding of the host response to *M. avium* infection. The production and balance of immunomodulatory cytokines and the activation of kinase cascades and transcription factors are key elements of the host cell response.

Infection of macrophages with *M. avium* induces the production of tumor necrosis factor-α (TNF-α) [9], which is one of the key cytokines responsible for mounting the host response to infection. Macrophages harboring *M. avium* undergo apoptosis [10] in a TNF-α-dependent manner [11], which helps in the reduction of successive bacterial growth and in the containment of infection. Understanding the mechanisms of TNF-α production should further our understanding of the host cell defense against *M. avium* and of the immunobiology of *M. avium* infection.

Mitogen-activated protein kinases (MAPKs) and their upstream kinases activate a number of transcription factors and signal the induction of a variety of inflammatory genes in response to lipopolysaccharide [12], cytokines [13] and mycobacterial lipoarabinomannan [14]. There are three principal MAPK family members: (i) p46 and p54 c-Jun NH_2-terminal kinase (JNK) or stress-activated protein kinase with multiple subisoforms, (ii) p38 MAPK with α, β, γ, and δ isoforms, and (iii) p42 and p44 extracellular signal-regulated kinase (ERK1 and 2). MAPKs are activated by specific upstream MAPK kinases (MKKs): (i)

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MKK4 and MKK7 activate JNK [15–17], (ii) MAPK/ERK kinase 1/2 (MEK1/2) activates the ERKs [18] and (iii) MKK3 and MKK6 activate p38 MAPK [19].

We asked whether MAPK signaling is involved in TNF-α secretion by macrophages challenged with *M. avium*. We made use of specific inhibitors of ERK1/2 and p38 MAPK as well as transfection with dominant-negative MEK1 to delineate the MAPK signaling response elicited by *M. avium* in murine macrophages. We demonstrate that TNF-α secretion induced by *M. avium* in macrophages occurs in a MEK1–ERK1/2-dependent manner. p38 MAPK is also activated by *M. avium*. We demonstrate that p38 MAPK negatively regulates ERK1/2 activation as well as TNF-α secretion in macrophages. We speculate that one-way cross-talk between p38 MAPK and ERK1/2 MAPK is crucial in determining *M. avium*-mediated TNF-α secretion from macrophages, and therefore in the overall biological response leading to destruction or survival of the bacterium within its host.

2. Materials and methods

2.1. Reagents

Antibodies against total MAPKs, phospho-specific antibodies and the Elk–glutathione *S*-transferase (GST) fusion protein were purchased from Cell Signaling Technology, Beverly, MA, USA. PD98059, SB203580, U0126, LY-294002, wortmannin and protease inhibitors were obtained from CN Biosciences (San Diego, CA, USA).

2.2. Isolation of human peripheral blood mononuclear cells (PBMCs)

Blood was drawn from healthy adult volunteers into heparinized (100 U ml^{-1}) syringes, layered on Ficoll-Hypaque (Amersham Biosciences, UK) and isolated by centrifugation according to the manufacturers' instructions. Cells were incubated in dishes containing RPMI 1640 medium supplemented with 25 mM HEPES, 2% penicillin and streptomycin, 1% glutamine and 10% fetal bovine serum for 1 h at 37°C in a humidified CO_2 (5%) incubator. Dishes were washed to remove non-adherent cells. Cells were $\geq$90% adherent as determined by trypan blue dye exclusion.

2.3. Culture of the murine macrophage-like cell line J774A-1

The murine macrophage cell line J774A-1 was obtained from the National Centre for Cell Science, Pune and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified atmosphere containing 5% CO_2.

2.4. Growth of M. avium

M. avium (NCTC 8562, a virulent strain) was obtained from the All India Institute of Medical Sciences, New Delhi, India. *M. avium* was grown in Middlebrook 7H9 broth (Difco, Detroit, MI, USA) supplemented with oleic acid–albumin–dextrose supplement and 0.04% Tween 80 until the mid-exponential phase, harvested, washed and resuspended in a small volume of phosphate-buffered saline (PBS) containing 0.04% Tween 80. The suspension was briefly sonicated until no bacterial clumps could be seen by microscopy. The preparation was diluted to a concentration of 2×10^8 colony-forming units ml^{-1} in 10% glycerol and stored in aliquots at −80°C. Repeated subculturing of *M. avium* in liquid medium was avoided in order to prevent loss of virulence.

2.5. Plasmid constructs and transient transfections

Constructs CMV β-gal, CMV-MEK-wt and CMV MEK2A (dn) were generously gifted by Dr. D.J. Templeton, Case Western Reserve University, Cleveland, OH, USA. Transient transfections were carried out using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. The β-gal reporter plasmid was used to normalize transfection efficiencies.

2.6. Infection and preparation of cell lysates

J774A-1 cells were cultured in 24-well tissue culture plates at 4×10^5 cells per well and were infected with *M. avium* at a multiplicity of infection (MOI) of 10. Cells were lysed with 1% Triton X-100 and the lysate was diluted and plated on 7H10 agar to quantitate the number of viable intracellular bacteria. The incubation of J774-1 with *M. avium* at a bacteria:macrophage ratio of 10:1 for 6 h led to the infection of 50% of the cells. The viability of the infected monolayers versus an uninfected control was monitored by the trypan blue exclusion method. The viability was $\geq$95% in all of the experiments described. After incubation with *M. avium*, the wells were washed with ice-cold PBS to remove free bacteria. Cells were lysed with lysis buffer (20 mM Tris–HCl pH 7.4, 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 137 mM NaCl, 20 mM NaF, 1 mM EDTA, 40 mM Na-β-glycerophosphate, 4 μg ml^{-1} each of leupeptin, pepstatin and aprotinin, 1 mM Na_3VO_4, 1 mM pefabloc, 1 mM benzamidine) on ice for 15 min. Cell lysates were boiled for 5 min after the addition of 5× Laemmli sample buffer and subjected to Western blotting. Where necessary, J774A-1 cells were first treated with pharmacological inhibitors or vehicle (DMSO) alone, prior to incubation with *M. avium*.

2.7. Western blotting

Whole cell lysates were separated on SDS–polyacryl-

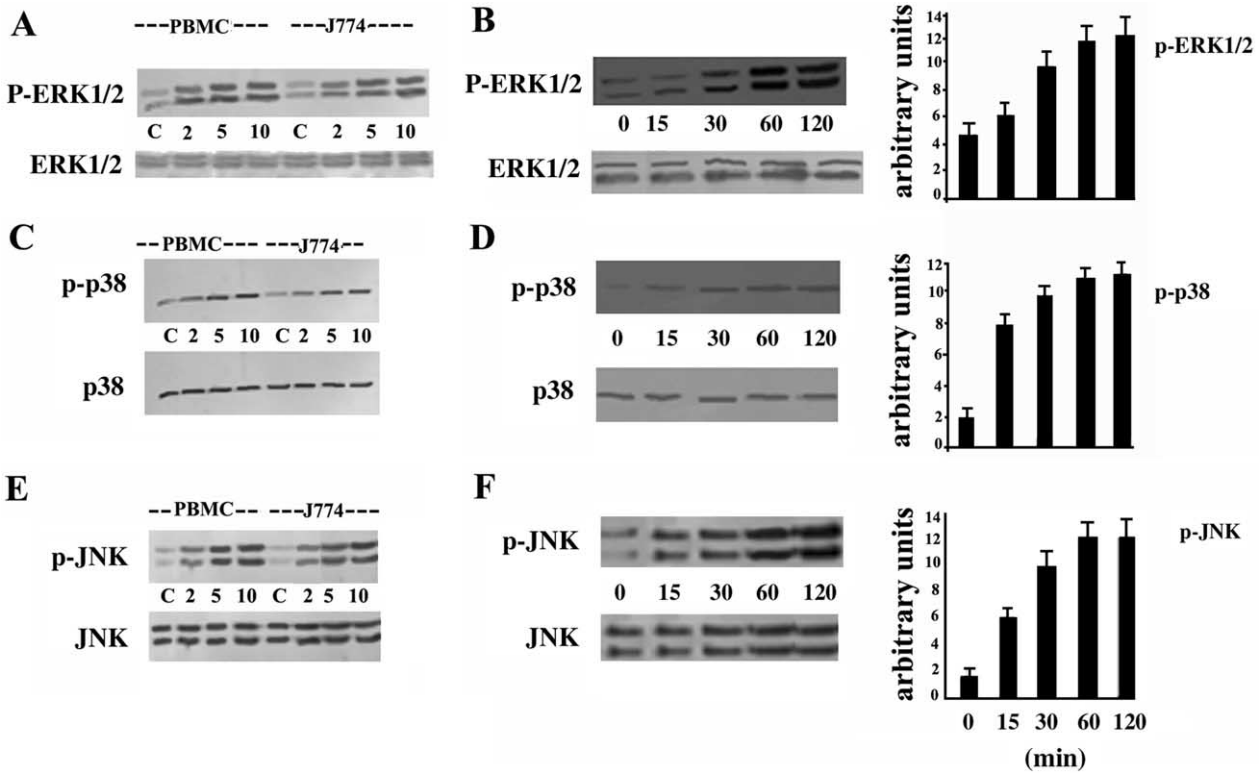

Fig. 1. ERK, p38 and JNK MAPKs are activated in *M. avium*-infected macrophages. Human PBMCs or J774A-1 macrophages were left untreated (lanes C) or treated at different ratios (2, 5 and 10) of bacteria:host cell for 45 min (A, C and E). J774A-1 cells were treated with *M. avium* (bacteria: host cell, 10:1) for various lengths of time (indicated in minutes in B, D and F). Cellular extracts were analyzed by Western blotting for the presence of phospho-MAPKs. Each of the blots was also probed with antibodies against total ERK1/2, p38 and JNK MAPK antibodies to ensure that these were present in equal amounts in all lanes. The graphs to the right of B, D and F show the corresponding densitometric analyses of the blots probed with anti-phospho-MAPK antibodies (data from three independent experiments; bars represent the mean ± S.D.).

amide gels and the proteins transferred to PVDF membranes. The blots were blocked with 5% non-fat dry milk powder in Tris-buffered saline (TBS) for 1 h at room temperature, then incubated overnight at 4°C with primary antibodies diluted 1:1500 in TBST (TBS with 1% (v/v) Tween 20) plus 5% bovine serum albumin. Following three washes of 5 min each with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology) in blocking buffer for 1 h at room temperature. After three washes with TBST, the blots were developed with BM chemiluminescence reagent (Roche Molecular Biochemicals) and exposed to X-ray film (Kodak XAR5).

2.8. In vitro kinase assay

ERK1/2 MAPK activity was determined by a solid phase kinase assay using GST–Elk1 (Cell Signaling Technology) as the substrate. After treatment, cells were lysed in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na-pyrophosphate, 1 mM Na-β-glycerophosphate, 1 mM Na_3VO_4) for 5 min on ice. After centrifugation at 10 000 rpm (Sorvall SS-34) for 5 min at 4°C, supernatants were incubated with immobilized phospho-ERK1/2 MAPK monoclonal antibody (Cell Signaling Technology) according to the manufacturer's protocol. The beads were washed twice with lysis buffer, followed by two washes with kinase reaction buffer (25 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 5 mM Na-β-glycerophosphate, 2 mM dithiothreitol, 100 μM Na_3VO_4). The beads were then incubated with 2 μg GST–Elk1 fusion protein in 20 μl kinase reaction buffer containing 200 μM ATP. After incubation for 30 min at 30°C, the reaction was terminated by the addition of 5× Laemmli sample buffer. Phosphorylated proteins were resolved on an SDS–polyacrylamide (10%) gel followed by Western blotting. Blots were probed with anti-phospho-Elk antibody and visualized using a chemiluminescence detection kit.

2.9. TNF-α measurement in culture supernatants

J774A-1 cells were either left untreated or were treated with vehicle (DMSO), U0126, PD98059 or SB203580 followed by incubation with *M. avium*. The conditioned medium was removed and assayed for TNF-α by enzyme-linked immunosorbent assay using the murine BIOTRAK TNF-α assay kit (Amersham Biosciences, UK) according to the manufacturer's protocol. The limit of detection was 10 pg TNF-α ml^{-1}.

2.10. Statistical analysis

Data obtained from three independent experiments are represented as mean ± S.D.; comparison was by the Student's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. M. avium infection leads to the activation of ERK, JNK and p38 MAPKs in macrophages

The phosphorylation of the three members of the MAPK family was considered to be an index of MAPK activation. Human PBMCs or the murine J774A-1 (macrophage-like) cell line were challenged with *M. avium* at different ratios of bacteria to host cell. Phosphorylation of all three members of the MAPK family occurred in both cell types and increased with increasing ratios of bacteria to host cell (Fig. 1A, C and E). Time-dependent phosphorylation of all three members of the MAPK family was observed on challenging J774A-1 cells with *M. avium*. *M. avium* stimulated phosphorylation of the MAPKs within 30–60 min of exposure (Fig. 1B, D and F). The activation time courses of all three MAPKs were similar.

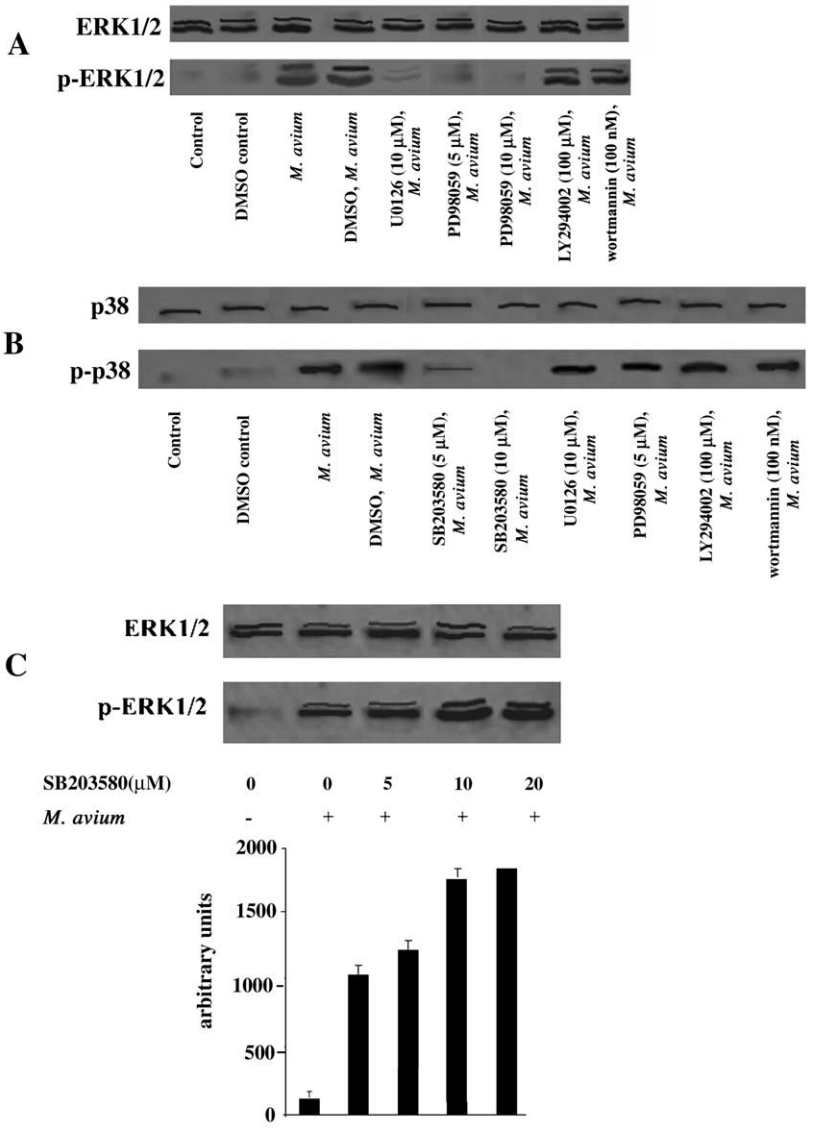

Fig. 2. Effect of pharmacological inhibitors of ERK and p38 MAPK on *M. avium*-induced MAPK activation. J774A-1 macrophages were treated with just medium (control) or vehicle (DMSO control) or with the MAPK-specific inhibitors PD98059, U0126 and SB203580, or with the non-specific inhibitors LY294002 and wortmannin, for 30 min prior to incubation (45 min) with *M. avium*. Cells were lysed and subjected to Western blot analysis with anti-phospho-ERK1/2 (A and C) and anti-p38 MAPK (B). Each of the blots was also probed with antibodies against total ERK1/2 (A and C), and p38 (B) MAPK antibodies to ensure that these were present in equal amounts in all lanes. A densitometric analysis of the blots of panel C probed with anti-phospho-ERK1/2 antibodies is shown (data from three independent experiments; bars represent the mean ± S.D.).

3.2. Specific inhibitors block M. avium-stimulated MAPK activation

The pharmacological inhibitors U0126 [20] and PD98059 [21,22] specifically inhibit MEK without inhibiting other MAPK pathways. Macrophages were treated with vehicle only or with these inhibitors for 30 min prior to challenge with *M. avium*. Extracts were then analyzed by Western blotting for phosphorylated ERK1/2. U0126 and PD98059 both blocked ERK1/2 phosphorylation mediated by *M. avium* (Fig. 2A), while exerting no effect on p38 MAPK. We also examined the effect of SB203580, which specifically inhibits p38 MAPK [23]. Pretreatment of cells with SB203580 (10 μM) caused complete inhibition of the phosphorylation of p38 MAPK (Fig. 2B). On the other hand, treatment with LY294002 or wortmannin, which do not inhibit MAPKs, had no effect on *M. avium*-induced MAPK activation.

SB203580 treatment led to a significant increase in the phosphorylation of ERK1/2 MAPK in a dose-dependent manner (Fig. 2C) as determined by immunoblot analysis with antibodies specific to phospho-ERK1/2 and densitometric scanning of the blots. Maximum activation of ERK1/2 was observed at 10 μM SB203580, which showed maximum inhibition of p38 MAPK.

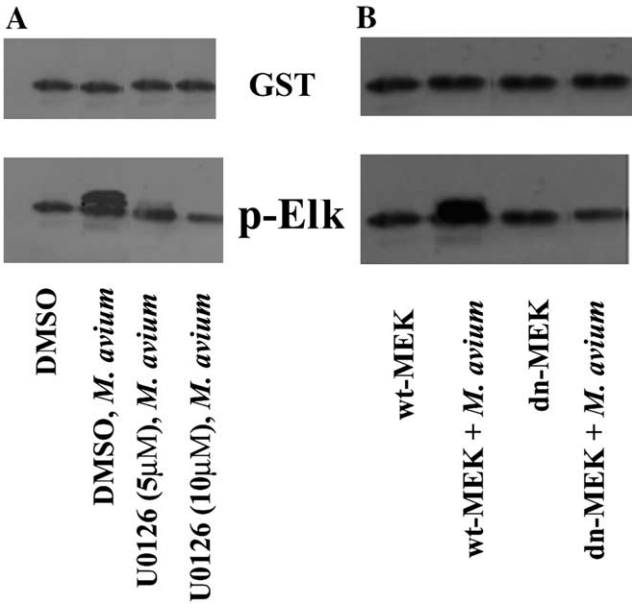

Fig. 3. Dominant-negative MEK1 abrogates *M. avium*-stimulated activation of ERK1/2. A: J774A-1 cells were treated with DMSO or U0126 for 30 min prior to incubation with *M. avium*. Cellular extracts were prepared and subjected to immunoprecipitation with immobilized anti-phospho-ERK1/2. Immunoprecipitates were added to an in vitro kinase assay using purified GST–Elk1 as substrate, and analyzed by Western blotting using anti-phospho-Elk1 antibody. Probing with anti-GST antibody verified equal loading in all lanes. The data shown are representative of three independent determinations. B: J774A-1 cells were transfected with wild-type (wt-MEK) or dominant-negative (dn-MEK) MEK1 prior to incubation with *M. avium* as described above. The immunoprecipitation/kinase assay using GST–Elk1 as substrate was performed as described for panel A. Data shown are representative of three independent experiments.

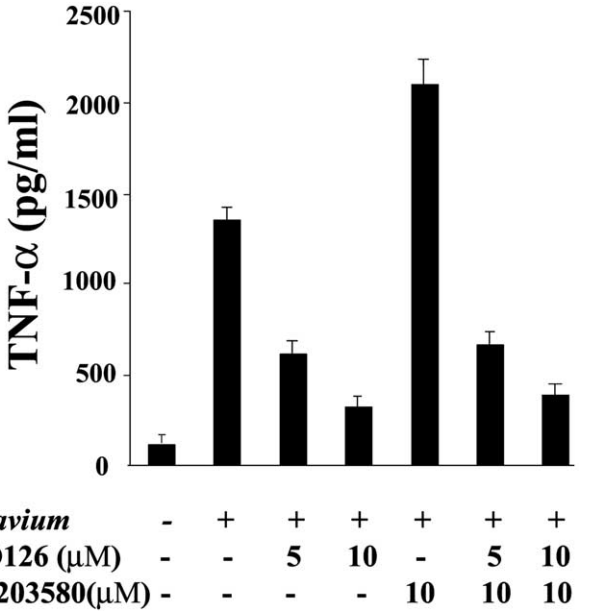

Fig. 4. *M. avium* stimulates TNF-α secretion in J774A-1 macrophages in a MAPK-dependent pathway. Monolayers of J774A-1 macrophages were cultured alone or with *M. avium*, with and without a 30-min pretreatment with the MEK1 inhibitor U0126 and/or the p38 MAPK inhibitor SB203580. The release of TNF-α was measured in the conditioned medium after 6 h. Data are from three independent experiments; bars represent the mean ± S.D.

3.3. M. avium activates ERK1/2 in a MEK1-dependent pathway

Phosphorylation of ERK by MEK activates this kinase, resulting in the phosphorylation and activation of the transcription factor Elk1. We determined ERK activity in J774A-1 cells incubated with *M. avium* using an in vitro kinase assay. Cell extracts were prepared and subjected to immunoprecipitation using an immobilized antibody directed against phosphorylated ERK (residues Thr-202 and Tyr-204). The immunoprecipitated proteins were subjected to an in vitro kinase assay using purified GST–Elk1 fusion protein as a substrate. Anti-GST antibody was used to confirm that equal amounts of Elk1 were present in all reactions. *M. avium*-mediated ERK activation led to the phosphorylation of Elk1. This could be inhibited by the pretreatment of cells with the MEK1 inhibitor U0126 in a dose-dependent manner (Fig. 3A). Further evidence that *M. avium* mediates ERK1/2 activation in a MEK1-dependent pathway was provided by the observation that the transfection of cells with MEK2A (dn) led to the abrogation of ERK1/2 kinase activity (Fig. 3B).

3.4. M. avium stimulates release of TNF-α from J774A-1 macrophages in a ERK1/2-dependent pathway

M. avium stimulated the secretion of TNF-α (Fig. 4).

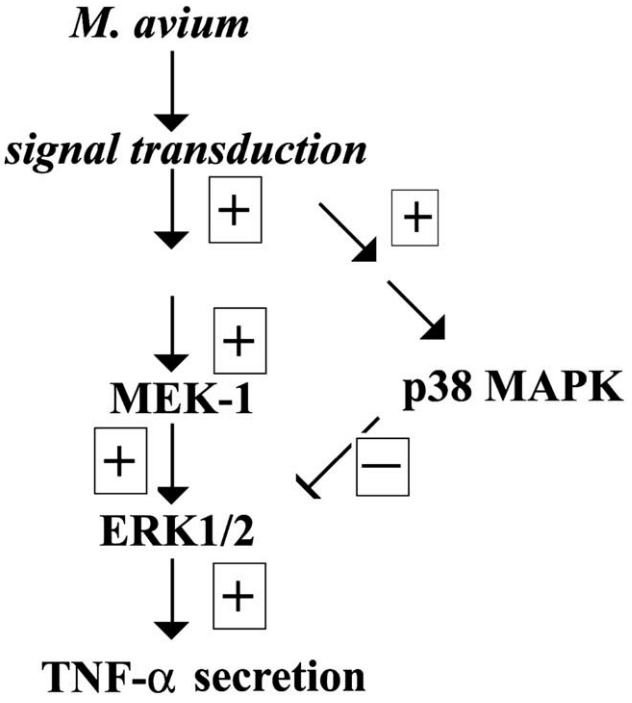

Fig. 5. Model of the regulation of TNF-α secretion through ERK1/2 and p38 MAPK signaling by *M. avium* in J774A-1 macrophages.

Maximal secretion occurred after 6–8 h of incubation of *M. avium* with J774A-1 macrophages at an MOI of 10 (data not shown). All subsequent experiments were performed by incubation of J774A-1 macrophages for 6 h with *M. avium*. In order to understand whether the MAPK pathway regulates *M. avium*-mediated TNF-α release, we studied TNF-α release both in the absence and in the presence of MAPK inhibitors.

We observed that inhibition of ERK1/2 MAPK by U0126 (10 μM) led to a four-fold reduction in TNF-α release (Fig. 4). On the other hand, treatment of cells with the p38 MAPK inhibitor SB203580 led to an increase in TNF-α release which could be offset by treatment of cells with U0126 in combination with SB203580. Inhibition of TNF-α release mediated by the combination of SB203580 and *M. avium* supports the proposal that TNF-α release occurs in an ERK1/2-dependent manner.

4. Discussion

In the present study we provide evidence that synthesis of TNF-α occurs as an early response to the challenge of the murine macrophage-like cell line J774A-1 by *M. avium*. We asked whether the MAPK signaling pathways were involved in TNF-α synthesis by *M. avium*-infected macrophages.

J774A-1 is a mouse cell line that possesses characteristics typical of macrophages [24]. In recent years, J774A-1 cells have been used extensively in the study of the infection and intracellular survival/apoptosis of many bacteria including mycobacteria [6,7,25,26]. J774A-1 was therefore chosen as a model to understand *M. avium*-induced activation of all three MAPK pathways. In addition, MAPK activation by *M. avium* was also tested in human PBMCs. Activation of all three MAPKs occurred in both cell types and was dependent upon the ratio of bacteria to host cell. Further studies were carried out in J774A-1 cells. Activation of the ERK1/2 MAPKs by *M. avium* was inhibited by the MEK-specific inhibitors PD98059 and U0126, suggesting that the MEK1–ERK1/2 pathway was stimulated by *M. avium* challenge. This argument was strengthened by the observation that the transfection of cells with MEK2A (dn) abrogated phosphorylation of ERK1/2 by *M. avium*. In addition, it was observed in in vitro kinase assays that *M. avium* stimulated Elk1 phosphorylation which could be abrogated when cells were transfected with MEK2A (dn), suggesting that *M. avium* stimulates the MEK1–ERK1/2 pathway in murine macrophages.

M. avium activation of the p38 MAPK pathway was inhibited by the p38-specific pyridinyl imidazole compound SB203580 [23], a cell-permeable inhibitor that binds to the ATP-binding site of p38. In addition, SB203580 treatment of cells led to an enhanced activation of ERK1/2 phosphorylation mediated by *M. avium*, suggesting that the p38 MAPK pathway negatively regulates the activation of ERK1/2.

TNF-α is thought to mediate pathologic inflammatory reactions [27] and protective responses to bacterial infections [28]. It is principally expressed in macrophages [29]. We have presented evidence that stimulation of the MAPK pathway by *M. avium* is causally linked to TNF-α secretion in J774-1 macrophages. The ERK1/2 MAPK activation correlated directly with induction of TNF-α secretion elicited by *M. avium*. The treatment of cells with SB203580 activated the *M. avium*-stimulated secretion of TNF-α. Activation of TNF-α release by SB203580 could be reversed when cells were treated with a combination of SB203580 and U0126. Taken together, these data suggest that *M. avium*-mediated ERK1/2 activation is directly related to the stimulation of TNF-α synthesis in murine macrophages, while p38 MAPK inhibits ERK1/2 activation and thereby negatively regulates TNF-α synthesis. The regulation of TNF-α release is unlikely to follow a common pathway, but is more likely to be strain-, cell type- and stimulus-specific. Several recent reports support this proposal. While SB203580 reduces TNF-α synthesis induced by lipopolysaccharide in macrophages [23], it enhances TNF-α secretion from stimulated mast cells [30]. p38 MAPK has been reported to inhibit low density lipoprotein receptor expression through inhibition of the ERK1/2 signaling pathway in HepG2 cells [31]. In the present study, the effect we saw of SB203580 on *M. avium*-stimulated TNF-α release argues in favor of the view that p38 MAPK negatively regulates ERK1/2 signaling and TNF-α release stimulated by *M. avium* in murine

macrophages. It is worth noting in this context that p38 MAPK inhibition resulted in increased TNF-α release in murine models of *M. tuberculosis* infection [32]. This was accompanied by severely reduced bacterial clearance. A recent report by Chan et al. [14] showed that SB203580 does not block, but rather enhances lipoarabinomannan-induced nitrite production in macrophages. The present study demonstrates that p38 MAPK-mediated inhibition of ERK1/2 is a one-way cross-talk between the two pathways, since the ERK1/2 MAPK does not regulate p38 MAPK.

In conclusion, we have demonstrated that *M. avium* stimulates TNF-α secretion from macrophages in a MEK1–ERK1/2-dependent manner (Fig. 5). While this work was in progress, Reiling et al. [33] demonstrated that *M. avium*-induced TNF-α secretion from peripheral blood monocytes is regulated by MAPKs. Tse et al. [34] have also shown that both the avirulent and virulent morphotypes of *M. avium* activate all three MAPKs in macrophages. These results support our findings. Tse et al. observed that only the growth of the avirulent morphotype was controlled by the activation of ERK1/2 and the production of TNF-α. We have demonstrated for the first time that a one-way cross-talk between p38 and ERK1/2 MAPKs is responsible for the p38 MAPK-mediated inhibition of ERK1/2, and therefore of TNF-α secretion. Since *M. avium* stimulates both ERK1/2 and p38 MAPK, the interplay between these signaling cascades and their dynamic balance may be critical for determining whether TNF-α secretion upon the challenge of J774A-1 macrophages with *M. avium* leads to macrophage apoptosis and containment of the bacterial infection, or whether suppression of TNF-α secretion mediated by the p38 MAPK pathway leads to prolonged survival of *M. avium* within macrophages. This distinct usage of MAPK pathways needs to be taken into careful consideration when defining targets along the MAPK pathway for therapeutic intervention of mycobacterial infection. Detailed studies into the inhibition of individual MAPK pathways and the survival of avirulent and virulent morphotypes are also warranted.

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