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Execution of Macrophage Apoptosis by *Mycobacterium avium* through Apoptosis Signal-regulating Kinase 1/p38 Mitogen-activated Protein Kinase Signaling and Caspase 8 Activation*

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Asima Bhattacharyya, Shresh Pathak, Chaitali Basak, Sujata Law, Manikuntala Kundu‡, and Joyoti Basu§

From the Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla, Chandra Road, Kolkata 700 009, India

Macrophage apoptosis is an important component of the innate immune defense machinery (against pathogenic mycobacteria) responsible for limiting bacillary viability. However, little is known about the mechanism of how apoptosis is executed in mycobacteria-infected macrophages. Apoptosis signal-regulating kinase 1 (ASK1) was activated in *Mycobacterium avium*-treated macrophages and in turn activated p38 mitogen-activated protein (MAP) kinase. *M. avium*-induced macrophage cell death could be blocked in cells transfected with a catalytically inactive mutant of ASK1 or with dominant negative p38 MAP kinase arguing in favor of a central role of ASK1/p38 MAP kinase signaling in apoptosis of macrophages challenged with *M. avium*. ASK1/p38 MAP kinase signaling was linked to the activation of caspase 8. At the same time, *M. avium* triggered caspase 8 activation, and cell death occurred in a Fas-associated death domain (FADD)-dependent manner. The death signal induced upon caspase 8 activation linked to mitochondrial death signaling through the formation of truncated Bid (t-Bid), its translocation to the mitochondria and release of cytochrome *c*. Caspase 8 inhibitor (z-IETD-FMK) could block the release of cytochrome *c* as well as the activation of caspases 9 and 3. The final steps of apoptosis probably involved caspases 9 and 3, since inhibitors of both caspases could block cell death. Of foremost interest in the present study was the finding that ASK1/p38 signaling was essential for caspase 8 activation linked to *M. avium*-induced death signaling. This work provides the first elucidation of a signaling pathway in which ASK1 plays a central role in innate immunity.

Mycobacterium avium is an intracellular facultative bacterium that proliferates inside host macrophages (1, 2). It causes severe pulmonary and disseminated disease in immunocompromised hosts, especially in individuals with AIDS (3). The bacterium poses a threat in these individuals particularly in view of the fact that 40–50% of all patients with *M. avium* infection are refractory to conventional antibiotics and antimycobacterial drugs (4). The innate immune response to mycobacteria is initiated when the bacteria interact with cell surface receptors. This results in subsequent internalization of the mycobacterium within the macrophage. The macrophage represents the first line in innate immune defense against mycobacteria. In macrophages infected with pathogenic mycobacteria, apoptotic death is associated with a reduction in bacillary viability (5–8). On the other hand, the success of virulent mycobacteria as pathogens is attributed largely to their ability to survive and multiply within macrophages for extended periods of time. Mycobacteria live and proliferate within phagosomes, which unlike those containing other bacteria, are arrested in their maturation (9–11), lack lysosomal markers (12) and fail to be acidified (13). The balance between the pathogen-eliminating mechanisms employed by the macrophage, and the survival pathways used by the mycobacterium determines the fate of the organism. In this context it is of foremost importance to understand the pathways of macrophage cell death upon challenge with mycobacteria and the strategies used by the pathogen to offset cell death and prolong its own survival within macrophages.

Apoptosis is an orchestrated suicide program, that among its other functions, enables an organism to remove invading pathogens without causing an inflammatory response. The key morphological alterations of apoptosis are mediated by a family of cysteine proteases called the caspases, which are activated by proteolytic cleavage (14–16). Activation of caspases is achieved via two principal signaling pathways, namely the extrinsic and intrinsic death pathways (17, 18). The extrinsic death pathway involves the ligation of death receptors (CD95/Fas; tumor necrosis factor receptor) that leads to the recruitment of adaptor molecules such as Fas-associated death domain (FADD),[1] and pro-caspase 8 or procaspase 10 into a death-inducing signaling complex (DISC) (19, 20). Adaptor molecules such as FADD and TRADD activate caspase 8 through proximal interactions between their death effector domains (DED) (21). Activated caspase 8 can then cleave and activate downstream effector caspases such as caspase 3 and caspase 7 (22–24). In the intrinsic (or mitochondrial) death pathway, mitochondria release several death-promoting fac-

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‡ To whom correspondence may be addressed. Fax: 91-33-23506790; E-mail: mani18@hotmail.com.

§ To whom correspondence may be addressed. E-mail: joyoti@vsnl.com.

[1] The abbreviations used are: FADD, Fas-associated death domain; ASK1, apoptosis signal-regulating kinase 1; MAP, mitogen-activated protein; DED, death effector domains; cyt *c*, cytochrome *c*; t-Bid, truncated Bid; DISC, death-inducing signaling complex; NAC, *N*-acetylcysteine; AFC, 7-amino-4-trifluromethyl coumarin; FMK, fluoromethyl ketone; z-DEVD-FMK, z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-FMK; z-LEHD-FMK, z-Leu-Glu(OMe)-His-Asp(OMe)-CH_2F; z-IETD-FMK, z-lle-Glu(OMe)-Thr-Asp(OMe)-FMK; Ac-DEVD-*p*NA, Ac-Asp-Glu-Val-Asp-*p*NA; Ac-LEHD-AFC, Ac-Leu-Glu-His-Asp-AFC; Ac-IETD-AFC, Ac-lle-Glu(OMe)-Thr-Asp(OMe)-AFC; MOI, multiplicity of infection; Me_2SO, dimethylsulfoxide; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonic acid; MBP, myelin basic protein; PBS, phosphate-buffered saline.

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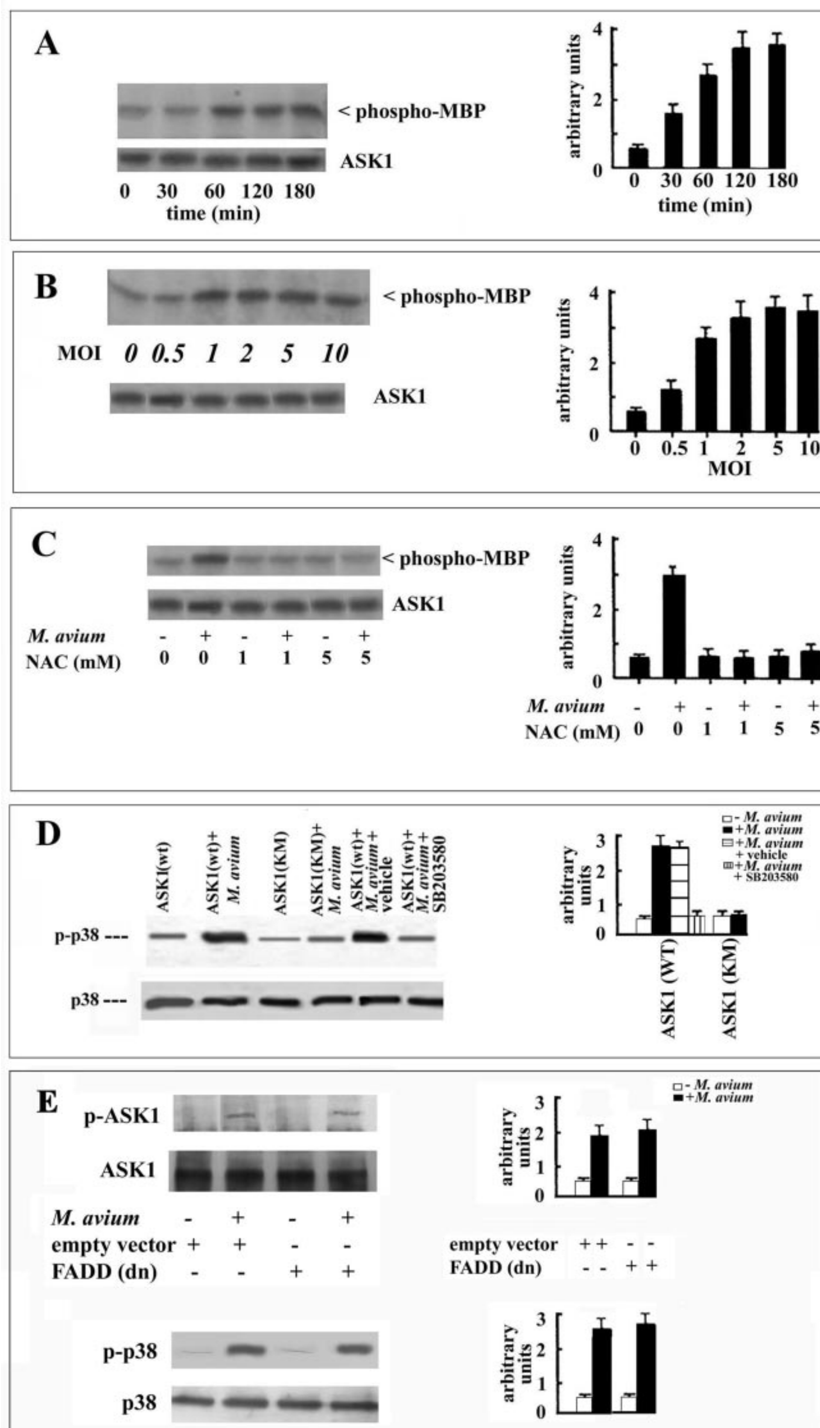

FIG. 1. **ASK1 and p38 MAP kinase are activated upon challenge of J774A-1 macrophages with *M. avium*.** J774A-1 macrophages were incubated with *M. avium* at a MOI of 10 for different periods of time (*panel A*), or J774A-1 macrophages were incubated with *M. avium* at different MOI for 3 h (*panel B*). Whole cell lysates were prepared, ASK1 was immunoprecipitated, and *in vitro* ASK1 kinase activity was determined using MBP as substrate as described under "Experimental Procedures." *Panel C*, J774A-1 macrophages were left untreated or treated with NAC at different concentrations for 60 min. Cells were washed and then incubated without or with *M. avium* at an MOI of 10 for 3 h. ASK1 activity was determined in the lysates as described above. In each case the *bottom panel* is a representative Western blot with anti-ASK1 to show that the same amount of ASK1 was present in each sample. *Panel D*, J774A-1 macrophages were transfected with wild-type ASK1(WT) or a catalytically inactive mutant of ASK1(KM) followed by incubation without or with *M. avium* at an MOI of 10 for 3 h. In separate experiments cells transfected with ASK1(WT) were treated with vehicle or with SB203580 (10 μM) for 60 min before incubation with *M. avium*. Whole cell lysates were prepared, followed by immunoblotting with anti-phospho-p38 MAP kinase antibodies. The lower blot shows that the same amount of p38 MAP kinase was present in each sample. *Panel E*, J774A-1 macrophages were transfected with empty vector or dominant-negative FADD (FADD(dn)) followed by incubation without or with *M. avium* at an MOI of 10 for 3 h. Immunoblots of whole cell lysates were probed with anti-phospho-ASK1 or anti-phospho-p38 MAP kinase antibodies. The *right hand panels* in each case represent the densitometric analysis of autoradiograms (*Error bars* represent S.E. from three independent experiments).

tors including cytochrome *c* (cyt *c*) (25), apoptosis-inducing factor (AIF) (26), and Smac (DIABLO) (27, 28) in response to death-inducing signals via the Bcl-2 family of proteins (29). In the cytosol, cyt *c* interacts with apoptotic protease-activating factor (Apaf-1). On binding cyt *c*, an ATP/dATP-binding oligomerization domain within Apaf-1 mediates Apaf-1 oligomerization (30, 31). The oligomerized complex (apoptosome) then binds procaspase 9 and facilitates processing of caspase 9 zymogens (32). Activated caspase 9 subsequently activates effector caspases (33).

There is a cross-talk between the extrinsic and intrinsic death pathways. One mechanism for this cross-talk is the cleavage of Bid by caspase 8. Bid is a BH3-domain-only member of the Bcl-2 family of death-regulating proteins (34). The BH3 domain-only family are upstream activators of apoptosis that signal to downstream proapoptotic Bcl-2 family members such as Bax or Bak leading to their oligomerization at the mitochondria. Activated caspase 8 can cleave p22 Bid to generate a p15 active truncated Bid (t-Bid) fragment, which is then targeted to the mitochondria (35). Cleavage of Bid to the mitochondrially active, t-Bid, is a feature of caspase 8-mediated apoptosis induced via death receptors (36, 37) which enables amplification of the apoptotic signal through the mitochondrial release of cyt *c* (38)

The mitogen-activated protein (MAP) kinase cascade is one of the evolutionarily conserved phosphorylation-regulated protein kinase cascades, which is involved in controlling the decision of cell survival or cell death. Apoptosis signal-regulating

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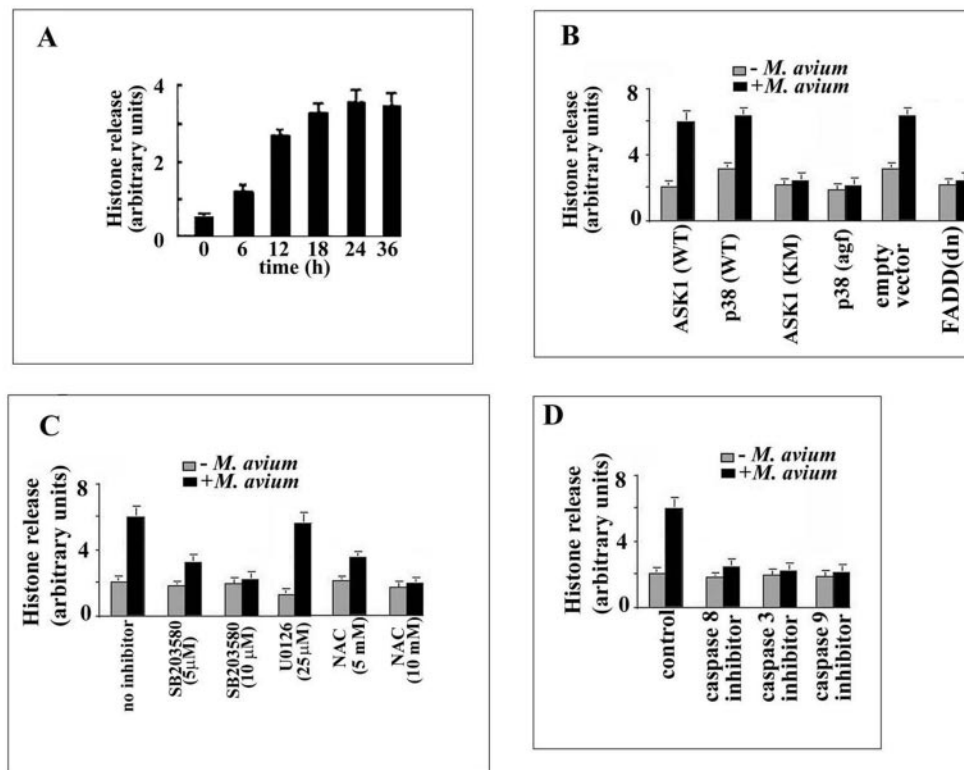

FIG. 2. ***M. avium*-induced cell death of J774A-1 macrophages measured as the release of histone using ELISA.** *Panel A*, cells were incubated with *M. avium* at an MOI of 10 and histone release was measured after different periods of time using the Cell Death ELISA kit from Roche Applied Science as described under "Experimental Procedures." *Panel B*, J774A-1 macrophages were transfected with: ASK1: WT or KM; p38: WT or agf; empty vector or FADD(dn); followed by incubation without or with *M. avium* at an MOI of 10 for 18 h. *Panel C*, cells were left untreated or treated with the cell-permeable MAP kinase inhibitors U0126 or SB203580, or with NAC at the indicated concentrations for 60 min; followed by removal of the inhibitors and incubation without or with *M. avium* at an MOI of 10 for 18 h. *Panel D*, cells were left untreated or treated with the cell-permeable irreversible caspase inhibitors (50 μM); z-IETD-FMK (for caspase 8), or z-DEVD-FMK (for caspase 3), or z-LEHD-FMK (for caspase 9) for 60 min followed by removal of the inhibitors and incubation without or with *M. avium* at an MOI of 10 for 18 h. In each case, bacteria were removed, cells were washed, lysed, and cell death was measured using the Cell Death ELISA kit from Roche Applied Science as described under "Experimental Procedures." Values are given as the means ± S.E. of three independent experiments.

kinase 1 (ASK1) is a member of the MAP kinase kinase kinase (MAPKKK) family, which plays a role in stress-induced apoptosis principally through activation of the JNK or the p38 MAP kinase signaling cascades (39–42)

Mycobacterium tuberculosis and *M. avium* are both facultative intracellular bacteria that reside and replicate inside the macrophages of the infected host. The relationship between the course of mycobacterial infection *in vivo* and the pro- or anti-apoptotic cell signaling within the host is still unclear. However, *in vitro* studies suggest that induction of apoptosis in macrophages is the host cell strategy to eliminate the invading pathogen, whereas prevention of programmed cell death can result in the possibility of prolonged intracellular survival of mycobacteria. The organism therefore tries to commandeer the host cell signaling machinery to offset cell death (43). The mechanisms of apoptotic cell death in macrophages challenged with mycobacteria are largely unknown, other than reports of apoptotic signaling involving both Fas and TNF receptor (5, 58). The present study was undertaken with the objective of understanding the signaling pathways mediating macrophage apoptosis upon challenge with *M. avium*. Our results suggest that *M. avium*-induced apoptosis involves FADD-mediated activation of caspase 8, cleavage of t-Bid, release of cyt *c* and activation of caspases 9 and 3. Further, we identify the ASK1/p38 signaling pathway as being critical to caspase 8 activation and subsequent *M. avium*-induced cell death.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies against total MAP and phosphospecific kinases, cleaved caspase 3, PARP, and caspase 9 were purchased from Cell Signaling Technology, Beverley, MA. Antibodies against ASK1, cyt *c*, β-actin, and Bid, and protein A/G (plus)-agarose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-ASK1-specific antibody was a gift from Hidenori Ichijo, Tokyo Medical and Dental University, Tokyo, Japan. *N*-acetylcysteine (NAC), SB203580, U0126, z-DEVD-FMK, z-LEHD-FMK, z-IETD-FMK, Ac-DEVD-pNA, Ac-LEHD-AFC, Ac-IETD-AFC, and protease inhibitors were from CN Biosciences (San Diego, CA).

Culture of the Murine Macrophage-like Cell Line J774A-1—Media and supplements were purchased from Invitrogen. 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 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Dishes were washed to remove non-adherent cells. Adherent cells were ≥95% viable as determined by trypan blue dye exclusion.

Growth of M. avium—*M. avium* (NCTC 8562) was obtained from the All India Institute of Medical Sciences New Delhi), grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with oleic acid-albumin-dextrose (OADC) supplement and 0.04% Tween 80 until mid-log phase, harvested, washed, and resuspended in a small volume of phosphate-buffered saline containing 0.04% (v/v) Tween 80. The suspension was briefly sonicated until no bacterial clumps were visualized by microscopy. The preparation was diluted to a concentration of 2×10^8 CFU/ml in 10% glycerol and stored in aliquots at −80 °C. Freshly thawed aliquots were used no more than once.

Plasmid Constructs and Transient Transfections—HA-tagged wild-type ASK1 (ASK1, WT) and a catalytically inactive mutant (K709M) of ASK1 (ASK1, KM) were obtained from Hidenori Ichijo. FLAG-tagged p8 MAPK and its dominant-negative mutant (p38 (agf)) were obtained from Roger Davis, University of Massachusetts Medical School, Worcester, MA, and dominant-negative FADD (FADD (dn)) was from Claudius Vincenz, University of Michigan, Ann Arbor, MI. 2×10^6 cells were

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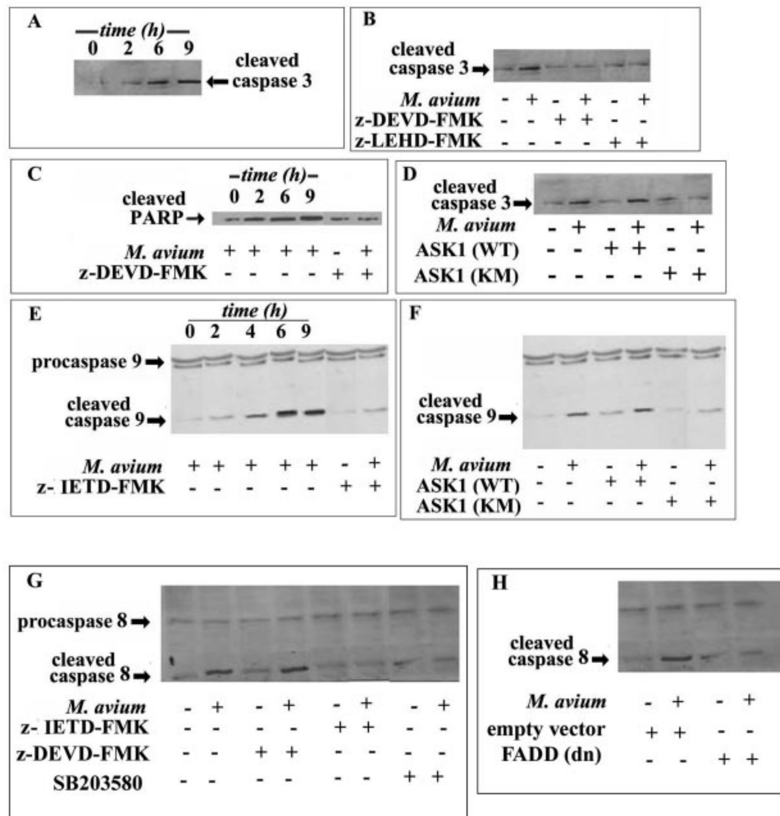

FIG. 3. **Western blot analysis of *M. avium*-induced caspase activation in J774A-1 macrophages.** J774A-1 macrophages were incubated with *M. avium* at an MOI of 10 for different periods of time (*panel A*), or J774A-1 macrophages were treated with z-DEVD-FMK (50 μM) or z-LEHD-FMK (50 μM) for 60 min prior to incubation without or with *M. avium* (*panel B*) at an MOI of 10 for 6 h. Cell lysates were prepared as described under "Experimental Procedures." Cleaved caspase 3 (17 kDa) was detected by Western blotting using antibodies against cleaved caspase 3. *Panel C*, J774A-1 macrophages were incubated with *M. avium* at an MOI of 10 for different periods of time, or J774A-1 macrophages were treated with z-DEVD-FMK (50 μM) 60 min (last two lanes) prior to incubation without or with *M. avium* at an MOI of 10 for 6 h. Cell lysates were prepared as described under "Experimental Procedures." Cleaved PARP (89 kDa) was detected by Western blotting using antibodies against cleaved PARP. *Panel D*, J774A-1 macrophages were either not transfected (first two lanes) or transfected with ASK1(WT) or with ASK1(KM) followed by incubation without or with *M. avium* at an MOI of 10 for 6 h. Cleaved caspase 3 (17 kDa) was detected as described under *panel A*. *Panel E*, J774A-1 macrophages were incubated with *M. avium* at an MOI of 10 for different periods of time or J774A-1 macrophages were treated with z-IETD-FMK (50 μM) for 60 min (last two lanes) prior to incubation without or with *M. avium* at an MOI of 10 for 6 h. Cell lysates were prepared as described under "Experimental Procedures." Procaspase 9 (52 kDa) and cleaved caspase 9 (37 kDa) was detected by Western blotting using antibodies against caspase 9. *Panel F*, J774A-1 macrophages were either not transfected (first two lanes), or transfected with ASK1(WT) or with ASK1(KM) followed by incubation without or with *M. avium* at an MOI of 10 for 6 h. Caspase 9 was detected as described under *panel E*. *Panel G*, J774A-1 macrophages were treated with z-IETD-FMK (50 μM) or z-DEVD-FMK (50 μM) or SB203580 (10 μM) for 60 min prior to incubation without or with *M. avium* at an MOI of 10 for 6 h. Cell lysates were prepared as described under "Experimental Procedures." Procaspase 8 (55 kDa) and cleaved caspase 8 (20 kDa) were detected by Western blotting using antibodies against caspase 8. *Panel H*, J774A-1 macrophages were transfected with empty vector or with FADD (dn) followed by incubation without or with *M. avium* at an MOI of 10 for 6 h. Caspase 8 was detected as described under *panel G*.

plated in each well of 6-well plates, and cells were transfected with 2 μg of plasmid (empty vectors or recombinants) using the Effectene reagent (Qiagen) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum according to the manufacturer's instructions. β-Galactosidase reporter plasmid was used to normalize transfection efficiencies.

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 or as stated under the legends to figures. Cells were lysed with 1% (v/v) Triton X-100, and the lysate was diluted and plated on Middlebrook 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 infection of 50% of the cells. The viability of the control monolayers was monitored during the experiments by the trypan blue exclusion method. The viability was ≥95% in all 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 sodium β-glycerophosphate, 4 μg/ml 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 (Me_2SO) alone, prior to incubation with *M. avium*. When performing Western blotting for detection of caspases, 6×10^6 cells were pelleted and freeze-thawed thrice in 20 μl of cell extraction buffer (50 mM PIPES/NaOH (pH 6.5), 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM pefabloc). The lysates were centrifuged at 10,000 × *g* for 5 min at 4 °C, and the supernatants were collected for detection of caspases.

Western Blotting—Proteins were separated on SDS-polyacrylamide gels and then transferred electrophoretically to polyvinylidene difluoride membranes. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and subsequently incubated overnight at 4 °C with primary antibodies in TBS-Tween 20 (1%, v/v) (TBST) with 5% (w/v) bovine serum albumin. Following three washes of 5 min each with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) (or appropriate secondary antibody) in blocking buffer for 1 h at room temperature. After three washes with TBST, the blots were developed with BM chemiluminescence reagent (Roche Applied Science) and exposed to x-ray film (Kodak XAR5).

ASK1 Kinase Assay—After treatment, cells were washed with ice-cold PBS, and lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1%(v/v) Triton X-100, 1% sodium deoxycholate, 0.5 mM sodium pyrophosphate, 1 mM sodium

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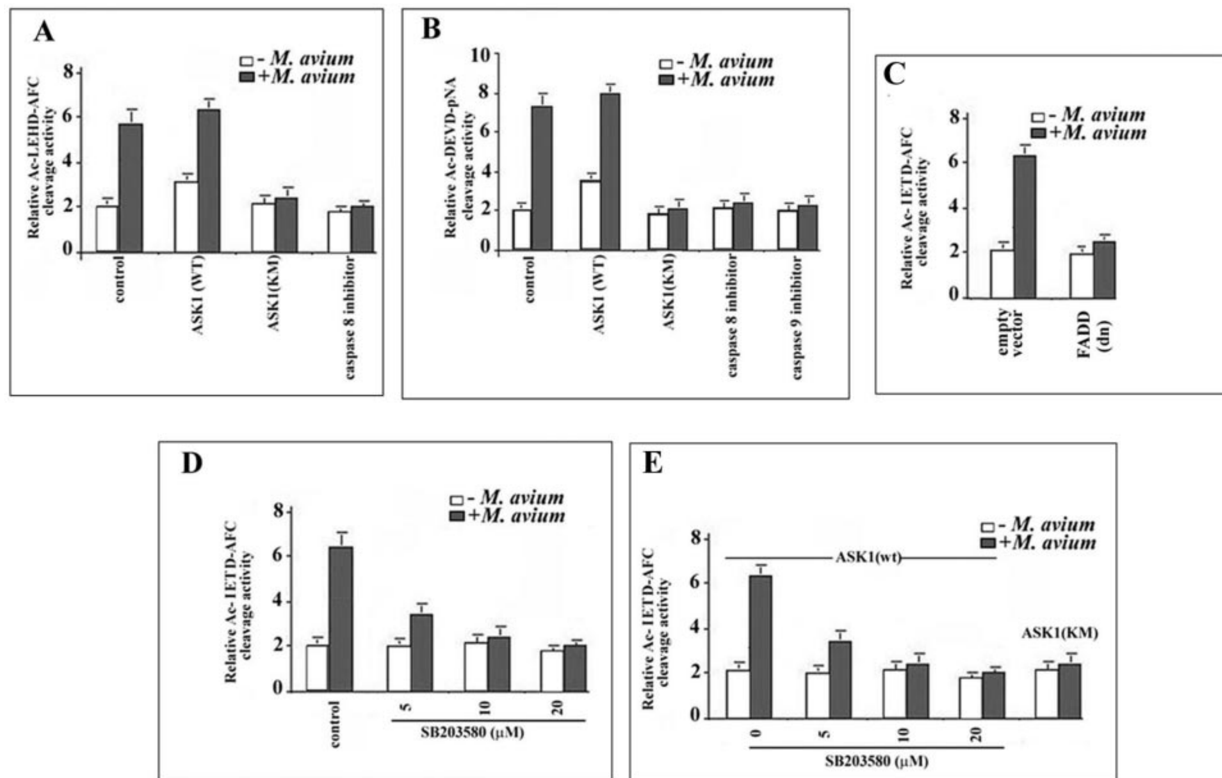

FIG. 4. ***M. avium*-induced caspase activity assays in J774A-1 macrophages.** J774A-1 macrophages were incubated without or with *M. avium* at an MOI of 10 for 6 h. Cell lysates were prepared and caspase activities were determined using 20 μg of protein with the fluorigenic substrates Ac-LEHD-AFC (for caspase 9) (*panel A*) Ac-IETD-AFC (for caspase 8) (*panels C*, *D*, and *E*) or the colorimetric substrate Ac-DEVD-pNA (for caspase 3) (*panel B*), as described under "Experimental Procedures." Cells were left untreated or treated with the caspase 8 inhibitor (z-IETD-FMK) (*panels A* and *B*) or the caspase 9 inhibitor (z-LEHD-FMK) (*panel B*) before challenge without or with *M. avium*. *Panel C*, cells were transfected with empty vector or FADD (dn) before incubation without or with *M. avium*. *Panel D*, cells were left untreated or treated with different concentrations of the p38 MAP kinase inhibitor SB203580 prior to incubation without or with *M. avium*. *Panel E*, cells were transfected with ASK1(WT) or ASK1(KM) followed by incubation without or with *M. avium*. Where indicated ASK1(WT)-transfected cells were treated with SB203580 at different concentrations prior to incubation without or with *M. avium*. Cleavage activities are expressed relative to that of uninfected and untreated cells. Results represent the mean of three separate experiments ± S.E.

β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin. Lysates were centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant (equivalent to 200 μg of protein) was incubated overnight at 4 °C on a rocker with rabbit polyclonal ASK1 antibody. 10 μl of protein A/G plus agarose was subsequently added and incubated at 4 °C for an additional 3 h. The beads were washed twice with lysis buffer and twice with kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM Na-β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO$_4$, 10 mM $MgCl_2$). The pellet was washed once with kinase buffer without protease inhibitors. The beads were then incubated in 20 μl of kinase buffer in the presence of 0.5 μCi of [γ-^{32}P]ATP (specific activity 3,000 Ci/mmol) with 1 μg of myelin basic protein (MBP) as substrate at 30 °C for 15 min. The reaction was stopped by adding protein gel denaturing buffer. After SDS-PAGE and autoradiography, the band intensities were quantified by densitometric scanning of the autoradiograms.

Cell Death ELISA—J774A-1 cells were plated (6 × 10^4 cells/plate) on 96-well plates. Cells were either treated with inhibitors or transfected with different plasmid constructs prior to incubation without or with *M. avium.* Cell death was detected with the cell death detection ELISA plus kit (Roche Applied Science) according to the manufacturer's protocol.

Caspase Activity Assays—Cells (6 × 10^5) were lysed by incubating with 25 μl of lysis buffer (25 mM HEPES (pH 7.5), 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM pefabloc) on ice for 15 min. The supernatant was kept frozen at −70 °C until used. Lysate (20–100 μg of protein) was taken in 100 μl of assay buffer containing 50 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM dithiothreitol, 10% glycerol, 0.1% CHAPS, and 200 μM substrate. For measuring caspase 3 activity, samples were incubated at 37 °C for 4 h with Ac-DEVD-pNA as substrate, and absorbance at 405 nm was measured. Caspase 8 and 9 activities were measured using the fluorigenic substrates Ac-IETD-AFC and Ac-LEHD-AFC respectively. Fluorescence of the released AFC was measured using excitation and emission wavelengths of 360 and 530 nm, respectively.

Isolation of Cytosol and Mitochondrial Fractions for Detection of Cyt c and Bid—After treatments, 18 × 10^6 cells were washed with ice-cold PBS, and cell pellets were resuspended in 400 μl of resuspension buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM pefabloc, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 150 mM sucrose). The cells were broken with 20 passages through a 26-gauge needle, and the homogenate was centrifuged at 750 × *g* for 10 min, to remove nuclei and unbroken cells. The mitochondrial pellet was obtained by centrifugation at 10,000 × *g* for 15 min and resuspended in 40 μl of resuspension buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM pefabloc, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The supernatant was centrifuged at 100,000 × *g* for 60 min at 4 °C. The supernatant obtained was the cytosolic fraction. Cytosolic and mitochondrial fractions (20 μg of protein) were used for quantification of cyt *c* and Bid, respectively. Prior to Western blotting, protein concentrations were assayed and all samples were normalized to equal protein concentrations. Blots were stripped and reprobed for β-actin expression.

RESULTS

M. avium Induces Activation of ASK1 and p38 MAP Kinases in J774A-1 Macrophages—Taking into account the facts that challenge of macrophages with *M. avium* leads to macrophage apoptosis (44) through a process in which oxidative stress plays a key role and that ASK1 is required for oxidative stress-induced apoptosis involving the stress-activated MAP kinases p38 and JNK, we asked the question whether ASK1 is activated in response to incubation of J774A-1 macrophages with *M. avium*. Incubation of macrophages with *M. avium* at an MOI of 10 led to a time-dependent increase in ASK1 kinase activity as evidenced by *in vitro* phosphorylation of MBP (Fig. 1, *panel A*). ASK1 kinase activity increased directly with increasing MOI (Fig. 1, *panel B*). Pretreatment of cells with NAC

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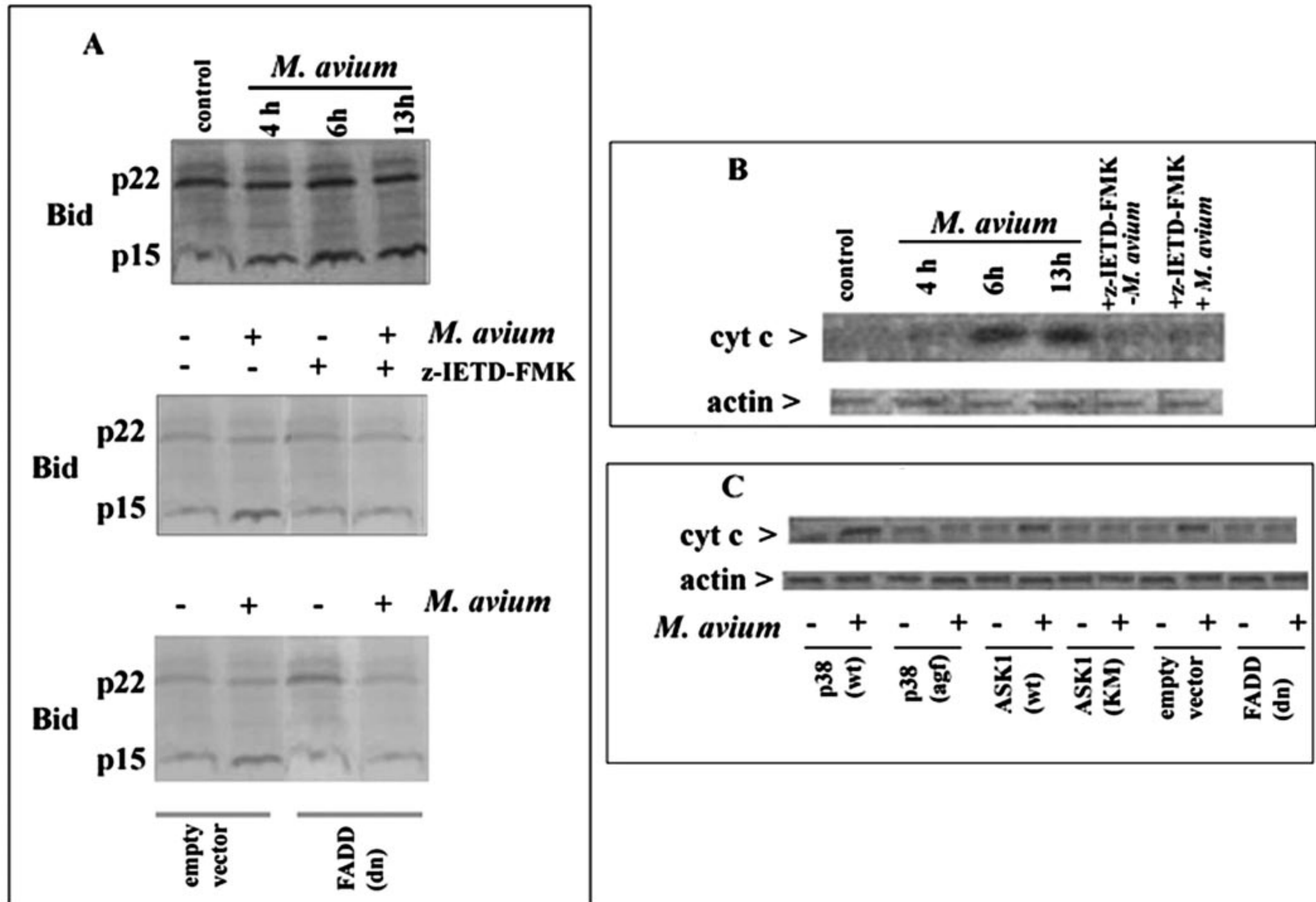

FIG. 5. ***M. avium*-induced cleavage of Bid and release of cyt *c* from the mitochondria.** *Panel A*, J774A-1 macrophages were incubated without (control) or with *M. avium* at an MOI of 10 for different periods of time (*upper blot*). In separate experiments, cells were either treated with the caspase 8 inhibitor z-IETD-FMK (50 μM) (*middle blot*) or transfected with empty vector or FADD (dn) (*lower blot*) prior to incubation without or with *M. avium* at an MOI of 10 for 6 h. Cells were processed to obtain mitochondrial fractions as described under "Experimental Procedures," and Bid was detected by Western blotting. *Panel B*, untreated cells or cells treated with caspase 8 inhibitor were incubated with *M. avium* as described under *panel A*. Cyt *c* and actin were detected in the cytosolic fraction by SDS-PAGE followed by Western blotting. *Panel C*, cells were transfected with p38 (wt) or p38 (agf), ASK1(WT) or ASK1(KM)) (*panel C*), FADD (dn), or empty vector followed by incubation without or with *M. avium* at an MOI of 10 for 6 h. The blot was developed as described for *panel B*. Blots are representative of results obtained from three separate experiments.

prior to challenge with *M. avium* abrogated the *M. avium*-induced ASK1 activation (Fig. 1, *panel C*), suggesting the probable involvement of reactive oxygen species (ROS) in ASK1 activation mediated by *M. avium*. Control experiments showed that equal amounts of precipitated ASK1 were used to assay kinase activity. In subsequent experiments, cells were transfected with wild type or a catalytically inactive mutant (KM) of ASK1, prior to challenge with *M. avium*. Whereas p38 MAP kinase activation was observed in ASK1(wt)-transfected cells challenged with *M. avium* as evidenced for phosphorylation of p38 MAP kinase (Fig. 1, *panel D*), no activation of p38 MAP kinase was evident in cells transfected with ASK1(KM). This suggested that ASK1 mediates the activation of p38 MAP kinase in *M. avium*-challenged macrophages. This effect was abrogated when cells were pretreated with the pharmacological inhibitor of p38 MAP kinase, SB203580 (45) (Fig. 1, *panel D*).

M. avium-induced Macrophage Cell Death—Taking into account previous reports that *M. avium* induces apoptosis of macrophages, and our results showing *M. avium*-induced ASK1 activation, we asked the question whether ASK1-dependent signaling is linked to death of J774A-1 macrophages challenged with *M. avium*. *M. avium* induced macrophage cell death in a time-dependent manner (Fig. 2, *panel A*) as measured using histone ELISA. Transfection of cells with catalytically inactive ASK1 abrogated *M. avium*-induced cell death (Fig. 2, *panel B*). Dominant-negative p38 MAP kinase exerted a similar effect, supporting the view that cell death was dependent on ASK1/p38 MAP kinase signaling. This was further supported by the fact that SB203580 could inhibit *M. avium*-induced cell death in a dose-dependent manner (Fig. 2, *panel C*), whereas U0126, an inhibitor of ERK 1/2 MAP kinase was without effect. In order to evaluate the role of FADD-mediated signaling in *M. avium*-induced apoptosis, cells were transfected with dominant-negative FADD (FADD(dn)) prior to challenge with *M. avium*. FADD (dn) abrogated *M. avium*-induced cell death (Fig. 2, *panel B*). FADD was not upstream of ASK1/p38 MAP kinase signaling since *M. avium*-induced ASK1 and p38 MAP kinase activation (assessed by probing Western blots with phospho-ASK1 and phospho-p38-specific antibodies) occurred even in FADD (dn)-transfected cells (Fig. 1, *panel E*). Cell death was also inhibited by treatment with NAC (Fig. 2, *panel C*). It is presently unclear how NAC regulates ASK1 activation and cell death in this case. ROS may oxidize thioredoxin thus dissociating it from ASK1 leading to ASK1 activation (46). It is also possible that a sequential signaling pathway links ROS production to ASK1 activation.

Members of the caspase family are crucial mediators of apoptosis. Therefore the role of caspases, in *M. avium*-mediated cell death was evaluated using specific inhibitors for different caspases. As evident from Fig. 2 (*panel D*), the involvement of

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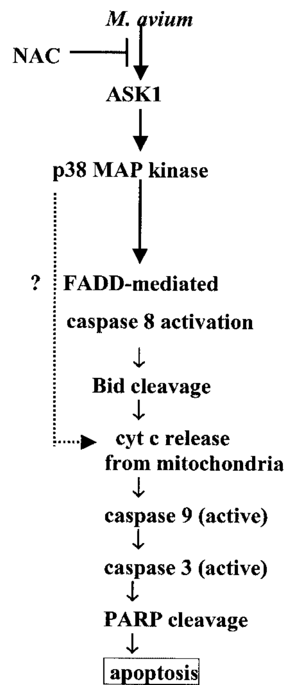

FIG. 6. **Proposed model for *M. avium*-induced ASK1- and caspase 8-dependent cell death.**

caspases 8, 9, and 3 was evident from the death-inhibitory effects z-IETD-FMK, z-LEHD-FMK, and z-DEVD-FMK, respectively.

Caspase Activities in M. avium-challenged J774A-1 Macrophages—Western blot analysis for cleaved, active caspases was undertaken to determine caspase activation. Consistent with the effect of caspase inhibitors on *M. avium*-induced cell death, *M. avium* was found to activate the executioner caspase 3 (Fig. 3, *panel A*) and the initiator caspase 9 (Fig. 3, *panel E*) in a time-dependent manner as evidenced by detection of the cleaved, activated form of the caspases by Western blotting. Caspase 3 activation was also supported by the detection of the cleaved form of the caspase 3 substrate PARP in *M. avium*-challenged macrophages (Fig. 3, *panel C*). The generation of cleaved caspase 3 could be blocked by pretreatment with the caspase 3-specific inhibitor z-DEVD-FMK as well as the caspase 9-specific inhibitor z-LEHD-FMK (Fig. 3, *panel B*). z-DEVD-FMK could also block the generation of cleaved PARP in *M. avium*-challenged macrophages (Fig. 3, *panel C*). Generation of cleaved caspase 3 and cleaved caspase 9 could be blocked by transfection of cells with ASK1(KM) (Fig. 3, *panels D* and *F*, respectively). Generation of cleaved caspase 9 could also be blocked by pretreating cells with the caspase 8-specific inhibitor z-IETD-FMK (Fig. 3, *panel E*). These data supported the role of ASK1 as well as caspase 8 in the activation of caspase 9 and subsequent activation of caspase 3 in *M. avium*-challenged macrophages. The role of caspase 8 as an initiator of *M. avium*-induced death signaling was supported by Western blotting analysis of pro- and active caspase 8. Cleaved caspase 8 was detected in *M. avium*-challenged macrophages (Fig. 3, *panel G*). This could be blocked in cells pretreated with the caspase 8-specific inhibitor z-IETD-FMK but not with the caspase 3-specific inhibitor z-DEVD-FMK. *M. avium*-induced caspase 8 activation was also blocked by pretreatment with the p38 MAP kinase inhibitor SB203580 (Fig. 3, *panel G*) suggesting a role of p38 MAP kinase in caspase 8 activation. The role of FADD-mediated signaling was suggested by the fact that caspase 8 activation was blocked in cells transfected with FADD (dn) (Fig. 3, *panel H*). The data obtained from Western blotting was further supported by caspase activity assays. *M. avium*-induced activation of caspase 9 was abrogated in cells transfected with ASK1(KM) or pretreated with the caspase 8 inhibtor z-IETD-FMK (Fig. 4, *panel A*). Caspase 3 activation could also be abrogated in cells transfected with ASK1(KM) or pretreated with the caspase 8 inhibtor z-IETD-FMK or the caspase 9 inhibtor z-LEHD-FMK (Fig. 4, *panel B*). Transfection with FADD (dn) blocked caspase 8 activation (Fig. 4, *panel C*). Western blotting and activity assays therefore suggested that *M. avium*-triggered caspase 8 activation likely involves FADD-dependent signaling. Of particular interest was the observation that caspase 8 activation was blocked in cells transfected with ASK1(KM) (Fig. 4, *panel E*). The p38 MAP kinase inhibitor SB203580 also inhibited caspase 8 activation in a dose-dependent manner both in untransfected (Fig. 4, *panel D*) as well as in ASK1-transfected (Fig. 4, *panel E*) cells. These results suggested that ASK1/p38 MAP kinase and FADD signaling lead to caspase 8 activation.

Generation of Truncated Bid and Release of Cyt c—The activation of caspase 9 in *M. avium*-challenged macrophages suggested a role of the mitochondrial pathway in *M. avium*-induced cell death. Taking into consideration that caspase 8 was also activated in *M. avium*-treated macrophages it appeared likely that caspase 8 activation was linked to the mitochondrial death pathway. Linkage of caspase 8 to mitochondrial death signaling occurs through the caspase 8-mediated cleavage of Bid and generation of t-Bid, which translocates to the mitochondria and promotes the release of cyt *c*. We tested whether *M. avium*-induced caspase 8 activation leads to signaling to the mitochondria by the generation of t-Bid. *M. avium*-treated macrophages showed time-dependent increase of t-Bid in the mitochondrial fraction (Fig. 5, *panel A*, *upper blot*). Generation of t-Bid was abrogated in caspase 8 inhibitor-treated- or in FADD(dn)-transfected cells (Fig. 5, *panel A*, *middle* and *lower blots*), suggesting that t-Bid formation is dependent both on caspase 8 activation and on FADD. Concomitant with this, cyt *c* was detected in the cytosol in a time-dependent manner although the content of actin remained the same (Fig. 5, *panel B*). Death signaling originating from FADD-dependent caspase 8 activation was likely amplified by mitochondrial cyt *c* release since pretreatment with the caspase 8 inhibitor z-IETD-FMK (Fig. 5, *panel B*) or transfection with FADD (dn) (Fig. 5, *panel C*) abrogated *M. avium*-induced cyt *c* release. Cyt *c* release was also blocked in cells transfected with dominant-negative p38 MAP kinase (agf) or ASK(KM) (Fig. 5, *panel C*), suggesting a role of ASK1/p38 MAP kinase signaling not only in caspase 8 activation but also in mitochondrial cyt *c* release in *M. avium*-challenged macrophages.

DISCUSSION

Previous reports have shown that macrophages infected with mycobacteria undergo apoptosis and that apoptosis serves as a mechanism of reducing bacillary viability. However, the signals leading to mycobacteria-induced host macrophage apoptosis remain largely unknown. We have attempted to dissect the mechanistic principles of mycobacteria-induced cell death in the murine macrophage cell line J774A-1 that possesses characteristics typical of macrophages (47). It has, in recent years, been extensively used to study interactions of mycobacteria with macrophages (12, 49–50).

The diverse signals associated with apoptosis converge upon the activation of procaspases which play a central role in the initiation and execution of apoptosis. In the present study we demonstrate that caspases 3, 8, and 9 are activated after challenge of macrophages with *M. avium* and that these are all required for mycobacteria-induced death of J774A-1 macrophages. Inhibition of any one of these caspases with caspase-

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specific peptide inhibitors was sufficient to block cell death.

Interestingly, we observed that the stress-activated MAP kinase kinase kinase ASK1 is activated in *M. avium*-challenged macrophages. NAC inhibited *M. avium*-induced cell death by inhibiting the activation of ASK1. However, it remains to be elucidated whether the effect of NAC was due to ROS-triggered sequential signaling leading to ASK1 activation or due to dissociation of thioredoxin from ASK1. That ASK1 plays a central role in innate immunity against *M. avium* was supported by the observation that a catalytically inactive mutant of ASK1 could block *M. avium*-induced cell death. p38 MAP kinase appeared likely to be the downstream effector of ASK1, since ASK1 activated p38 MAP kinase and a dominant-negative mutant of p38 MAP kinase, or the p38 MAP kinase-specific inhibitor SB203580 could block *M. avium*-induced cell death. The link between ASK1/p38 MAP kinase signaling and caspase activation was evident from the observation that a catalytically inactive mutant of ASK1 could prevent activation of caspases 8, 9, and 3. Treatment with SB203580 was also associated with the inhibition of caspase 8 activation. Our results suggest a role of ASK1/p38 signaling in caspase 8 activation. The release of cyt *c* from the mitochondria could be blocked by caspase 8 inhibitor, suggesting that the death signal triggered by caspase 8 activation is amplified by the mitochondrial release of cyt *c*. Cyt *c* release could also be blocked in p38 (agf)-transfected cells suggesting that ASK1/p38 MAP kinase signaling plays a crucial role not only in activation of caspase 8 but also in downstream events leading to the release of cyt *c* and activation of caspases 9 and 3 (Fig. 6). Caspase 3 inhibition did not affect the activation of caspase 8 ruling out caspase 3 as the mediator of caspase 8 activation.

In order to examine the role of FADD in *M. avium*-induced signaling leading to cell death, we transfected cells with FADD (dn) and examined downstream events. The role of FADD-dependent signaling was demonstrated by the abrogation of *M. avium*-induced caspase 8 activation and cell death in FADD (dn)-transfected cells. However, FADD was not upstream of ASK1/p38 MAP kinase activation since FADD (dn) transfection did not affect *M. avium*-induced ASK1 and p38 MAP kinase activation. Since death signals are in several instances amplified through the mitochondrial pathway by the caspase 8-mediated cleavage of Bid and its translocation to the mitochondria, we analyzed the status of Bid in the mitochondrial fractions of *M. avium*-challenged macrophages. Western blotting showed an increase in the fraction of t-Bid in the mitochondria as a function of time of challenge with *M. avium*, whereas caspase 8 inhibitor or FADD(dn) transfection could block the generation of t-Bid in the mitochondrial fraction, suggesting that FADD-dependent, and caspase 8-mediated Bid cleavage activates the mitochondrial death pathway in this case. This was supported by the concomitant detection of cyt *c* in the cytosolic fraction.

Our results provide new insight into the role of ASK1 signaling in cell death in macrophages and differ from the results obtained with mink lung epithelial cells where ASK1-induced apoptosis has been reported to occur through mitochondria-dependent caspase activation in a caspase 8-independent manner (51). Similar to our observations Raoul *et al.* (52) have demonstrated the involvement of ASK1 and p38 MAP kinase together with the classical FADD/caspase-8 pathway in the Fas-triggered death of embryonic motor neurons, whereas Gilot *et al.* (53) have demonstrated that hepatoprotection results from both inhibition of the caspase cascade and prevention of ASK1-JNK/p38 MAP kinase signaling.

We hypothesize that upon *M. avium* challenge, FADD-mediated caspase 8 activation occurs downstream of ASK1/p38 MAP kinase signaling (Fig. 6). ASK1/p38 MAP kinase signaling also leads to the release of cyt *c*. Caspase 8-mediated cleavage of Bid and its translocation to the mitochondria is probably associated with the release of cyt *c* leading to the formation of an apoptosome, activation of caspase 9 and finally to the execution of apoptosis mediated by activation of caspase 3. Critical to the activation of caspase 8 is the formation of the DISC. Since ASK1 activates caspase 8, it is possible that ASK1 exerts its effects upstream of the mitochondria probably at the level of the formation of the DISC. Progression of the apoptotic signal depends on an interplay of protein-protein interactions at different levels. Among these, cellular FLICE-like inhibitory protein (c-FLIP) prevents FADD-mediated apoptotic transduction through inhibition of caspase 8 maturation (54–56). It appears possible that ASK1 acts at the level of regulating c-FLIP activity. This could be at the level of synthesis or degradation of c-FLIP or through its phosphorylation (57) as suggested in recent reports. On the other hand, ASK1/p38 MAP kinase signaling could also possibly regulate caspase 8 activity by phosphorylating caspase 8 itself. However, to the best of our knowledge, there are to date no reports of phosphorylation-dependent activation of caspases. It is also possible that ASK1/p38 MAP kinase signaling could lead to increased death receptor ligand expression as documented in the case of p38 MAP kinase-dependent overexpression of Fas ligand in intestinal epithelial cells undergoing anoikis (48). Testing of these possibilities is the subject of a separate investigation. The importance of the present investigation lies in the fact that it is the first elucidation of a signaling pathway through which ASK1 plays a central role in innate immunity and therefore in the containment of bacterial infection. It also one of the few recent reports of the ASK1-dependent regulation of caspase 8 activation.

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