Endothelial MKK3 Is a Critical Mediator of Lethal Murine Endotoxemia and Acute Lung Injury

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Sepsis is a leading cause of acute lung injury and death in critically ill patients in the United States. It is estimated that sepsis develops in 750,000 people, of whom >210,000 die every year (1). No curative therapy is available—only supportive care. Furthermore, the incidence of sepsis is predicted to increase with the aging of our expanding population. Targeted biologic therapies are urgently needed. Our study offers new insights into the critical role of endothelial MAPK kinase 3 (MKK3), a component of the p38 MAPK pathway, in lethal sepsis.

MAPK pathways are core components of signal transduction in the cell. They are intracellular signaling pathways that mediate cell survival and death; proliferation; and differentiation in response to a wide variety of signals, such as cytokines, growth factors, UV light, osmotic stress, and LPS. Three distinct MAPK subfamilies exist: ERK, JNK, and p38. The p38 MAPK subfamilies are activated by various cellular stimuli, including cytokines, growth factors, stress, and inflammatory mediators. The p38 MAPK subfamily consists of three members: p38α, p38β, and p38γ. The p38 MAPK pathway plays a critical role in the regulation of cell growth, differentiation, survival, and death; proliferation; and differentiation in response to stress stimuli. The p38 MAPK pathway has been implicated in the regulation of inflammation, apoptosis, and cell proliferation.

MKK3 is a critical mediator of lethal murine endotoxia and acute lung injury. This work was supported by American Heart Association Grant AHA 09FTF2090019 (to P.J.L.) and by National Institutes of Health/National Heart, Lung, and Blood Institute, Grants R01 HL090660 and R01 HL071595 (to P.J.L.). Address correspondence and reprint requests to Dr. Patty J. Lee, Pulmonary and Critical Care Medicine, Department of Internal Medicine, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208057, New Haven, CT 06520-8057. E-mail address: patty.lee@yale.edu

Abbreviations used in this article: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAL, bronchoalveolar lavage; BUN, blood urea nitrogen; CT, threshold cycle; MDA, malondialdehyde; MKK3, MAPK kinase 3; MPO, myeloperoxidase; Nox, NADPH oxidase; ROS, reactive oxygen species; siRNA, small interfering RNA; WT, wild-type.

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Materials and Methods

Generation of MKK3+/− mice

MKK3+/− mice were generated by deletion of exons 8 and 9, which encode aa 217–221 of the murine MKK3 protein, as previously described (7).
MKK3+/− mice expressed normal levels of MKK6, MKK4, JNK, and p38 MAP kinases, and, thus there were no compensatory changes in the expression of these other kinases as a consequence of MKK3 deficiency. The MKK3+/− mice were provided by R. Davis (University of Massachusetts Medical School, Worcester, MA) and R. Flavell (Yale University, New Haven, CT) and have been backcrossed onto a C57BL/6 background for >15 generations.

**LPS exposure**

For survival and injury studies, the mice were given 40 mg/kg and 5 mg/kg LPS i.p., respectively (Escherichia coli055:B5; Sigma–Aldrich). Body-surface temperature was measured using an Infrascan infrared thermometer (La Crosse Technologies). Bronchoalveolar lavage (BAL) was performed by tracheal cannulation, and whole-lung lavage was performed twice with a total volume of 1.8 ml ice-cold PBS. BAL was centrifuged at 3000 g, and the protein concentration of the supernatant was determined using the BCA Protein Assay (Pierce Labs). Mouse serum troponin I was measured using an ELISA kit (Life Diagnostics) according to the manufacturer’s protocol.

**Irradiation and bone marrow transplantation**

Whole-body irradiation of recipient mice and harvesting of donor bone marrow were performed as described previously (17). Briefly, donor bone marrow was flushed from the femora, tibiae, and humeri of mice. Cells were pelleted at 300 g for 10 min at 4˚C before counting. Recipient mice at 6 wk of age underwent whole-body irradiation (1000 GY), followed by i.v. injection of whole bone marrow cells (9 × 106 cells in 0.2 ml PBS). After bone marrow transplantation, mice were maintained until 3 mo of age under specific pathogen–free conditions at the Yale University School of Medicine animal facility and fed acidic water.

**Isolation of primary lung endothelial cells**

Endothelial cells were isolated as described by Kuhlencordt et al. (18), with some modifications. Briefly, lungs were extracted, minced, and digested for 1 h with 0.1% collagenase (Roche Diagnostics). The digest was passed through a 100-μm cell strainer and pelleted at 200 g for 5 min; resuspended in endothelial medium containing 20% FBS, 40% DMEM, and 40% F12 with 100 U/ml penicillin G and 100 μg/ml streptomycin; and plated onto 0.1% gelatin-coated T75 flasks. Cells were cultured for 2–4 d and resuspended in 2% FBS containing 10 μl biotin-labeled rat anti-mouse CD31 (PECAM-1) Ab (BD Biosciences–Pharmingen). After incubation on ice for 30 min, the cells were washed with streptavidin magnetic beads (New England BioLabs). Cells were washed with 2% FBS, resuspended in 5 ml 2% FBS, and incubated on ice for 30 min. The cells were then pelleted onto the magnet for 5 min; unbound cells were removed, and bound cells were resuspended in medium and plated onto 0.1% gelatin-coated T25 flask. We confirmed with CD31 staining and flow cytometry that >95% of the cells were endothelial cells.

**Small interfering RNA knockdown of MKK3**

ON-TARGETplus SMARTpool small interfering RNA (siRNA) against MKK3 and scrambled siRNA were obtained from Thermo Scientific (formerly Dharmacoyn RNAi Technologies). Endothelial cells were seeded onto six-well plates 1 d prior to transfection, using 40% DMEM and 40% F12 tissue culture medium supplemented with 20% FBS, without antibiotics. At the time of transfection with the specific siRNA, the cells were 50–60% confluent. Lipofectamine 2000 Reagent (Invitrogen) was used as the transfection agent. After 48 h of incubation, the cells were collected and subjected to assays.

**TUNEL assay**

We used the In Situ Cell Death Detection Kit according to the manufacturer’s protocol (Roche Applied Science). Sections of formalin-fixed, paraffin-embedded lung tissue were deparaffinized and rehydrated, rinsed with PBS, and digested with proteinase K (Roche Applied Science) at a concentration of 20 μg/ml for 20 min. After PBS washes, sections were incubated with TUNEL reaction mixture at 37˚C for 3 h, then incubated with antifluorescein conjugated with alkaline phosphatase at 37˚C for 30 min. Sections were washed twice with PBS and stained with NBT/5-bromo-4-chloro-3-indolyl phosphate substrate solution before counterstaining with nuclear fast-red. Apoptotic and normal cells were observed under a light microscope. Normal nuclei exhibited red counterstaining, whereas TUNEL-positive cells, indicating cell death/apoptosis, exhibited purple nuclear staining. A total of 500 cells were counted for each sample, and the number of apoptotic cells is expressed as a percentage of the total counted.

**Myeloperoxidase assay**

Myeloperoxidase (MPO) levels were assessed as follows. Lung tissue was homogenized in 50 mM phosphate buffer (pH 6.0). Then, after centrifugation at 10,000 g for 15 min, the pellet was resuspended in 50 mM hexadecyltrimethylammonium bromide (Sigma–Aldrich) in 50 mMol/l potassium phosphate buffer, pH 6.0, before sonication for 20 s in an ice bath. The samples were freeze thawed three times, after which sonication was repeated. Suspensions were then centrifuged at 10,000 g for 10 min. MPO activity was assayed spectrophotometrically by mixing 0.1 ml supernatant with 2.9 ml 50 mMol/l phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride (Sigma–Aldrich) and 0.0005% hydrogen peroxide (Sigma–Aldrich). The change in absorbance at 460 nm was measured using a spectrophotometer (SmartSpec300; Bio-Rad) periodically for 3 min. MPO activity was then derived from the observed change in absorbance per minute.

**Western blot analysis**

Protein extracts were taken from cells using a radioimmunoprecipitation assay buffer, electrotransferred, and immunoblotted with primary Abs. Detection was performed with an HRP Western Blot Detection System (Cell Signaling Technology). Equivalent sample loading was confirmed by stripping membranes with Blot Restore Membrane Rejuvenation solution (Thermo Scientific) and probing for actin, α-tubulin, or lamin A/C. All of the Abs were obtained from Santa Cruz Biotechnology, except p-IκBα/β (Cell Signaling Technology). Nuclear and cytoplasmic fractions of endothelial cells were obtained using an NE-PER kit (Thermo Scientific) according to the instructions. Western blots of nuclear and cytoplasmic fractions were then performed as detailed before.

**EMSA**

Nuclear extracts were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Scientific) according to the manufacturer’s protocol. The AP-1 site was synthesized as complementary oligodeoxyribonucleotide strands. The sequence of AP-1 consensus oligonucleotides was 5'-CCG TTT ATG ACT CAG CCG GAA-3' (Sigma–Aldrich). The DNA binding ability of AP-1 in the nuclear extracts was assessed by EMSA with biotin-labeled, double-stranded AP-1. EMSA was carried out using the LightShift Chemiluminescent EMSA Kit (Pierce). Specific binding was confirmed using a 200-fold excess of an unlabelled probe as a specific competitor. Protein–DNA complexes were separated using a 6% non-denaturing acrylamide gel electrophoresis and then transferred to positively charged nylon membranes and cross-linked by UV irradiation. Gel shifts were visualized with streptavidin HRP according to standard protocols.

**Oxidant assays**

Malondialdehyde (MDA) was measured using the Lipid Peroxidation Assay Kit (Calbiochem; EMD Biosciences) according to the manufacturer’s instructions. CM-H2DCFDA and MitoSOX Red (Invitrogen) were used to determine levels of ROS in endothelial cells. Cells were seeded onto six-well non–tissue culture plates 1 d before the experiment. On the next day cells were stimulated with LPS (1 μg/ml, 6 h). Cells were washed and exposed to CM-H2DCFDA (5 μM) or MitoSOX Red (2.5 μM) in regular medium and kept at 37˚C for 25 min. The cells were washed with PBS and then detached gently with 0.4% EDTA and analyzed on a BD FACSCalibur machine. CM-H2DCFDA was detected in the FL-1 channel, and MitoSOX Red was detected in the FL-3 channel. Rotenone was purchased from Sigma–Aldrich, and Mito-TEMPO was purchased from Santa Cruz Biotechnology.

**Real-time RT-PCR**

Total RNA was extracted from one-half of one lung or cells using TRIzol reagent according to the manufacturer’s protocol (Life Technologies BRL). First-strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) with random hexamers; conditions were 10 min at 25˚C, 30 min at 48˚C, and 5 min at 95˚C. Real-time RT-PCR reactions were carried out with the Power SYBR Green PCR Master Mix (Applied Biosystems) and an ABI Prism 7000 Sequence Detection System (Applied Biosystems). GAPDH was amplified as a control. Real-time PCR conditions were 95˚C for 10 min and 40 cycles of 95˚C for 15 s, followed by 60˚C for 1 min. The relative quantification values for these gene expressions were calculated from the accurate threshold cycle (CT), which is the PCR cycle at which an increase in reporter fluorescence from SYBR green dye can first be detected above a baseline signal. The CT values for GAPDH were subtracted from the CT values for ICAM-1 and MKK3 in each well, to calculate ΔCT. The ΔCT values for each sample were averaged. To calculate the fold induction over controls (ΔΔCT), the average ΔCT values calculated for wild-type (WT) animals or cells were subtracted.
from ΔCT values calculated for MKK3 \(^{-/-}\) animals or cells. Next, the fold induction for each well was calculated using the \(2^{-\Delta\Delta CT}\) formula. The fold induction values for replicate wells were averaged, and data were presented as the mean ± SEM of triplicate wells.

FIGURE 1. MKK3 \(^{-/-}\) mice have less cell death after systemic LPS. (a) WT and MKK3 \(^{-/-}\) mice were given i.p. LPS (40 mg/kg), and TUNEL staining was performed on lung sections. Arrows point to TUNEL-positive cells. The number of TUNEL-positive cells was quantified and expressed as a percentage of the total number of lung cells counted on each section, mean ± SEM. \(n = 3\), *\(p < 0.05\). (b) WT and MKK3 \(^{-/-}\) mice were given IP LPS (40 mg/kg), and TUNEL staining was performed on organ sections.
Results

MKK3−/− mice are resistant to lung and organ injury after systemic LPS

We initially sought to determine whether MKK3-deficient mice are resistant to organ injury after LPS exposure. We observed lower levels of cell death in the lungs and vasculature of MKK3−/− mice, as shown by TUNEL staining (Fig. 1a). Other organs such as kidney,
spleen, liver, and heart also showed substantially fewer TUNEL-positive cells in MKK3−/− mice (Fig. 1b). BAL cell counts were not elevated in WT and MKK3−/− mice, indicating absence of inflammatory cell influx into the alveolar space, typical of i.p. LPS, as reported by others (19). However, parenchymal lung inflammation was markedly increased in WT mice, as observed in representative histopathologic sections of the lung (Fig. 2A). BAL protein levels, a measure of lung endothelial barrier disruption, were significantly elevated in WT mice compared with MKK3−/− mice (Fig. 2B). Lung MPO levels, a measure of neutrophil recruitment, were significantly decreased in MKK3−/− mice compared with WT mice after i.p. LPS. We found similar differences in MPO levels in kidney and liver, key target organs of systemic LPS (Fig. 2C). We measured serum markers of organ injury in mice given LPS. We found significantly higher levels of transaminases (AST, ALT) and BUN in WT mice given LPS, compared with MKK3−/− mice, indicating higher liver and kidney damage, respectively. We also checked levels of creatinine, another marker of kidney injury, and troponin I, a marker of myocardial injury (Fig. 2D). Although a trend toward higher creatinine and troponin I levels in septic WT mice was noted, the differences did not reach statistical significance (data not shown). Collectively, these data showed that MKK3-deficient mice are resistant to lung and systemic organ injury after LPS exposure.

Nonhematopoietic cells are important in MKK3-mediated endotoxemia

Next, we determined whether decreased organ injury in MKK3−/− mice correlated with changes in survival and the relative contribution of MKK3 in cells of hematopoietic versus nonhematopoietic lineages to the responses. Survival studies showed that MKK3−/− mice transplanted with WT bone marrow were still protected against lethal LPS, suggesting that the loss of MKK3 in nonhematopoietic cells is sufficient for improved survival (Fig. 2A). WT mice transplanted with MKK3−/− bone marrow appeared to have a trend toward improved survival after i.p. LPS, compared with WT mice transplanted with WT bone marrow, but this trend was not statistically significant (Fig. 3A). For the purposes of this article, we focused on the role of endothelial MKK3, because of the significant survival advantage that MKK3 deficiency in nonhematopoietic lineages provided. Using the idea that the body temperature of mice is an accurate marker of survival in various models of sepsis (20), we found that MKK3−/− mice transplanted with WT bone marrow exhibited body temperatures similar to those of MKK3+/− mice transplanted with MKK3−/− bone marrow after LPS, indicating that MKK3 deficiency in nonhematopoietic cells plays a more important role in protection than does MKK3 deficiency in hematopoietic cells. WT mice transplanted with MKK3−/− bone marrow did not have a statistically significant recovery of body temperatures 6 h after LPS, consistent with the survival data (Fig. 3B). Of note, the basal body temperatures were similar in all groups. These data suggest that the loss of MKK3 in primarily nonhematopoietic cells has sufficient protective effects against lethal LPS to override any deleterious effects that MKK3 reconstitution, at least in bone marrow, may have. On the basis of these data and the known critical role of endothelium in sepsis, we chose to focus our subsequent in vitro studies on lung endothelial cells.

ICAM-1 expression is lower in MKK3−/− organs and endothelial cells after LPS

Compared with WT lungs, MKK3−/− lungs had decreased inflammatory influx after LPS (Fig. 2). Given that endothelial adhesion and chemokine molecules are important in inflammatory cell recruitment, we analyzed MKK3−/− lungs for ICAM-1, VCAM-1, selectin E, CXCL-1, and CXCL-2. In the molecule profile we analyzed, ICAM-1 expression was notably different in MKK3−/− lungs after LPS, compared with WT lungs (Fig. 4A). Levels of VCAM-1 and CXCL-2 were also lower in MKK3−/− lungs, but we decided to focus on ICAM-1, given the strong association of ICAM-1 with septic responses in previous reports (21). ICAM-1 mRNA was decreased in the lungs, kidney, and liver of MKK3−/− mice after LPS (Fig. 4B). As ICAM-1 is expressed by endothelial cells and function in inflammatory cell recruitment (22), we proceeded to study ICAM-1 in primary mouse lung endothelial cells. We found that ICAM-1 mRNA and protein levels were decreased in MKK3−/− cells at baseline and after LPS stimulation (Fig. 4C, 4D). We examined surface expression of ICAM-1 by flow cytometry and, consistent with our cell lysate data, we found decreased surface expression of ICAM-1 in MKK3−/− endothelial cells (Fig. 4E). We confirmed specific regulation of ICAM-1 by MKK3, using siRNA against MKK3. An ~50% reduction in MKK3, using siRNA in WT endothelial cells, caused a small but significant reduction of ICAM-1 after LPS exposure (Fig. 4F). These studies show for the first time, to our knowledge, that MKK3 is an upstream regulator of ICAM-1 in organs and endothelial cells and a viable therapeutic target.

Activation of ICAM-1–related transcription factors was lower in MKK3−/− endothelial cells after LPS

To identify the mechanism whereby MKK3 regulates ICAM-1, we explored the role of NF-κB and its upstream kinase, IKKα/β. NF-κB has been demonstrated to regulate ICAM-1 via the IKK path-
FIGURE 4. ICAM-1 expression is lower in M KK3\(^{-/-}\) organs and endothelial cells after LPS. (A) WT and M KK3\(^{-/-}\) mice were given i.p. LPS (40 mg/kg), and ICAM-1, VCAM-1, selectin E, CXCL-1, and CXCL-2 mRNA were measured by real-time PCR in lung lysates. The values are expressed as mean fold induction over untreated mice ± SEM. n = 5, *p < 0.05. (B) WT and M KK3\(^{-/-}\) mice were given i.p. LPS (40 mg/kg), and ICAM-1 mRNA was measured by real-time PCR in lung, kidney, and liver lysates. The values are expressed as mean fold induction over untreated mice ± SEM. n = 5, *p < 0.05. (C) Primary cultures of lung endothelial cells were exposed to LPS (1 μg/ml), and mRNA levels of ICAM-1 were measured at different time points. The values are expressed as mean fold induction over unstimulated cells ± SEM. *p < 0.05. The results are representative of at least three independent experiments. (D) Lung endothelial cells were exposed to LPS (1 μg/ml), and protein levels of ICAM-1 were assessed at different time points. Actin was detected as a loading control. The results are representative of at least three independent experiments. (E) Lung endothelial cells were exposed to LPS (1 μg/ml), and surface expression of ICAM-1 was measured using flow cytometry. The values are expressed as mean fluorescent intensity ± SEM. *p < 0.05. The results are representative of at least three independent experiments. (F) Knockdown of M KK3 in WT lung endothelial cells was achieved using siRNA, and M KK3 or ICAM-1 mRNA expression was detected by real-time PCR after LPS stimulation (1 μg/ml). The values are expressed as mean fold induction over unstimulated cells ± SEM. *p < 0.05. The results are representative of at least three independent experiments.
way (23, 24). We found reduced translocation of NF-κB to the nucleus in \(MKK3^{-/-}\) endothelial cells at baseline and after LPS, as shown by Western blots of NF-κB in nuclear and cytoplasmic fractions (Fig. 5A). The loss of NF-κB nuclear translocation is known to be caused by the inability of the upstream kinase, IKKα/β, to be phosphorylated and subsequently inactivated. Therefore, we determined whether IKKα/β phosphorylation is altered in \(MKK3^{-/-}\) cells. Consistent with our NF-κB Western blots, we found reduced phosphorylation of IKKα/β in \(MKK3^{-/-}\) endothelial cells compared with WT cells at baseline and in response to LPS (Fig. 5B). ICAM-1 transcription is also dependent on the transcription factor AP-1, which appears to cooperatively interact

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Activation of ICAM-1 transcription factors was lower in \(MKK3^{-/-}\) endothelial cells after LPS. (A) After WT and \(MKK3^{-/-}\) lung endothelial cells were exposed to LPS (1 μg/ml), cell nuclear and cytoplasmic fractions were isolated and analyzed for the p65 subunit of NF-κB by Western blots. α-Tubulin was detected as cytoplasmic protein loading controls. Lamin A/C was detected as nuclear protein loading controls. The results are representative of at least three independent experiments. (B) WT and \(MKK3^{-/-}\) lung endothelial cells were exposed to LPS (1 μg/ml), and lysates were analyzed for phosphorylated (p)-IKKα/β by Western blots. α-Tubulin was detected as protein loading controls. The results are representative of at least three independent experiments. (C) Nuclear extract prepared from the control or mouse lung endothelial cells transfected with MKK3 siRNA and followed by LPS treatment (1 μg/ml) was mixed with biotin-labeled oligonucleotide containing AP-1 motif. Bound complexes were analyzed by electrophoresis. The results are representative of at least three independent experiments. (D) Competitive inhibition of AP-1 binding with nonlabeled probe. Nuclear extract prepared from the control or mouse lung endothelial cells transfected with MKK3 siRNA and followed by LPS treatment (1 μg/ml) was mixed with biotin-labeled oligonucleotide containing AP-1 motif along with 200× unlabeled probe. Bound complexes were analyzed by electrophoresis. The results are representative of at least 3 independent experiments. (E) Control or WT mouse lung endothelial cells were transfected with MKK3 siRNA and treated with LPS (1 μg/ml). Cell lysates were analyzed by Western blot to determine efficacy of MKK3 inhibition. The results are representative of at least three independent experiments.
with NF-κB in ICAM-1 gene regulation (25, 26). Hence, we examined whether AP-1 activity is altered in MKK3−/− cells. We found by EMSAs there was less AP-1 binding to the target sequence in MKK3−/− cells compared with WT cells at baseline and after LPS (Fig. 5C–E). These results show that MKK3 regulates both basal and LPS-induced AP-1 and NF-κB–IKKα/β activation and that a consequence of MKK3 deficiency is decreased AP-1, NF-κB, and ICAM-1 expression. To our knowledge, our studies are the first to identify a regulator of constitutive AP-1, NF-κB, and ICAM-1 expression in endothelial cells.

**ROS production was lower in MKK3−/− mice and endothelial cells after LPS**

LPS is known to induce ROS and activate NF-κB in neutrophils (27). To investigate whether MKK3 is involved in LPS-induced ROS generation, we checked MDA levels in the serum of LPS-exposed mice. MDA assay measures lipid peroxidation and is a marker of ROS excess. We found that MDA levels were significantly lower in MKK3−/− mice compared with that of WT mice after LPS (Fig. 6A). We also detected intracellular ROS in endothelial cells by measuring CM-H2DCFDA, an indicator of H₂O₂, a major component of ROS. We found that CM-H2DCFDA levels were significantly lower in MKK3−/− endothelial cells at baseline and after LPS exposure (Fig. 6B), indicating that the absence of MKK3 is associated with decreased ROS. The major sources of ROS in cells are either the cytoplasmic families of NADPH oxidases (Nox) or related family of dual oxidases (Duox) or the mitochondria. To determine the source of ROS, we initially checked mRNA expression of Nox 1–4 and Duox 1,2 by PCR in lungs and endothelial cells and did not find any difference between WT and MKK3−/− mice and endothelial cells. Furthermore, when we specifically inhibited Nox, using diphenylene iodonium, we did not see any difference in ICAM-1 expression in WT endothelial cells (data not shown). We then considered the mitochondria as a source and investigated the levels of mitochondrial ROS, using MitoSOX Red, a fluorescent

![MDA assay](image1.png)

**A** WT and MKK3−/− mice were given i.p. LPS (40 mg/ml), and MDA levels were measured in serum. n = 5–8, mean ± SEM shown, *p < 0.05. **B** WT and MKK3−/− lung endothelial cells were exposed to LPS (1 μg/ml). Cells were stained with CM-H2DCFDA, which detects H₂O₂; levels were measured by flow cytometry. The values are expressed as mean fluorescent intensity ± SEM. *p < 0.05. The results are representative of at least three independent experiments. Representative fluorescence histograms of LPS-exposed cells are shown. **C** WT and MKK3−/− lung endothelial cells were exposed to LPS (1 μg/ml). Cells were stained with MitoSOX Red, which detects mitochondrial ROS; levels were measured by flow cytometry. The values are expressed as mean fluorescent intensity ± SEM. *p < 0.05. The results are representative of at least three independent experiments. Representative fluorescence histograms of LPS-exposed cells are shown.
dye specific for mitochondrial ROS (28). We found that levels of mitochondrial ROS were lower in MKK3<sup>−/−</sup> than in WT endothelial cells at baseline and in response to LPS (Fig. 6C).

**Mitochondrial ROS is upstream of ICAM-1**

Next, we determined whether causative links existed between reduced mitochondrial ROS and ICAM-1 expression in MKK3<sup>−/−</sup> endothelial cells. We asked if specific induction of mitochondrial ROS can restore ICAM-1 expression in MKK3<sup>−/−</sup> endothelial cells. Cells were exposed to rotenone, a mitochondrial respiratory chain inhibitor that induces production of mitochondrial ROS. We found that ICAM-1 mRNA was induced in WT and MKK3<sup>−/−</sup> endothelial cells after rotenone exposure, suggesting not only that mitochondrial ROS is sufficient for ICAM-1 upregulation but that depressed mitochondrial ROS can account for decreased ICAM-1 expression in MKK3<sup>−/−</sup> endothelial cells (Fig. 7A). To confirm these results, we used Mito-TEMPO, an antioxidant specific to the mitochondria (29), to determine the contribution of mitochondrial ROS to ICAM-1 expression. We found that in WT endothelial cells Mito-TEMPO reduced significantly the expression of ICAM-1 mRNA at baseline and after LPS exposure. In contrast, MKK3<sup>−/−</sup> endothelial cells showed no difference in ICAM-1 expression after Mito-TEMPO exposure (Fig. 7B). The latter was expected because MKK3<sup>−/−</sup> endothelial cells already have reduced mitochondrial ROS and addition of Mito-TEMPO has no added benefit. In conclusion, we show that endothelial MKK3 is an important regulator of mitochondrial redox status with subsequent regulation of AP-1, NF-κB, and ICAM-1 during LPS exposure. Collectively, these studies identify a novel role for MKK3 in lethal LPS responses and provide new therapeutic targets against sepsis and acute lung injury.

**Discussion**

Sepsis remains a critical problem, with significant mortality and morbidity despite intense efforts to find effective therapies. The prevailing theory of sepsis is an unregulated inflammatory response...
or cytokine storm results in organ damage and death (30). However, multiple trials of anti-inflammatory or anticytokine therapies have been disappointing failures (31). Most of the unsuccessful therapies try to inhibit mediators acting far downstream to the initial stimulus that set in motion the inflammatory response. By identifying more proximal mediators, we may have a better chance of halting the inflammatory response before deleterious consequences ensue. Our study identifies MKK3, a proximal-activating kinase in the p38 MAPK pathway, as a potential therapeutic target. We demonstrate for the first time, to our knowledge, that MKK3 deficiency leads to protection against LPS-induced lung injury through the reduction of endothelial mitochondrial ROS, AP-1, NF-κB activation, and ICAM-1 expression, ultimately reducing tissue inflammation.

Mitochondria are ancient bacterial endosymbionts in eukaryotic cells that are responsible for oxidative phosphorylation and generation of ATP. Mitochondria are also a major source of ROS and inducers of apoptosis. New evidence suggests that mitochondria play a critical role in inflammatory responses (32, 33). Mitochondrial dysfunction is associated with greater severity and worse outcomes in patients with sepsis and lung injury (34, 35). In this article, we show that MKK3-deficient mice have lower mitochondrial ROS (at baseline and after LPS) and are protected against endotoxemia and lung injury. ICAM-1 is predominantly transcriptionally regulated, under the control of AP-1 and NF-κB transcription factors, key players in the inflammatory responses of sepsis (36). AP-1 and NF-κB are redox-sensitive transcription factors and cooperatively influence the expression of ICAM-1 in endothelial cells (37–40). The mechanism of regulation of AP-1 and NF-κB by oxidant signaling remains undetermined. IKK is considered the major proximal redox-modulated regulatory kinase for NF-κB signaling (41). Our studies indicate that MKK3 may influence the phosphorylation of IKK through the mitochondrial redox status in endothelial cells, in response to LPS. Our studies establish novel links between MKK3, mitochondrial redox status, AP-1, IKK–NF-κB signaling, and ICAM-1 expression in endotoxemia and lung injury (Fig. 8).

TLR4 is reported as the canonical LPS-responsive pathway. Mice that are deficient in TLR4 function are highly resistant to endotoxic shock (42–44). MKK3 appears to be an important part of LPS responses and may function in tandem with TLR4 as part of the innate immune response. The specific contribution of MKK3 in innate immunity has not been well characterized, and we show for the first time, to our knowledge, that MKK3 mediates critical responses to LPS. We believe that MKK3+/− mice are not completely deficient in TLR4 responses, as the increase in ICAM-1 in response to LPS was not completely abolished and MKK3+/− mice retained the ability to induce TLR4-induced cytokines, such as IL-6 and TNF-α (not shown). There was a general reduction in other adhesion and chemokine markers, such as VCAM-1 and CXCL-2, in MKK3-deficient mice. Hence MKK3 likely has both TLR4-dependent and TLR4-independent effects. Future studies will help us delineate the specific contribution of MKK3 to TLR4 function. We show dramatic protection against injury in LPS-challenged MKK3−/− mice. Apart from TLR4-deficient mice, only a few other studies show substantial protection against endotoxic shock using knockout mice. Mice deficient in IL1-β–converting enzyme (caspase-1), poly(ADP-ribose) polymerase 1, and ICAM-1 were completely protected against death after LPS exposure (21, 45, 46). Notably, we demonstrate that MKK3−/− endothelial cells and mice are deficient in ICAM-1 after LPS exposure, which identifies MKK3 as a critical upstream regulator of molecules that determine survival after LPS. Our identification of MKK3 as a major mediator of LPS-induced injury provides new insights into innate immune pathways and serves as a basis for new therapies against sepsis.

Vascular inflammation is a sentinel event in sepsis. The endothelium is central to the pathogenesis of sepsis through effects on inflammation, leukocyte recruitment, vascular tone, coagulation, and thrombosis. Key endothelial changes in sepsis are disruption of

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**FIGURE 8.** Summary of MKK3 signaling. We postulate that endothelial MKK3 is required for LPS-induced mitochondrial ROS generation, IKKα/β phosphorylation, NF-κB and AP-1 activation, and ICAM-1 expression, ultimately leading to inflammatory recruitment.
the endothelial barrier and promotion of leukocyte adhesion. Leukocyte accumulation is orchestrated by coordinated expression of chemokine and adhesion molecules. ICAM-1 on endothelial cells attaches to β2 integrin receptors on leukocytes, leading to firm binding and transmigration (47). We show that MKK3 deficiency prevents the loss of endothelial barrier function and prevents inflammatory influx by decreasing ICAM-1 expression.

MKK3 has not been reported to be involved in ICAM-1 regulation, but our studies point to a pivotal role for MKK3 during LPS challenge. Lung epithelial cells express ICAM-1 in response to LPS (48). Reduced epithelial ICAM-1 expression in MKK3-deficient mice may also contribute to the protection in sepsis, as our bone marrow chimera mice suggest that MKK3 expression in nonhematopoietic cells is important. In future studies, we will examine the role of epithelial cells in determining septic responses in MKK3-deficient mice.

Finally, it is notable that even the baseline levels of mitochondrial ROS, ICAM-1, AP-1, and NF-κB activation in MKK3−/− lung and endothelial cells are lower than those of WT. It is known that ICAM-1 is expressed constitutively in specific vascular beds, with the highest expression in the lung, and that the upregulation of ICAM-1 expression in the lung increases after LPS exposure (49). This observation suggests that the pulmonary vasculature may serve as an active gateway for inflammatory cell recruitment and is primed to adhere and recruit neutrophils in response to a proinflammatory stimulus by rapidly upregulating ICAM-1 expression. Similar mechanisms of regulation may be in effect in organs, such as liver and kidney, with high populations of endothelial cells. We show that MKK3 plays an important role in this regulation and demonstrate that MKK3−/− mice have less ICAM-1 expression and tissue damage in lung, liver, and kidney. It is noteworthy that the lung, kidney, and liver are the most common organs to fail in sepsis and that multiorgan failure is the major cause of death in sepsis-induced lung injury. Our studies have identified MKK3 as a promising therapeutic target for sepsis-induced multiorgan failure.

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Disclosures

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References


