ORIGINAL RESEARCH

Macrophage Migration Inhibitory Factor: A Novel Inhibitor of Apoptosis Signal-Regulating Kinase 1–p38–Xanthine Oxidoreductase–Dependent Cigarette Smoke–Induced Apoptosis

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Abstract

Cigarette smoke (CS) exposure is the leading cause of emphysema. CS mediates pathologic emphysematous remodeling of the lung via apoptosis of lung parenchymal cells resulting in enlargement of the airspaces, loss of the capillary bed, and diminished surface area for gas exchange. Macrophage migration inhibitory factor (MIF), a pleiotropic cytokine, is reduced both in a preclinical model of CS-induced emphysema and in patients with chronic obstructive pulmonary disease, particularly those with the most severe disease and emphysematous phenotype. MIF functions to antagonize CS-induced DNA damage, p53-dependent apoptosis of pulmonary endothelial cells (EndoCs) and resultant emphysematous tissue remodeling. Using primary alveolar EndoCs and a mouse model of CS-induced lung damage, we investigated the capacity and molecular mechanism(s) by which MIF modifies oxidant injury. Here, we demonstrate that both the activity of xanthine oxidoreductase (XOR), a superoxide-generating enzyme obligatory for CS-induced DNA damage and EndoC apoptosis, and superoxide concentrations are increased after CS exposure in the absence of MIF. Both XOR hyperactivation and apoptosis in the absence of MIF occurred via a p38 mitogen-activated protein kinase–dependent mechanism. Furthermore, a mitogen-activated protein kinase kinase kinase family member, apoptosis signal–regulating kinase 1 (ASK1), was necessary for CS-induced p38 activation and EndoC apoptosis. MIF was sufficient to directly suppress ASK1 enzymatic activity. Taken together, MIF suppresses CS-mediated cytotoxicity in the lung, in part by antagonizing ASK1–p38–XOR–dependent apoptosis.

Keywords: migration inhibitory factor; apoptosis signal–regulating kinase 1; xanthine oxidase; cigarette; apoptosis

Clinical Relevance

This study establishes a role for migration inhibitory factor (MIF) in controlling reactive oxygen species (ROS) responses to cigarette smoke (CS) by regulating p38-dependent xanthine oxidoreductase activity, and it links MIF as a direct inhibitor of apoptosis signal–regulating kinase 1, upstream of p38 in CS-induced endothelial cell (EndoC) injury. The identification of this pathway provides additional therapeutic targets directed at ameliorating ROS production and EndoC apoptosis, driving forces behind tissue injury and remodeling in emphysema.

Five million Americans suffer from emphysema (1), a morbid disease characterized histologically by the irreversible destruction of gas-exchanging surfaces in the lung. Cigarette smoke (CS) exposure is the main cause of emphysema (2), and triggers apoptosis within the lung parenchyma (3–5), resulting in enlargement of the airspaces, loss of the capillary bed, and diminished surface area for gas exchange. As we and others have previously shown, endothelial cell (EndoC) apoptosis represents an early (6), necessary (7), and sufficient (8) event in emphysematous tissue destruction. Thus, we set forth to identify novel regulators of

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EndoC apoptosis and survival in the context of CS exposure, postulating that these would represent potential determinants of disease severity and needed therapeutic targets.

Emphysema is characterized by increased oxidative stress. CS amplifies the volume of inhaled oxidants (9) and also increases reactive oxygen species (ROS) produced by cells of the airway, alveoli, and immune system (10–12). The superoxide (O_2^-) and hydrogen peroxide–generating enzyme xanthine oxidoreductase (XOR) is an important cellular source of oxidative stress. XOR activity is elevated in patients with emphysema compared with control subjects (13, 14), and XOR activity is increased in the lungs of animals exposed to CS (15). Importantly, XOR activity drives CS-induced DNA damage, specifically double-stranded DNA breaks, p53 expression, and EndoC apoptosis (15).

Macrophage migration inhibitory factor (MIF) is significantly reduced in the serum of patients with severe emphysematous chronic obstructive pulmonary disease (COPD) (16) and in the lungs of mice exposed to chronic smoke (16, 17). MIF is an endogenous regulator of CS-induced EndoC apoptosis (18) with the capacity to antagonize CS-induced p53 expression and p53-dependent apoptosis. Acute exposure to CS is associated with a rise in total lung and lung microvascular EndoC-associated MIF (*see* Figure E1 in the online supplement) (17, 18), suggesting a potential cytoprotective role that is lost over time. MIF deficiency ($Mif^{-/-}$) exacerbates the toxicity of CS *in vivo*, accelerating emphysematous tissue remodeling, and preferentially sensitizes EndoCs to CS-induced DNA damage and apoptosis (16). Thus, the cytoprotective effects of MIF act upon EndoCs to modify emphysema severity. The mechanism(s) by which MIF antagonizes CS-induced DNA damage are unknown. Because MIF deficiency exacerbates DNA damage and EndoC apoptosis, and potentiates airspace enlargement (16), and XOR activity contributes significantly to these events, we hypothesized that MIF impacts on CS-mediated cytotoxicity in the lung by regulating ROS production, in part, via XOR.

In the present study, we demonstrate that MIF deficiency potentiates superoxide generation and XOR activation in response to CS. This occurs via a p38 mitogen-activated protein kinase (MAPK)–dependent mechanism. Furthermore, we demonstrate that the apoptosis signal–regulating kinase 1 (ASK1; a.k.a., MAPK kinase [MKK] kinase [MAP3K] 5) is upstream of p38 in response to CS, and both kinases are required for EndoC apoptosis. Importantly, MIF suppresses CS-induced p38 activation and directly antagonizes ASK1 kinase activity. Thus, MIF antagonizes ROS formation and subsequent EndoC apoptosis by repressing ASK1-p38 kinase signaling upstream of XOR activation and increased ROS production.

Materials and Methods

Reagents and Cells

Human and rat lung microvascular EndoCs were cultured and maintained in microvascular endothelial cell growth media (EGM-2MV) (Lonza, Walkersville, MD) or Basal Media supplemented with M1266 (Cell Biologics, Chicago, IL) from passage 4–9. Recombinant MIF (rMIF; R&D Systems, Minneapolis, MN), on-target control small interfering RNA (siRNA) (Ctrl siRNA), ASK1 siRNA (Dharmacon, Lafayette, CO), and CS extract (CSE) were used as previously described (18). Briefly, at 50% confluence, cells were washed with PBS and bathed with optimem reduced serum media (Life Technologies, Grande Island, NY) for transfection of siRNAs. Geneporter 2 transfection reagent (GenLantis, San Diego, CA) was optimized for lipid-based transfections and, after 4 hours, complete media were added to the cells. Naive cells, transfected cells, or cells receiving rMIF were exposed to CSE for 5–45 minutes, and 4, 8, 24, and 48 hours, after which lysates and supernatants were collected for analysis.

Kinase Assay

MIF and ASK1 interactions were tested using a cell-free ASK1 kinase enzyme system (Promega, Madison, WI), whereby ASK1 in the presence of a substrate protein, myelin basic protein (MBP), and ATP will phosphorylate MBP, converting ATP to ADP. After quenching any remaining ATP after a reaction period, the remaining ADP is converted back to ATP and coupled to a luciferase/luciferin reaction-producing light (ADP-Glo assay; Promega). The manufacturer's protocol was followed for setting up controls and determining the half-maximal inhibitory concentration values for inhibitors. With this assay system rMIF was titrated across several dilutions in triplicate with ASK1, and the formation of ADP was subsequently measured.

Animals and Treatments

All animal protocols were conducted as approved by the Johns Hopkins University Institutional Animal Care and Use Committee. $Mif^{-/-}$ mice with a C57BL/6 background were generated as previously described (19). At 8–10 weeks of age, mice were exposed to CS or filtered air for 3 days or 6 months. CS exposure was performed as previously described (20) with minimal adjustments (detailed in the online supplement).

Human Subjects

Lung tissue sections from deidentified human subjects with and without emphysema (BioChain, Newark, CA) were used to assess phosphorylation status of ASK1, as detailed in the online supplement.

Pterin Assay

The measurement of XOR enzymatic activity was performed on mouse lung homogenates using a fluorometric assay based on the conversion of pterin to its fluorescent product, isoxanthopterin, as previously described (21). Lung tissue was homogenized with pterin buffer containing 50 mM KH_2PO_4, 10 mM DTT, and 0.18 mg/ml PMSF. Lysates were centrifuged at 13,000 × *g* for 15 minutes at 4°C, and the supernatants were used in the assay immediately before measurement.

Lung Caspase 3 Activity

After CS exposure, $Mif^{+/+}$ and $Mif^{-/-}$ lung homogenates were collected and directly plated in triplicate on a white-bottom, 96-well plate. Homogenates were probed for caspase 3/7 activity using the Caspase-Glo 3/7 assay (G8091; Promega) following the manufacturer's guidelines. Briefly, the Caspase-Glo substrate and buffer were combined and equal volumes of this Glo reagent and lung sample were mixed in each well. In the presence of the Glo reagent, active caspase 3/7 will cleave from the substrate and generate luminescent signal, produced by luciferase.

Immunohistochemistry and Western Blotting

Immunohistochemistry and Western blotting protocols and reagents are detailed in the supplemental MATERIALS and METHODS.

MIF ELISA

Supernatants from human microvascular EndoCs exposed to 4, 8, 24, and 48 hours of CSE or PBS were analyzed using a solid-phase sandwich MIF ELISA (R&D Systems), as previously described (16) and per the manufacturer's specifications.

Statistical Analysis

For comparisons among groups of normally distributed datasets, the student's *t* test or ANOVA with *post hoc* Bonferroni correction was used. For nonnormally distributed data, the Mann-Whitney or Kruskal-Wallis test with *post hoc* Bonferonni correction was used. Values are presented as means (±SE).

Results

MIF Is a Determinant of CS-Induced XOR Activity and ROS Production

ROS are produced by multiple cellular sources in the lung, including the pulmonary vascular EndoCs, the major target of CS-induced damage in the context of MIF deficiency (16). Important vascular sources of ROS are XOR, uncoupled nitric oxide synthase, and nicotinamide adenine dinucleotide phosphate oxidase (22–24). More specifically, XOR is a source of superoxide and hydrogen peroxide within pulmonary EndoCs. Importantly, XOR protein and activity are elevated in human CS-induced lung disease (14, 25). We have previously established that C57BL/6 mice exposed to CS have increased pulmonary XOR activity compared with filtered air–exposed controls (15). To test the hypothesis that MIF modifies CS-induced XOR activation, lung protein homogenates were collected from $Mif^{+/+}$ and $Mif^{-/-}$ mice exposed to 3 days of CS or filtered air, and XOR activity was measured as detailed in Materials and Methods. XOR activity was increased in response to CS in both genotypes relative to air controls; however, activity was significantly greater in $Mif^{-/-}$ mice in comparison with their $Mif^{+/+}$ counterparts (Figure 1A). XOR activity did not differ at baseline between genotypes. In addition, XOR protein levels, determined by Western blotting, did not differ at baseline or in response to acute CS (Figures E2A and E2B), suggesting that the increases in XOR activity observed in response to CS were unlikely due to the effects of MIF on XOR protein expression. Rather, these data provide evidence that MIF antagonizes CS-induced increases in XOR activity by modifying the activation status of XOR.

In further support of this, intracellular O_2^- concentrations were also assessed qualitatively using the lipophilic cell-permeable dye, dihydroethidium (26). In the presence of MIF ($Mif^{+/+}$ wild-type mouse lungs), 72 hours of CS exposure resulted in a mild increase in dihydroethidium (DHE) fluorescence (Figure 1B). In contrast, CS exposure produced a robust increase in DHE staining in $Mif^{-/-}$ animals. Air-exposed $Mif^{-/-}$ mice had higher basal superoxide concentrations than wild-type controls, despite similar basal XOR activity, suggesting an XOR-independent mechanism to account for this difference. *Ex vivo* treatment with the thiol donor, *N*-acetyl cysteine (NAC), antagonized DHE fluorescence, demonstrating specificity (data not shown). Thus, the absence of MIF alters the redox status within the lung and potentiates CS-induced ROS production concurrently with increased XOR activity.

The Role of p38 in CS-Induced XOR Activity

An established mechanism for post-translational activation of XOR is via p38-dependent phosphorylation (27, 28). p38 MAPK activity has been linked to enhanced apoptosis (29), CS-induced endothelial permeability changes (30), human COPD (31), and emphysema susceptibility in mice (32). In addition, p38 activation is increased with chronic CS in our preclinical model. Using immunohistochemistry directed at phospho-p38, a surrogate for its activation status, we detected increased activation of p38 in the microvasculature of the lungs in both genotypes, with more intensive staining observed in the $Mif^{-/-}$ mice exposed to chronic CS (Figure E3). The contribution of the p38 signaling pathway in XOR activation in response to CS *in vivo* has not yet been established. We postulated that the increased XOR activation in $Mif^{-/-}$ mice exposed to CS may be mediated by p38. To test the effect of the p38 signaling pathway in XOR activation, we exposed $Mif^{+/+}$ and $Mif^{-/-}$ mice to CS for 3 days after treatment with a p38 inhibitor (SB203580) or its vehicle, given intraperitoneally before CS exposure. Dose and route were chosen based on the literature (28). Subsequently, lung homogenates were collected and XOR was measured as detailed in Materials and Methods. In response to CS, $Mif^{-/-}$ mice had significantly higher XOR activity compared with $Mif^{+/+}$ mice, which was completely abrogated with SB203580

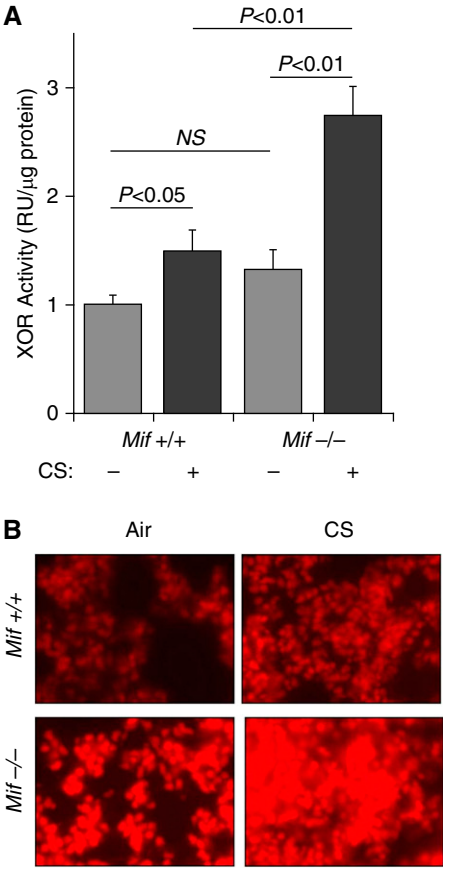

Figure 1. Migration inhibitory factor (MIF) is a determinant of cigarette smoke (CS)–induced xanthine oxidoreductase (XOR) activity and reactive oxygen species (ROS) production. $Mif^{+/+}$ and $Mif^{-/-}$ mice were exposed to 3 days of CS, after which lung homogenates or fresh-frozen lung cross-sections were respectively collected in buffer and immediately probed for XOR activity using a fluorometric readout or histologically probed for dihydroethidium (DHE) as a measure of intracellular ROS. There was significant interaction between genotype and exposure (two-way ANOVA, $P = 0.02$). Both genotypes displayed significant increases in XOR activity in response to CS; however, $Mif^{-/-}$ mice responded with significantly higher XOR activity (*A*). NS, no significant difference, $P = 0.1$ versus $Mif^{+/+}$ mice; RU, relative units. Whereas $Mif^{+/+}$ mice showed a modest increase in CS-induced DHE staining, $Mif^{-/-}$ mice had higher baseline and more robust CS-induced DHE staining when compared with wild-type mice (*B*) ($n = 5$ for each condition). Values are presented as means (±SE).

treatment (Figure 2), returning to levels comparable to those of *Mif*$^{+/+}$ mice. Thus, the enhanced XOR activity observed in the absence of MIF is dependent on p38 MAPK activity.

MIF Antagonizes CS-Induced and p38-Dependent EndoC Apoptosis

Activation of p38 and other MAPKs, such as the c-Jun N-terminal protein kinase (JNK), are mediated through stress-inducing signals, including oxidative stress (33), a key component of CS-induced cellular injury. MIF deficiency potentiates p38 signaling responses in the context CS *in vivo*, as demonstrated here by enhanced XOR activation and ROS production, suggesting a protective role for MIF as a modifier of ROS. To test the hypothesis that p38 is activated in response to CS and to evaluate the potential role of MIF in blocking CS-induced activation of p38, we used an *in vitro* approach. EndoCs were preincubated with recombinant MIF protein or its carrier, treated with CSE, and probed for p38 phosphorylation by Western blot. In response to CSE, p38 is activated, as demonstrated by changes in its phosphorylation status in primary human lung microvascular EndoCs (Figure 3A). Similar findings were observed in primary rat pulmonary EndoCs (Figure E4A). Total p38 protein did not differ across time points or with rMIF treatment. Pretreatment with exogenous MIF (rMIF) compared with carrier significantly abrogated the phosphorylation of p38 in EndoCs, independent of species (Figure 3B and Figure E4B). Thus, MIF levels influence CSE-induced p38 activation.

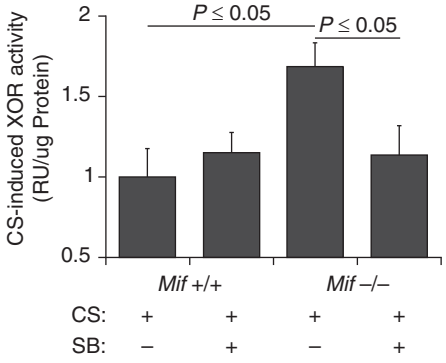

Figure 2. The role of p38 in CS-induced XOR activity. *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were treated with SB203580 (SB) or its vehicle and exposed to 3 days of CS, after which lung homogenates were collected and immediately probed for XOR activity. Data were normalized to the response of CS-treated *Mif*$^{+/+}$ mice. *Mif*$^{-/-}$ mice had a significant increase in XOR activity, and SB203580 treatment reduced this XOR activity to levels observed in *Mif*$^{+/+}$ mice ($n = 5$ for each condition). Values are presented as means (±SE).

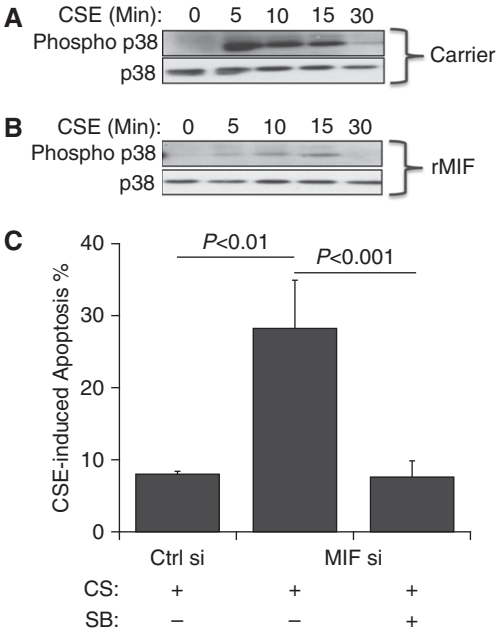

Figure 3. MIF antagonized CS extract (CSE)–induced and p38-dependent apoptosis. Primary human lung microvascular endothelial cells (EndoCs) were exposed to CSE for 5, 10, 15, and 30 minutes (Min) in the presence of recombinant MIF (rMIF) or its carrier and subsequently harvested for Western blot analysis or analyzed with Hoechst stain for apoptosis in the presence or absence of SB. In response to CSE, p38 activation (Phospho p38) was rapid (*A*). The addition of rMIF was sufficient to block the phosphorylation of p38 (*B*). Total p38 was unchanged. Pharmacological blockade of p38 with SB resulted in significantly reduced apoptosis (*C*) ($n = 3$–5 per condition). Values are presented as means (±SE). Ctrl si, control small interfering RNA.

To understand the functional outcome of p38 activation *in vitro*, EndoCs were subjected to CSE exposure in the presence or absence of the p38 inhibitor, SB203580, and analyzed for alterations in nuclear morphology consistent with apoptosis via Hoescht staining, as previously described (18). The observed increase in p38 activation in response to CSE was linked with increased apoptotic cell death (Figure E5) that was antagonized by pharmacologic blockade of p38.

Using a complementary approach to evaluate the antagonism of MIF on p38, EndoCs were transfected with siRNA directed against MIF or nontargeting siRNA (Ctrl siRNA), as previously described (34), and exposed to CSE in the presence of SB203580 or its vehicle. MIF-deficient EndoCs were sensitized to CS-induced apoptosis *in vitro* (Figure 3C). Preincubation with SB203580 was sufficient to significantly diminish CSE-induced apoptosis to the level observed with Ctrl siRNA. SP600125, a JNK inhibitor, did not have an effect on CSE-induced apoptosis (Figure E6), showing specificity and a dependence on p38 kinase activity. Together, these data provide strong evidence that: (*1*) exogenous MIF blocks p38 activation; and (*2*) increased CSE-induced apoptosis in the absence of MIF is p38 dependent.

p38 Inhibition Antagonizes DNA Damage in MIF-Deficient Mice

Our *in vitro* analysis revealed that cellular apoptosis in response to CSE is regulated by p38. Furthermore, we have previously shown that DNA damage and EndoC apoptosis are exacerbated in the absence of MIF *in vivo* (16). To address the role of p38 *in vivo* and in the context of MIF deficiency and CS exposure, *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to CS for 3 days with or without SB203580. Lung homogenates were collected and caspase 3/7 activity was measured as a marker of apoptosis. Baseline activity did not differ by genotype. In response to CS, both *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice had significantly increased

caspase 3/7 activity; however, this activity was significantly higher in CS-exposed *Mif*$^{-/-}$ mice compared with their *Mif*$^{+/+}$ counterparts. The increase in caspase 3/7 activity in *Mif*$^{-/-}$ mice was reduced in animals treated with SB203580, demonstrating a dependence on p38 (Figure 4A).

We have previously established that chronic CS exposure (2 weeks and 6 months) results in increased apoptosis in the lung of *Mif*$^{-/-}$ mice relative to *Mif*$^{+/+}$ or filtered air–exposed animals (35). Furthermore, the increased cell death observed in the absence of MIF was mainly due to apoptosis of alveolar EndoC (35). To further test the contribution of the p38 pathway on the alveolar compartment, lung sections were probed for caspase 3 (red) in tandem with the microvascular endothelial marker, thrombomodulin (green). Apoptotic EndoCs were apparent in CS-exposed mice of both genotypes, as determined by increased overlay of the above markers, which was abrogated by SB203580 treatment (Figure 4B).

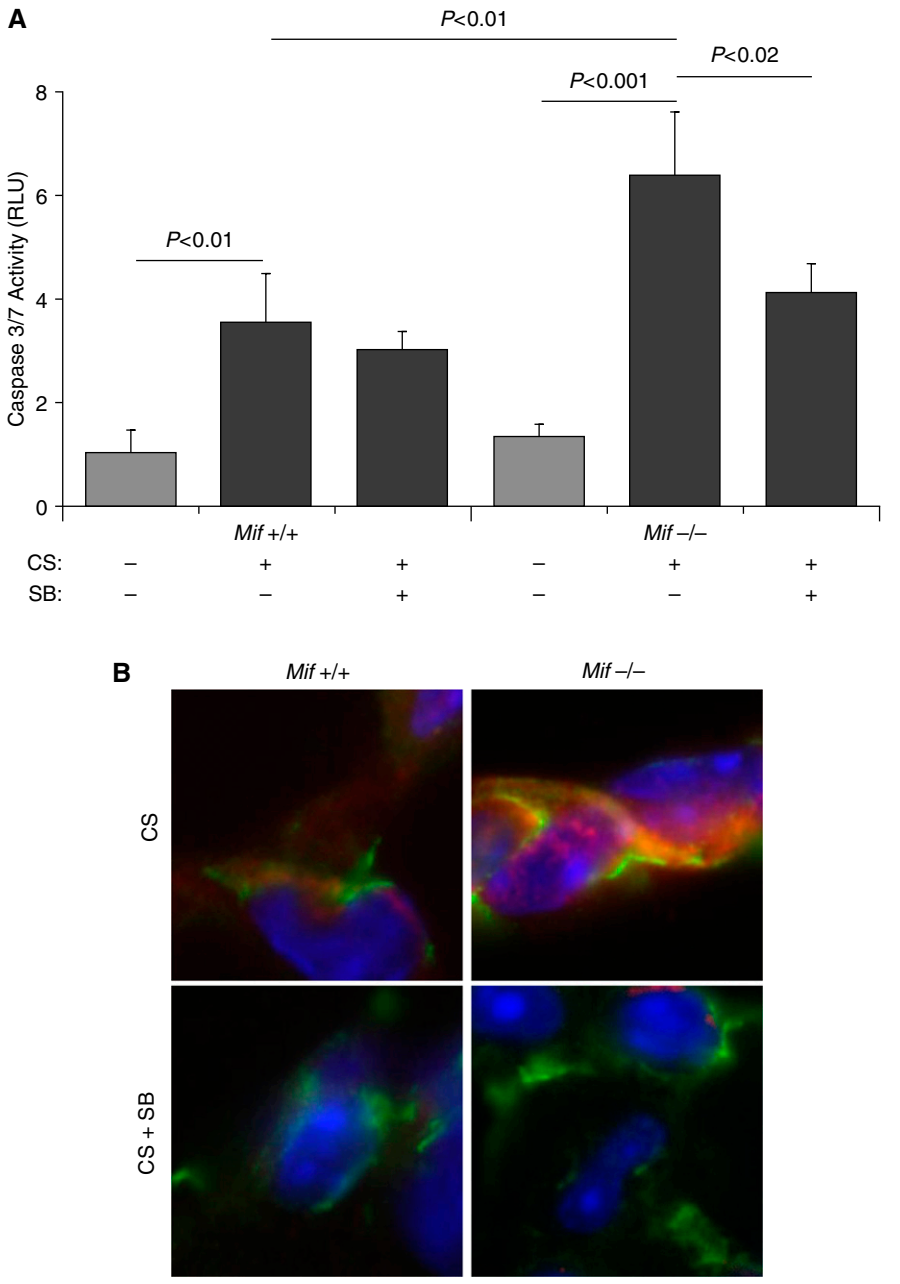

Figure 4. p38 inhibition antagonizes apoptosis in *Mif*$^{-/-}$ mice. *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to CS for 3 days after treatment with SB or its vehicle. Lung homogenates and tissue sections were collected and subjected to a luminescent caspase 3/7 activity assay, or immunohistochemistry, respectively. In response to CS, both *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice had significantly increased caspase 3/7 activity (*A*). RLU, relative-luminescence units. Activity was significantly higher in CS-exposed *Mif*$^{-/-}$ mice compared with their wild-type counterparts. Caspase 3/7 activity was significantly reduced with SB in *Mif*$^{-/-}$ mice ($n = 5$ per condition). Lung tissue sections were also probed for cleaved caspase 3 (*red*) in the presence of the EndoC marker thrombomodulin (*green*) (*B*). Both genotypes displayed noticeable EndoC and non-EndoC apoptosis, with *Mif*$^{-/-}$ mice exhibiting a visible increase in Endo death, which was dramatically abrogated by SB treatment. Nuclei are counterstained *blue* ($n = 3$ per condition). Values are presented as means (±SE).

CSE-Induced p38 Activation Is ASK1 Dependent

Having established that MIF antagonizes p38 phosphorylation, we questioned whether the ability of MIF to block p38 activation occurred by a direct or indirect interaction. There is no evidence that MIF possesses kinase/phosphatase activity. However, it is well established that p38 activation is mediated by the MKKs, MKK3 and MKK6 (36), which are, in turn, regulated by MAP3K. The MAP3K, ASK1, is activated and autophosphorylated in a redox-sensitive fashion (37). In quiescent cells, the redox-sensitive thioredoxin (Trx) protein, which possesses a thiol-protein oxidoreductase (TPOR) motif similar to MIF, is known to bind and inhibit ASK1 (38, 39). In the face of increased ROS, TPORs undergo oxidation, thereby releasing ASK1. Based on functional homology and oxidoreductase activity, we postulated that MIF may be a novel inhibitor of ASK1 activity. To test this hypothesis, we transfected EndoCs with ASK1 or Ctrl siRNA and treated cells with CSE or its vehicle, PBS. Sufficient knockdown of ASK1 was achieved by 24 hours (Figure 5A), as demonstrated by Western blotting. In response to CSE, p38 phosphorylation was not observed in ASK1-deficient EndoCs (Figure 5B), whereas cells derived from the control transfections showed rapid p38 phosphorylation.

To test the functional consequence of diminished ASK1, apoptotic responses to CS were quantified by nuclear morphology in EndoCs transfected with Ctrl or ASK1 siRNA. Knockdown of ASK1 led to decreased CS-induced apoptosis compared with Ctrl si

transfectants (Figure 5C). The reduction in p38 activation in the absence of ASK1 (Figure 5) mimics the effects of pretreatment with exogenous rMIF (Figure 3), suggesting that MIF may be acting to antagonize CS-induced p38 activation by blocking its upstream activator, ASK1.

MIF Has the Capacity to Directly Block ASK1 Kinase Activity

It is well recognized that MIF can bind to or affect intracellular proteins: (*1*) as an endogenous protein; (*2*) via pinocytosis into the cell; or (*3*) through extracellular receptor ligand interactions (40–44). To test the hypothesis that MIF alters p38 through a direct interaction with ASK1, we used a cell-free assay designed to measure the kinase activity of ASK1 in the presence or absence of rMIF. In this assay, ASK1, ATP, and MBP (a nonspecific MAPK substrate) concentrations were held constant, whereas rMIF was added in a dilution series. The addition of rMIF was sufficient to block ASK1 kinase activity, as determined by a reduction in ADP formation, in a dose-dependent manner (Figure 6). Furthermore, the half-maximal inhibitory concentration of MIF was approximately 1.6 nM, well within the known biologically active range for MIF (45).

Interestingly, phosphorylation of ASK1 at threonine-845, which correlates with its activation, may also be enhanced with clinical emphysema (Figure E7), which we and others have previously established to be a state of relative MIF deficiency (16, 17). These data indicate a mechanism by which MIF, internalized or endogenous, directly inhibits ASK1 autoactivation, thereby diminishing downstream signaling, including p38 phosphorylation and XOR activity.

Discussion

CS-induced lung diseases, including emphysema, contribute to an estimated 2.5 million deaths worldwide and a healthcare cost approaching $38.8 billion annually in the United States alone (46). Emphysema is a debilitating disease characterized by the irreversible destruction of the lung architecture, with enlargement of the airspaces driven by enhanced EndoC apoptosis (47). The molecular regulators of EndoC fate in response to CS exposure are potential therapeutic targets and modifiers of disease phenotype. Despite this, no current therapies directly target tissue destruction in emphysema. Furthermore, our understanding of host factors predisposing to the emphysematous phenotype in humans is nascent (48). Here, we show that MIF acted to blunt the rise in intracellular ROS caused by CS exposure and antagonized CS-induced XOR activation and EndoC apoptosis in an ASK1–p38–dependent manner.

MIF is a 12.5-kD, evolutionarily conserved protein constitutively expressed by multiple cell types in the lung, including pulmonary microvascular EndoCs (34). MIF is found in the serum under basal conditions, and is elevated in acute and chronic inflammatory disease states (49). Specifically, serum MIF levels are significantly lower in patients with COPD compared with healthy, nonsmoking control subjects, and vary according to the severity of COPD, as estimated by the GOLD (Global Initiative for Chronic Obstructive Lung Disease) criteria. Thus, patients with severe COPD (GOLD stage IV) have significantly lower circulating MIF than subjects with less-severe COPD (GOLD II or III), implicating MIF in human disease severity and/or susceptibility (16). Similar findings are observed in a separate COPD cohort (17), independently validating this association. Thus, human COPD represents a relative MIF-deficient state, and there appears to be an inverse relationship between disease severity and MIF levels. In murine models, wild-type animals chronically exposed to CS have significantly less MIF protein and mRNA expression. Preclinical data also demonstrate that MIF deficiency accelerates CS-induced emphysema (16, 17). Taken together, these data provide evidence that MIF is key in regulating the nodal point in emphysematous disease progression whereby the cytoprotective effects of MIF are diminished, and oxidative stress, cell damage, and death by definition exceed repair. This protective effect is apparent in considering the results here, which

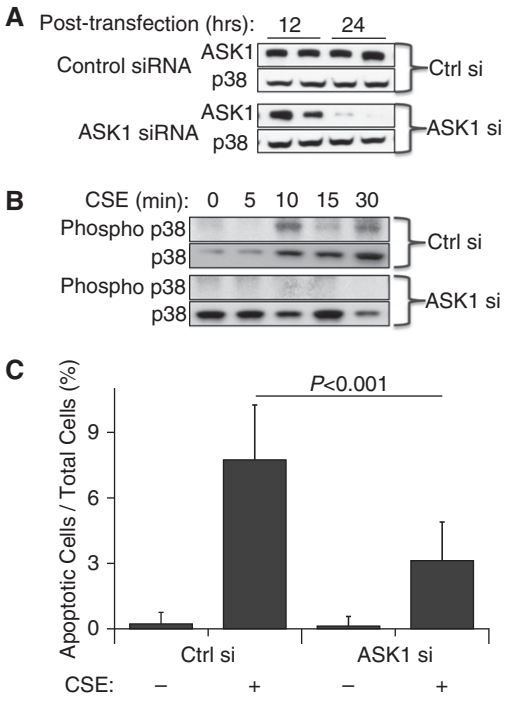

Figure 5. CSE-induced p38 activation is apoptosis signal–regulating kinase (ASK) 1 dependent. Primary rat microvascular EndoCs were transfected with small interfering RNA (siRNA) targeting ASK1 or control siRNA (Ctrl si), treated with CSE or PBS, and probed via Western blotting or analyzed with Hoechst staining. Sufficient knockdown of ASK1 was achieved by 24 hours (*A*). In response to CSE, EndoCs derived from the control transfections showed rapid p38 phosphorylation, which was not observed in ASK1-deficient cells (*B*). There was a significant reduction in apoptosis in ASK1-deficient cells compared with Ctrl transfectants (*C*) ($n = 3–5$ per condition). Values are presented as means (±SE).

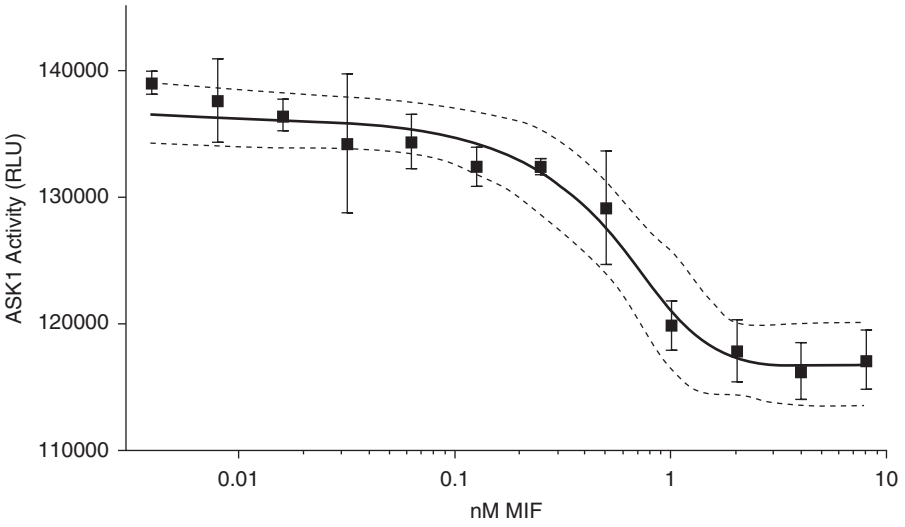

Figure 6. MIF antagonizes ASK1 kinase activity. A cell-free assay was used to measure the kinase activity of ASK1 in the presence or absence of rMIF. ASK1, ATP, and myelin basic protein (MBP; a nonspecific mitogen-activated protein kinase substrate) concentrations were held constant, whereas rMIF was added in a dilution series. rMIF was sufficient to block ASK1 kinase activity (ADP formation) in a dose-dependent manner. MIF half-maximal inhibitory concentration = 1.6 nM. ASK1 activity in the presence of ATP and MBP was used as a buffer control. Values are presented as means (±SE).

demonstrate reduced XOR activation and diminished ROS production in the presence of MIF (versus MIF deficiency). Our previous *in vitro* and *in vivo* data also provide strong support for MIF in blocking CS-induced p53-dependent apoptosis, specifically in EndoCs; p53 is directly regulated by MIF and exacerbated with human disease (18, 50). Acute increases in MIF, through paracrine or autocrine loops, also stabilize hypoxia-inducible factor 1-α (51, 52), which is necessary for vascular endothelial growth factor production and EndoC homeostasis, both of which are diminished in human emphysema (53). Furthermore, MIF as well as vascular endothelial growth factor, through extracellular receptor signaling, can enhance survival through protein kinase B (AKT) signaling, which is also dysregulated in human COPD (54–56). Taken together, this suggests that MIF is important in protecting cells from death on multiple levels, as well as regulating oxidative stress, both of which are significantly dysregulated in human CS-induced emphysema, skewing cell fate toward death and disease progression.

In the absence of MIF, alveolar EndoCs are predisposed to increased CS-induced DNA damage and apoptosis (16). We have identified MIF as an intrinsic, negative regulator of LPS- and CS-induced apoptosis in human pulmonary EndoCs (18, 34). MIF functions to antagonize EndoC apoptosis by blocking death signals from both the death receptor and mitochondrial apoptotic pathways (18, 34). Specifically, MIF is a novel positive regulator of the short isoform of the Fas-associated protein with death domain (FADD)-like interleukin-1β-converting enzyme (FLICE)-like inhibitor protein (FLICE-like inhibitor protein [FLIP]short), which functions to antagonize signals via the death receptor pathway in response to LPS (34). In addition, MIF functions to suppress CS-induced p53 expression and p53-dependent activation of the mitochondrial death pathway in CSE-challenged EndoCs (18). Thus, MIF has the unique capability of antagonizing death signals from multiple apoptotic pathways in EndoCs, acting to stabilize intrinsic inhibitors of the apoptosis cascade and suppressing promoters of apoptosis.

CS contains abundant oxidants and ROS, including short-lived superoxide radicals, nitric oxide, and longer-lived hydroquinones, which contribute to the persistent oxidative stress observed after smoke exposure (57). CS exposure modifies intracellular redox by altering both the expression and/or activity of pro- and antioxidant enzymes (58). Thus, we hypothesized that MIF could exert its cytoprotective effects by modifying redox responses in the lung in the context of CS exposure. An important endogenous source of CS-induced oxidative stress is XOR. XOR is elevated in human and murine CS-induced lung disease (14, 15, 21, 25). Moreover, XOR is sufficient for CS-induced EndoC DNA damage, p53 induction, and apoptosis, implicating it as a critical mediator of EndoC cytotoxicity in response to CS. Here, we demonstrate that XOR activity increased significantly in $Mif^{-/-}$ mice exposed to CS compared with their wild-type ($Mi^{+/+}$) counterparts without significant differences in XOR protein, implicating a post-translational mechanism involved in its acute activation. In addition, our previous work demonstrates that XOR protein is also increased with longer exposures, likely contributing to or stabilizing XOR activity (15). Here, we show that acute XOR hyperactivation in CS-exposed $Mif^{-/-}$ mice occurred via a p38-dependent mechanism. In considering that MIF expression may regulate a nodal point in emphysema development, therapeutically targeting p38 activity in the face of declining MIF levels and possibly increasing XOR levels may prove beneficial.

The link between XOR activity and p38 signaling is established in other models, including hypoxia (27) and ventilator-induced lung injury (28), where p38 signaling is linked to EndoC apoptosis and dysfunction (28, 29). Despite this established link between p38 signaling and XOR hyperactivation, potential upstream regulators of p38 in these models have not been established. Here, we show that the enhanced CS-induced apoptosis observed in the lungs of $Mif^{-/-}$ mice occurred via a p38-dependent mechanism, as demonstrated by its inhibition with the p38 inhibitor, SB203580. *In vitro* studies in primary lung microvascular EndoCs demonstrated that CS exposure is sufficient to activate p38, as assessed by its phosphorylation status. Antagonism of p38 prevented CS-induced apoptosis of EndoCs and was sufficient to prevent CS-mediated death of MIF-deficient EndoCs *in vitro*. Thus, MIF-deficient EndoCs undergo apoptosis via a p38-dependent mechanism both *in vivo* and *in vitro*.

In addition, we have demonstrated that exogenous MIF was sufficient to suppress p38 phosphorylation in response to CS. Because MIF does not possess intrinsic kinase/phosphatase activity, we speculated that MIF acts upstream of p38 to modify its activation state. ASK1 is a well recognized ROS-responsive kinase upstream of p38 MAPK and recently only speculated to be a

therapeutic target for COPD due to its regulation of p38 (31, 59) and possible involvement in bronchitis-related cell turnover, as determined in studies with bronchiolar cell lines (60). To assess the role of ASK1 in CS-induced p38 activation and apoptosis, we used a loss-of-function approach. Using siRNA directed against ASK1, we demonstrated that ASK1 was necessary for phosphorylation of p38 and EndoC apoptosis in response to CS *in vitro*. Thus, in EndoC, ASK1 lies upstream of p38 in response to CS and is obligatory for CS-mediated cytotoxicity. ASK1 activity in quiescent cells is regulated by protein–protein interactions, which mask its capacity to autophosphorylate and activate the kinase. One recognized negative regulator of ASK1 is Trx, which possesses a TPOR motif similar to MIF (38, 39). In the face of increased ROS, the TPOR residues undergo oxidation, causing disassociation from ASK1, disinhibiting the kinase. Based on functional homology between MIF and Trx, we predicted that MIF may represent a novel direct inhibitor of ASK1 and, thus, modify downstream signaling intermediates, including p38 MAPK. In support of this, we demonstrated that MIF was sufficient to directly inhibit the kinase activity of ASK1 at physiologically relevant concentrations. In addition, there are limited data suggesting that ASK1 activation is enhanced in emphysematous lung disease (Figure E7), a relative MIF-deficient state. Collectively, this work supports a model in which MIF protects EndoCs in the lung from CS-induced toxicity by inhibiting signaling via an ASK1–p38–XOR pathway, thereby identifying additional potential therapeutic targets and disease modifiers.

In potential contrast to our findings in EndoCs and the intact lung exposed to CS, others examining the relationship between MIF and the p38 axis implicate exogenous MIF as a positive regulator of p38 via a CD74-dependent mechanism (61, 62). CD74, in conjunction with CD44, is capable of transducing the extracellular binding of MIF into activation of intracellular kinase cascades, including JNK, extracellular signal–regulated kinase 1/2, and AKT (63–65). We have not been able to detect CD74 expression on human lung EndoCs (data not shown), and there are limited published data suggesting that CD74 plays a role in EndoC signaling during inflammation (66). Multiple factors may contribute to the differential role of MIF in the p38 axis under their experimental conditions, including differences in the cellular targets, role of ASK1 in the model, differences in both MIF receptors and/or p38 isoform expression in the target cell(s), dose of recombinant MIF, and kinetics of the analysis. Importantly, here, we demonstrate that CS-induced p38 activation was ASK1 dependent and that MIF directly blocked ASK1 kinase activity, suggesting that the effects of MIF on ASK1 can occur independently of CD74.

Although MIF has many potential intracellular binding partners, a direct interaction with ASK1 has yet to be demonstrated. The classic ASK1 inhibitor, Trx, is a redox-sensitive protein containing a TPOR motif (39) similar in structure to MIF. The TPOR site consists of two reactive cysteine residues in a CXXC motif, which responds to cellular stress. In MIF, the intact CXXC motif is necessary for its biological function, but does not appear to be required for binding to its intracellular receptor, COP9 signalosome subunit 5, also known as Jun activation domain-binding protein 1 (CSN5/Jab1) (41). Intriguingly, Trx has been demonstrated to bind to CSN5/Jab1 (67), and, although both proteins contain reactive cysteine residues, it is currently not known if these residues contribute to this interaction. These observations may suggest potential molecular redundancy with MIF and Trx, as has been implied in studies of lymphocytes (68). Further studies examining the mechanism(s) by which these TPOR-containing proteins inhibit and interact with ASK1 are needed, but are beyond the scope of this article.

Another MAPK that lies downstream of ASK1, implicated both in ROS signaling and in CS-mediated EndoC dysfunction, is JNK (69). We observed phosphorylation of JNK in response to CSE (Figure E6A), but inhibition of JNK was insufficient to abrogate CS-induced EndoC apoptosis (Figure E6B). Thus, although JNK may contribute to EndoC signaling, the activity of this kinase is not required for EndoC apoptosis.

Limitations of this study revolve around the kinetics of the molecular events as they relate to later markers of injury and remodeling. ASK1 autoactivation and p38 phosphorylation occur rapidly, making both justifiably difficult to assess *in vivo*. With such rapid activation of this pathway, the kinetics of activation in the presence of exogenous MIF could be altered or delayed rather than completely inhibited. The importance of this limitation is lessened by the use of complementary approaches, which demonstrate that ASK1 and p38 contributed significantly to CS-induced cytotoxicity, and that MIF directly antagonized ASK1 kinase activity. Inhibition of this pathway rescues MIF deficiency *in vitro* and *in vivo*, collectively arguing that these molecules interface on a common pathway. There are also limited data, presented here and elsewhere (70–72), regarding the role of ASK1 in human emphysema, and thus an indirect linkage with the development of clinical disease; however, the data here support the hypothesis. Further translational work will be necessary to confirm this. Other limitations may arise in that we used an acute CS model with genetic knockouts to identify molecular

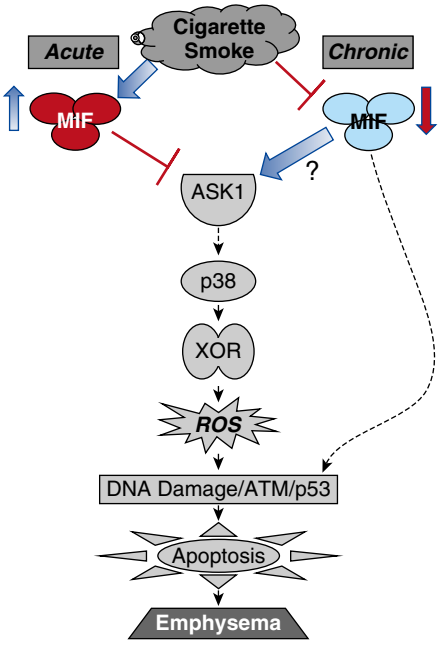

Figure 7. The role of MIF in CS-induced activation of the ASK1–p38–XOR pathway. Acute CS exposure resulted in increased MIF expression (Figure E1) (17, 18). This functions, in part, to antagonize CS-induced ASK1–p38–XOR activation and resulting ROS, p53 expression, and EndoC apoptosis. Chronic CS is associated with loss of MIF (16, 17). Activation of XOR, p38, and ASK1 are also increased in human and murine CS-induced lung disease, as we show here and as others have shown previously (15, 26, 32). When MIF is lost, we postulate that the ASK1–p38–XOR pathway is hyperactivated, resulting in increased EndoC apoptosis and emphysematous tissue destruction. ATM, ataxia telangiectasia mutated kinase.

mechanisms exacerbated by chronic CS exposure of wild-type animals and in humans. Our preclinical and *in vitro* models of MIF deficiency represent a critical point in emphysema progression, where the cytoprotective effects of MIF are diminished, and cell damage/death by definition exceed repair. Understanding that MIF has proinflammatory activity and that acute versus chronic inflammation may differ, we have probed for several inflammatory endpoints, and did not detect acute genotypic differences aside from increased lymphocytes in $Mif^{-/-}$ mice (16). Whereas, with chronic CS exposure, macrophages are increased in both genotypes, and increased lymphocytes are detected in $Mif^{-/-}$ mice (17), the lack of other inflammatory differences: (*1*) argues against a mechanism by which the proinflammatory effects of MIF play a role in COPD pathology; and (*2*) fails to account for the EndoC-specific alterations detected with chronic CS exposure (16).

Redox signaling and, more specifically, an imbalance in oxidant and antioxidant production is a well recognized contributing factor to disease progression. Our results provide strong support for a novel role of MIF as a determinant of ROS production in response to smoke exposure, impacting on the ASK1–p38 kinase cascade regulating the activity of the ROS-generating enzyme, XOR, and antagonizing ASK1–p38–dependent EndoC apoptosis (Figure 7). In our model, the acute production of MIF in response CS acts to partially repress signaling via the ASK1–p38–XOR pathway, accounting for its cytoprotective effects. After chronic exposure to CS, MIF expression is diminished (16, 17), potentiating injury signaling via this pathway and exacerbating emphysematous remodeling.

In summary, this study establishes a role for MIF in controlling ROS responses to smoke by regulating p38-dependent XOR activity, and it links MIF as a direct inhibitor of ASK1, upstream of p38 in CS-induced EndoC injury. The identification of this pathway provides additional therapeutic targets directed at ameliorating ROS production and EndoC apoptosis, driving forces behind tissue injury and remodeling in emphysema. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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