Peptide-Mediated Inhibition of Mitogen-Activated Protein Kinase–Activated Protein Kinase–2 Ameliorates Bleomycin-Induced Pulmonary Fibrosis

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Mitogen-activated protein kinase–activated protein kinase–2 (MAPKAPK2, or MK2), a serine/threonine kinase downstream of p38 mitogen–activated protein kinase, has been implicated in inflammation and fibrosis. Compared with pathologically normal lung tissue, significantly higher concentrations of activated MK2 are evident in lung biopsies of patients with idiopathic pulmonary fibrosis (IPF). Expression is localized to fibroblasts and epithelial cells. In the murine bleomycin model of pulmonary fibrosis, we observed robust, activated MK2 expression on Day 7 (prefibrotic stage) and Day 14 (postfibrotic stage). To determine the effects of MK2 inhibition during the postinflammatory/prefibrotic and postfibrotic stages, C57BL/6 mice received intratracheal bleomycin instillation (0.025 U; Day 0), followed by PBS or the MK2 inhibitor (MK2i; 37.5 μg/kg), administered via either local (nebulized) or systemic (intraperitoneal) routes. MK2i or PBS was dosed daily for 14 days subsequent to bleomycin injury, beginning on either Day 7 or Day 14. Regardless of mode of administration or stage of intervention, MK2i significantly abrogated collagen deposition, myofibroblast differentiation and activated MK2 expression. MK2i also decreased circulating TNF-α and IL-6 concentrations, and modulated the local mRNA expression of profibrotic cytokine *il-1β*, matrix-related genes *col1a2, col3a1*, and *lox*, and transforming growth factor–β family members, including *smad3*, *serpine1* (*pai1*), *and smad6/7*. *In vitro*, MK2i dose-dependently attenuated total MK2, myofibroblast differentiation, the secretion of collagen Type I, fibronectin, and the activation of focal adhesion kinase, whereas activated MK2 was attenuated at optimal doses. The peptide-mediated inhibition of MK2 affects both inflammatory and fibrotic responses, and thus may offer a promising therapeutic target for IPF.

Keywords: MK2; IPF; established fibrosis; transforming growth factor–β; SMAD

Idiopathic pulmonary fibrosis (IPF) is a fatal scarring disease of the lung with no known etiology or definite treatment modality, and a survival rate of 3 to 5 years. Although the onset of IPF symptoms (breathlessness and cough) is usually insidious, significant fibrotic damage is already present at the time of diagnosis. IPF afflicts approximately 128,100 people in the United States, with 48,000 new cases occurring annually (1). Although lung transplantation is considered the definitive therapy for IPF, the 5-year survival after lung transplantation is only 50%. Accordingly, even lung transplantation cannot be considered a "cure" for IPF.

CLINICAL RELEVANCE

The selective inhibition of a downstream tyrosine kinase target (such as mitogen-activated protein kinase–activated protein kinase–2, or MK2) leads to the effective mitigation of pre-existing fibrotic responses. The peptide-mediated inhibition of MK2 affects both inflammatory and fibrotic responses, and thus may offer a promising therapeutic target for idiopathic pulmonary fibrosis.

Histopathologically, IPF can be described as an accumulation of activated myofibroblasts in fibroblastic foci (2). The impaired apoptosis of myofibroblasts may result in a persistent and dysregulated repair process that culminates in tissue fibrosis. Arguably, inflammation also plays a critical role in IPF, perhaps through the cyclic acute stimulation of fibroblasts in response to epithelial injury. Therefore, effective lung-tissue repair and matrix remodeling subsequent to chronic injury may require the concomitant targeting of inflammatory and fibrotic processes.

Mitogen-activated protein kinase–activated protein kinase–2 (MAPKAPK2 or MK2) is an intracellular serine/threonine kinase substrate downstream from p38 mitogen-activated protein kinase (MAPK) that has been implicated in many inflammatory diseases that are complicated by scarring and fibrosis (3). MK2 controls gene expression at transcriptional and posttranscriptional levels (4, 5), and regulates cytoskeletal architecture (6). Activated MK2 increases the translation and stability of inflammatory cytokine mRNA, and causes actin reorganization. The inhibition of MK2 is associated with reduced inflammation (7) and myofibroblast differentiation, and MK2 inhibition also significantly reduces α–smooth muscle actin (α-SMA) protein expression (8). Accordingly, inhibiting MK2 activity may offer therapeutic potential in IPF.

The MK2 inhibitor MK2i (donated by Moerae Matrix Inc. [MMI]), known as MMI-0100, is a synthetic, 22 amino-acid cell-permeant peptide that enters cells through the action of a transduction domain. Once in the cell, the drug inhibits MK2 activity and reduces inflammatory cytokine expression, and also inhibits myofibroblast activation. We have reported on its effectiveness in inhibiting fibrosis occurring as a complication of vascular graft and abdominal surgeries (intimal hyperplasia and abdominal adhesions, respectively) (7, 8). Because fibrotic mechanisms in different organs may be shared, these reports suggest that inhibiting MK2 activity may offer an attractive therapeutic approach to preventing or treating pulmonary fibrotic disorders.

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MATERIALS AND METHODS

Human Studies

Frozen tissues from IPF patients were obtained from the Lung Tissue Research Consortium, sponsored by the National Institutes of Health–National Heart, Lung, and Blood Institute. Paraffin-embedded IPF and normal specimens were procured from the Department of Pathology at the Indiana University School of Medicine. All protocols were approved by the Institutional Research Board at the Indiana University School of Medicine.

Normal Primary Human Fetal Lung Fibroblasts

Cell culture methods are described in the online supplement.

Peptides, Antibodies, and Other Reagents

MMI-0100 was synthesized using standard fluorenylmethyloxycarbonyl group (FMOC) chemistry as previously described, with the peptide sequence YARAAARQARAKALARQLGVAA (molecular weight, 2.2836 kD) (9). Antibodies against α-SMA (Dako Corp., Carpinteria, CA), glyceraldehyde 3–phosphate dehydrogenase (GAPDH), and tyrosine (Tyr)-397–focal adhesion kinase (FAK; Invitrogen, Carlsbad, CA) and threonine (Thr)–334–MK2 (Cell Signaling, Boston, MA) were used for immunoblotting or immunostaining. Recombinant human platelet–derived transforming growth factor (TGF)–β1 was acquired from Roche Diagnostics (Indianapolis, IN). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Peptide compound was initially solubilized in dimethyl sulfoxide to achieve a 1 mg/ml stock concentration. For animal experiments, the peptide compound was dissolved in PBS at a concentration of 1 mg/ml, and the dose was calculated as previously described (10). The calculated dose (0.001 mg/injection) would achieve a 4.4-μM concentration of the drug in a volume (measured in milliliters) of distribution equivalent to the weight (measured in milligrams) of the mice, assuming 100% bioavailability of the drug and an average weight of 20 g for the mice. In early pilot experiments, bleomycin-injured mice received doses of 25, 50, and 75 μg/kg, and these doses were tolerated without evidence of toxicity.

Western Blotting, Immunofluorescence Staining, and Cytokine Profiling by Cytometric Bead Analysis

These methods are described in the online supplement.

Immunohistochemistry and Intensity Analyses

Sections obtained from paraffin-embedded, formalin-fixed lungs were immunostained as described previously (10). Immunostained sections were scanned, and the staining intensity was analyzed using an Aperio Imagescope, version 11.2.0.780 (Vista, CA). Results are expressed as percent positively stained pixels.

Animal Studies

The Animal Care and Use Committee at the Indiana University School of Medicine approved the animal protocols used in this study. C57BL/6J mice (6–8 wk of age; Jackson Laboratories, Bar Harbor, ME) were intratracheally instilled with bleomycin (0.025 U) as previously described (10), with minor modifications.

Murine Fibrosis PCR Microarrays

Murine lung mRNA was used to generate cDNA. The Mouse Fibrosis PCR Array–RT^2 Profiler PCR Arrays, version 3.0 (SABiosciences; Qiagen, Valencia, CA), were then used according to the manufacturer's instructions, and array data were analyzed using PCR Array Data Analysis software (Qiagen).

Statistical Analysis

Statistical analysis was performed according to the Student *t* test and one-way ANOVA with the Bonferroni *post hoc* test, using GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com), unless otherwise stated. Statistical significance was defined as $P < 0.05$.

Supplementary Material

We have described the nebulized drug-delivery technique in the online supplement. Immunostained intensity analyses of activated MK2 expression for the murine tissues described in Figures 4C and 5C are also presented in Figures E1A and E1B, respectively, in the online supplement. The dataset for the microarray analyses is included in the online supplement, and a clustergram of the entire murine fibrosis PCR array is presented as Figure E2.

RESULTS

Activated MK2 Is Differentially Expressed in IPF versus Non-IPF Human Lung Tissue, and Is Specifically Localized in Fibrotic Foci and Airway Epithelia

To investigate the potential clinical relevance of MK2 in IPF, we assayed activated MK2 expression in lung-tissue explants from IPF and non-IPF patient biopsies according to both Western blot and immunohistochemical analyses (Figure 1). Patient lung homogenates were immunoblotted using an antibody recognizing activated MK2 (phospho-Thr^{334} MK2) and total MK2, using GAPDH as a loading control. We observed strong, activated MK2 expression in IPF lung homogenates (Figure 1A). A densitometric analysis of immunoblots (normalized to GAPDH) comparing normal lung tissue ($n = 7$) and IPF lung tissue ($n = 20$) showed significantly higher activated MK2 expression in IPF lung tissue ($P < 0.05$; Figure 1B). An immunohistochemical analysis demonstrated higher activated MK2 expression in IPF lung tissue, specifically in the fibroblasts and epithelia. However, biopsy tissue harvested from patients without IPF displayed significantly less activated MK2 (Figure 1). Together, these data demonstrate a potential role for MK2 in the pathogenesis of IPF.

MK2 Inhibition Blocks TGF-β1–Induced Myofibroblast Differentiation and Matrix Synthesis

Sousa and colleagues (5) showed that TGF-β1–mediated myofibroblast differentiation and α-SMA expression are dependent on MK2. As reported in several earlier studies (11, 12), TGF-β1 is up-regulated in IPF, which leads to myofibroblast differentiation and an enhanced secretion of extracellular matrix that, in turn, leads to the development of fibrotic foci. Accordingly, the effects of MK2 inhibition via MK2i on TGF-β–stimulated myofibroblast activation were examined. To determine the temporal pattern of TGF-β1–stimulated MK2 activation in normal primary human fetal lung fibroblasts obtained from Coriell Institute for Medical Research (IMR, Camden, NJ), IMR-90 cells were cultured to 65–70% confluence, growth-arrested via serum starvation for approximately 36 hours, and treated with TGF-β1 (2 ng/ml) for specific periods of time. We observed an increase in α-SMA expression (a marker for myofibroblast differentiation) at 16 hours, which was sustained until 72 hours (Figure 2A). The activation of MK2 in response to TGF-β1 stimulation was observed at around 3–6 hours, and was sustained until 72 hours, whereas total MK2 expression levels remained unchanged (Figure 2A).

Next, to determine the effects of MK2i on MK2 activation, quiescent IMR-90 cells were pretreated with MK2i at several concentrations for 1 hour, and then cultured in the presence or absence of TGF-β1 (2 ng/ml) for 72 hours. We observed a significant decrease in activated MK2 (phospho-Thr^{334} residue) beginning at 2 μM MK2i, and a dose-dependent decrease in total MK2 expression (Figure 2B). We next evaluated the effect of MK2 inhibition on fibroblast differentiation. Cell lysates were immunoblotted for α-SMA, fibronectin, collagen Type I, and β-actin (loading control). Dose-dependent decreases in α-SMA, fibronectin, and collagen Type I expression were observed, with significant

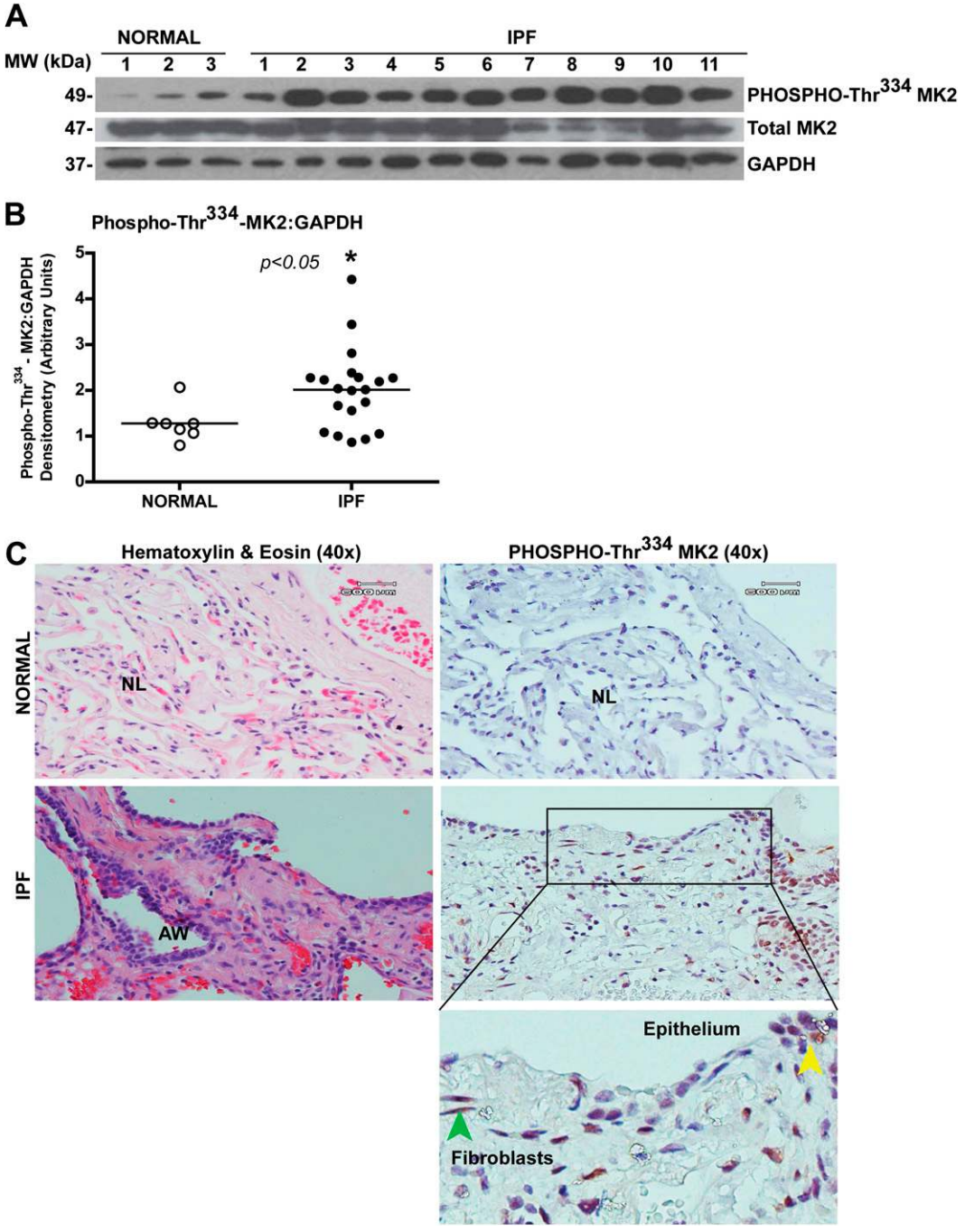

Figure 1. Activated mitogen-activated protein kinase–activated protein kinase–2 (MK2) is increased in idiopathic pulmonary fibrosis (IPF) versus non-IPF human lung biopsy tissue, with expression localized to fibrotic foci and airway epithelium. (*A*) Pathologically normal (non-IPF) and IPF lung biopsies were homogenized and then subjected to Western blotting, using antibodies against threonine (Thr)–334–MK2 and glyceraldehyde 3–phosphate dehydrogenase (GAPDH) as a loading control. (*B*) Densitometric analyses of individual band intensities showed significantly higher expression of activated Thr-334–MK2 when normalized to GAPDH. Values represent means ± SEM. Normal group, $n = 7$; IPF group, $n = 20$. $^*P < 0.05$, one-way ANOVA; *post hoc* test, Bonferroni. (*C*) Comparative immunohistochemical analysis of paraffin-embedded human IPF lung biopsy explants obtained during lung transplant and tissue resected from "normal" (non-IPF) lung tissue was performed using activated MK2 antibody and hematoxylin-and-eosin staining. *Top*: Normal lungs at the time of resection of other clinical diagnosis. Hematoxylin-and-eosin staining shows normal lung architecture, with no detectable activated MK2 expression. *Bottom*: IPF lung-tissue biopsy (expansion of enclosed area at *top*). Hematoxylin-and-eosin staining shows disrupted epithelium (*yellow arrowhead*) with fibroblasts (*green arrowhead*), with activated MK2 staining (3,3′-Diaminobenzidine DAB, *brown*) appearing in fibroblasts (*green arrowhead*) and epithelium (*yellow arrowhead*). Nucleus was counterstained with hematoxylin (*blue*). Original magnification, ×40. *Scale bar* = 600 μm. AW, airway; MW, molecular weight; NL, normal lung architecture. Representative lesions are presented subsequent to examining lung biopsies from three different patients.

effects beginning at 2 μM MK2i (Figure 2C). Decreased α-SMA expression was also confirmed by the immunofluorescent staining of cultured IMR-90 cells (Figure 2D). Collectively, these studies suggest that MK2 inhibition attenuates myofibroblast differentiation and matrix synthesis/secretion.

MK2 Inhibition Down-Regulates TGF-β1–Induced FAK Activation

The activation of FAK by TGF-β1 mediates the stable induction of myofibroblast differentiation (13) and survival (14). Recent studies demonstrated that the TGF-β1–induced phenotypic transition of fibroblasts to myofibroblasts is critically dependent on cell adhesion events and integrin signaling via FAK (10, 14). To evaluate the mechanism further by which MK2 inhibition blocks myofibroblast differentiation, the activation states of fibroblast prosurvival kinase FAK, which is also implicated in the pathogenesis of IPF, were examined. Quiescent IMR-90 cells were pretreated with MK2i at several concentrations for 1 hour, and then cultured in the presence or absence of TGF-β1 (2 ng/ml) for 16 hours. Cell lysates were immunoblotted for Tyr-397–FAK and β-actin (loading control). MK2i suppressed TGF-β1–induced FAK activation and myofibroblast differentiation at 2 μM (Figure 2E). These data suggest that MK2 activity plays a critical role in myofibroblast differentiation through the MK2/FAK signaling axis.

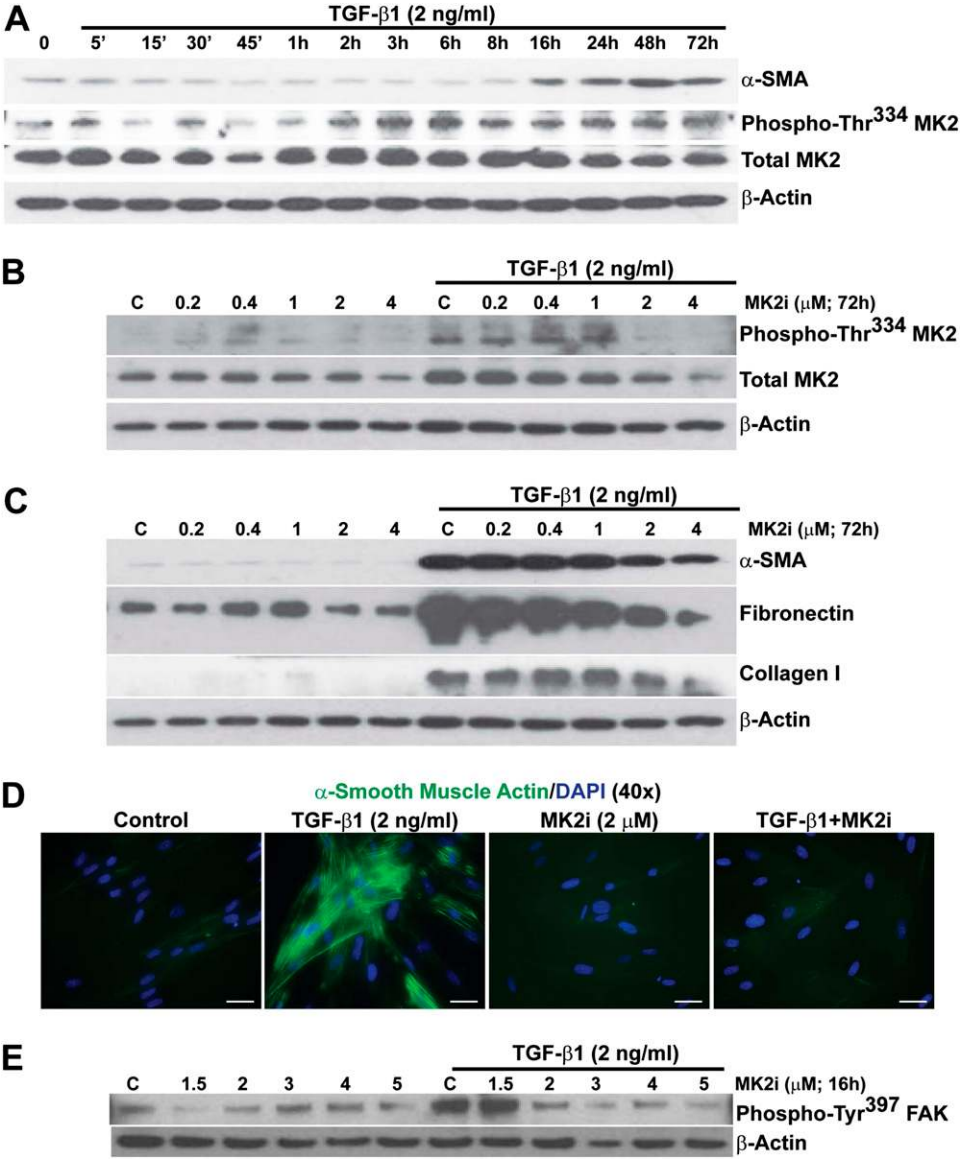

Figure 2. The MK2 inhibitor (MK2i) also inhibits transforming growth factor (TGF)–β1–induced myofibroblast differentiation, matrix secretion, and activation of focal adhesion kinase (FAK) in normal primary human fetal lung fibroblast cultures. IMR-90 cells were grown to 65–70% confluence and serum-starved for approximately 36 hours. Cell lysates were immunoblotted with antibodies recognizing α–smooth muscle actin (α-SMA) and β-actin (loading control). (*A*) When stimulated with TGF-β1, peak MK2 activation occurred between 3–6 hours of culture, with no changes in total MK2. Cells were then pretreated with several concentrations of MK2i for 1 hour, and cultured with and without TGF-β1 (2 ng/ml) for 72 hours. (*B*) MK2i down-regulated MK2 activation and total MK2 synthesis. (*C*) MK2i at 2 μM effectively down-regulated α-SMA, fibronectin, collagen Type I, and β-actin (loading control). (*D*) This down-regulation was confirmed by immunofluorescence staining with anti–α-SMA and DAPI (4′, 6-diamidino-2-phenylindole). Original magnification, ×40. *Scale bar* = 100 μm. The results are representative of three independent experiments. (*E*) IMR-90 cells were grown to 80% confluence and serum-starved for approximately 36 hours. Cells were then treated with several concentrations of MK2i for 1 hour, and cultured with and without TGF-β1 (2 ng/ml) for a period of 16 hours. Cell lysates were immunoblotted for tyrosine (Tyr)–397–FAK, with β-actin as a loading control (C). Results are representative of three independent experiments.

Temporal Expression Pattern of MK2 Activation in the Bleomycin Model of Lung Fibrosis

To simulate our observations of MK2 activation in clinical IPF tissues, we investigated the temporal activated MK2 expression pattern in the lungs of bleomycin-injured mice. We used a murine model of chronic lung injury with intratracheal instillation of PBS or bleomycin (0.025 U), and examined the lungs for activated MK2 expression on Days 7, 14, 21, and 28 after bleomycin injury. Similar to the differential activated MK2 expression observed in human IPF versus pathologically normal human lung tissue (Figure 1), activated MK2 expression was significantly robust on Day 7 and Day 14 after bleomycin injury (Figure 3A), compared with PBS-instilled mice. At the later time points, these concentrations were lower than those on Day 7 and Day 14 (Figure 3B). These results implicate early, robust bleomycin-induced MK2 activation in lung fibrosis.

Local and Systemic MK2 Inhibition Prevents Bleomycin-Induced Fibrosis

MK2 has been reported to play a key role in the regulation of lung fibroblast α-SMA expression and stability in the bleomycin model (5). To determine the effects of inhibiting MK2 in the development of fibrosis *in vivo*, the murine model of chronic lung injury with intratracheally instilled bleomycin, as already described, was used again, as illustrated in Figure 4A. At the stage of PBS or MK2i intervention in this model (Days 7–21), previous reports indicated that inflammatory responses subside (15), and fibrotic responses initiate and develop (16). Our previous observations (Figure 3) presented a rationale for using Day 7 as a starting point of MK2 intervention, because we observed high levels of activated MK2 expression compared with that in normal lungs. Inhibiting MK2 by either nebulizing MK2i locally into the lung or delivering it systemically via intraperitoneal injection inhibited fibrotic responses to bleomycin injury. Specifically, local or systemic MK2 inhibition resulted in significant reductions in total lung collagen content, as determined by hydroxyproline concentrations in whole lungs (Figure 4B). One of the hallmarks of IPF involves the activation of mesenchymal cells and the exuberant deposition of extracellular matrix, and specifically collagen. We observed that bleomycin-induced collagen deposition is significantly reduced, as indicated by trichrome staining (Figure 4C) that was consistent with the reduced collagen content determined by the hydroxyproline assay (Figure 4B). To determine the effects of MK2i on the expression of activated MK2, we used immunohistochemical staining methods. We

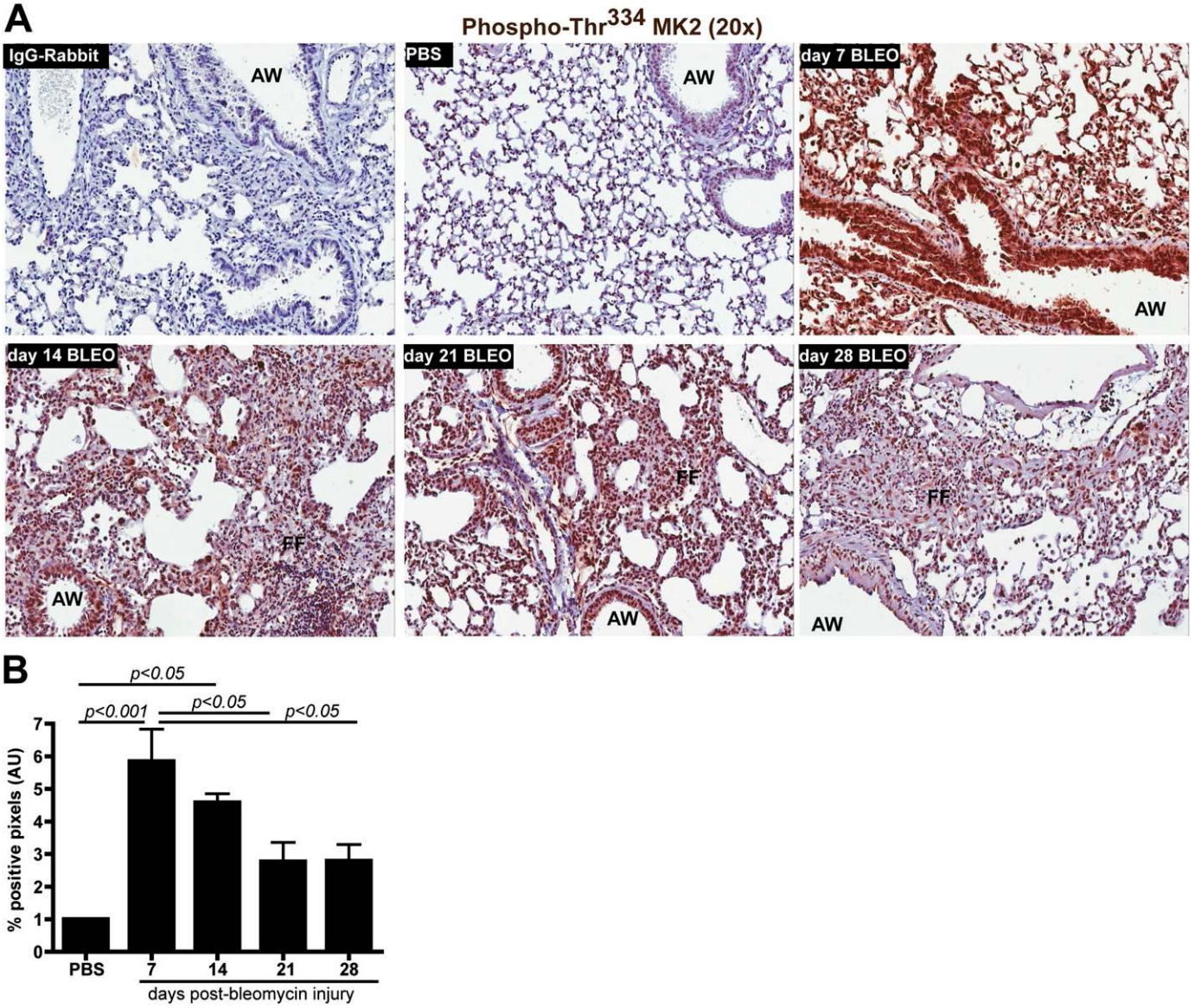

Figure 3. Temporal expression pattern of activated MK2 in the bleomycin (BLEO) model of lung fibrosis. C57-BL/6 mice were subjected to an intratracheal instillation of PBS or bleomycin (0.025 U) on Day 0. Lungs were harvested at the indicated time points after bleomycin injury. (*A*) Formalin-fixed lung-tissue sections were immunostained with antibodies recognizing phospho-threonine (Thr)^{334}–MK2. Control staining was applied with biotinylated secondary IgG antibody. Streptavidin-conjugated horseradish peroxidase was used, with 3,3′-diaminobenzidene as substrate (*brown*). Nuclei were counterstained with hematoxylin (*blue*). Original magnifications, ×20. (*B*) Staining intensity was analyzed using Imagescope (Aperio, Vista, CA), and is expressed as percent positive staining. Values represent the means ± SEMs ($n = 3$ mice per group). AU, arbitrary units; AW, airway; FF, fibrotic foci.

observed that MK2 activation was greater in bleomycin-injured lungs on Day 21, compared with PBS control lungs (Figure 4C and Figure E1A in the online supplement). With the administration of MK2i either by nebulizing it locally into the lung or by delivering it systemically via intraperitoneal injection, we observed a trend toward lower MK2 activation levels (Figure 4C and Figure E1A). These data demonstrate that inhibiting MK2 activity, either locally or systemically, during the postinflammatory/prefibrotic stage of the modeled disease significantly decreased collagen deposition, compared with the bleomycin control samples.

Local and Systemic MK2 Inhibition Ameliorates Established Fibrosis

Because patients with IPF have severely scarred lungs at the time of diagnosis, we next examined whether MK2 inhibition would reverse established fibrosis. To simulate this clinically relevant treatment paradigm, the bleomycin model of established fibrosis, described by Henderson and colleagues, was used wherein the intervention is begun on Day 14, and the lungs show significant fibrosis (Figure 5A) (17). Our previous observations (Figure 3) presented a rationale for using Day 14 as a starting point of MK2 intervention, because we observed high concentrations of activated MK2 compared with that in normal lungs. Regardless of whether MK2 was inhibited locally or systemically, the progression of fibrosis was arrested, as determined by collagen deposition quantitatively on the left lung, using a standard hydroxyproline assay (Figure 5B). Interestingly, at a total of 28 days after bleomycin injury, MK2 inhibitor–treated groups demonstrated significantly lower concentrations of collagen than did the bleomycin group. The 28-day bleomycin group exhibited significantly higher concentrations of collagen compared with the 14-day (baseline) bleomycin group, indicating the progression of fibrosis within that interval, when

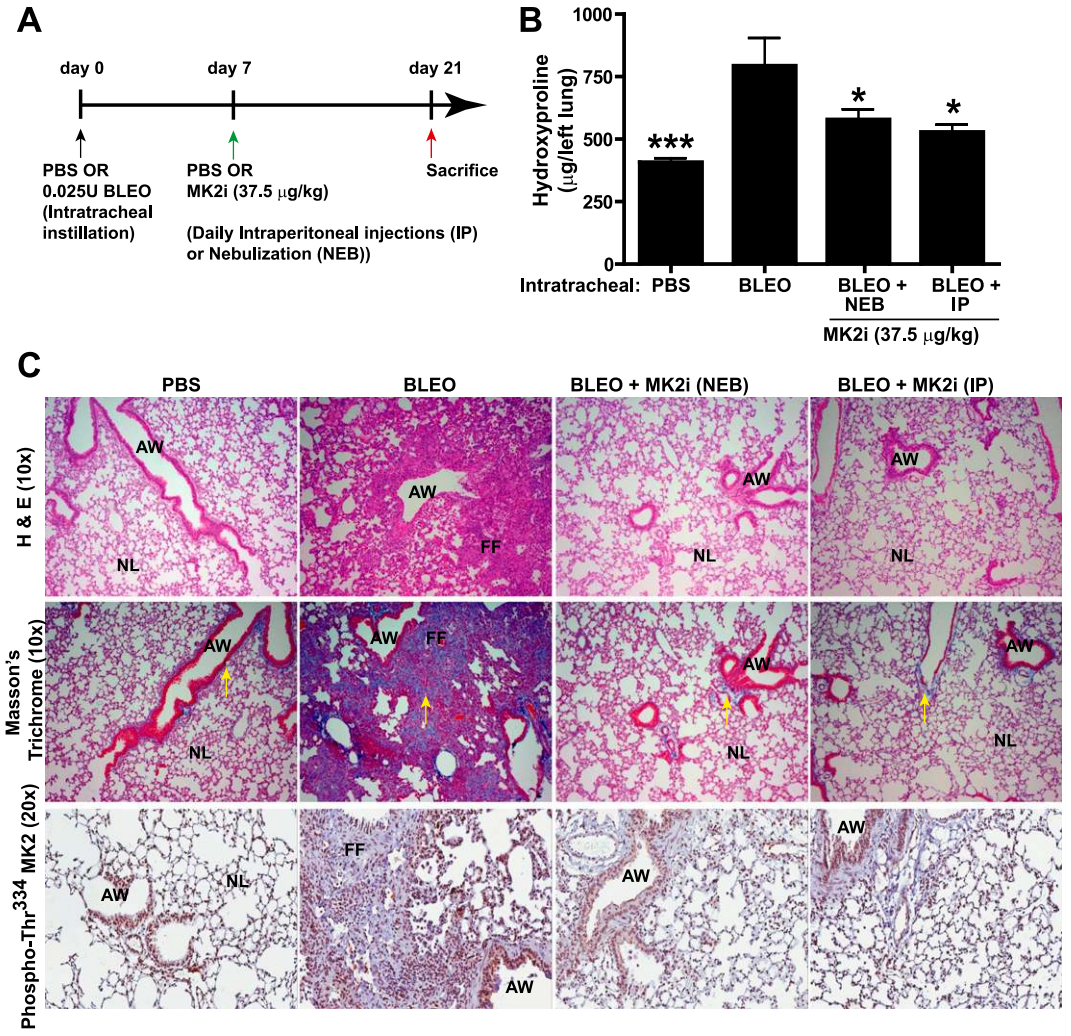

Figure 4. Both local, inhaled delivery and systemic administration of MK2i protect against the development of bleomycin-induced lung fibrosis in mice (prevention model). C57-BL/6 mice were subjected to bleomycin injury via intratracheal instillation on Day 0. (*A*) On Day 7, when significant inflammation was still present, we administered 37.5 μg/kg/day of MK2i daily, either locally to the lung via nebulizer (NEB), or systemically via intraperitoneal (IP) injection, until Day 21. (*B*) MK2i prevented the significant collagen deposition attributable to bleomycin injury. Total collagen (soluble and insoluble) deposition was assessed by analyzing hydroxyproline concentrations in murine lung tissue on Day 21 after bleomycin injury. Values represent means ± SEMs ($n = 5$ animals per group). $^*P < 0.05$ and $^{***}P < 0.001$, one-way ANOVA; *post hoc* test, Bonferroni. (*C*, *upper*) Hematoxylin-and-eosin (H & E) staining of representative murine lung tissues on Day 21 revealed grossly scarred lungs atributable to bleomycin injury. This effect was significantly reversed via both modes of MK2i administration. (*C*, *middle*) Masson blue trichrome staining of the same fields revealed extensive collagen deposition (*yellow arrows*) with bleomycin injury. MK2i lung tissue appeared to be protected and was similar to normal lung tissue, with collagen deposition limited to areas around airways and blood vessels. Original magnification, ×10. *Scale bar* = 600 μm. (*C*, *lower*) Formalin-fixed lung-tissue sections were immunostained with antibodies recognizing phospho-Thr^{334}–MK2. Control staining was applied with biotinylated secondary IgG antibody. Streptavidin-conjugated horseradish peroxidase was used with 3,3′-diaminobenzidine as substrate (*brown*). Nuclei were counterstained with hematoxylin (*blue*). Original magnifications, ×20. AW, airway; FF, fibrotic foci; NL, normal lung architecture. Results are representative of three independent experiments.

mice were not treated with MK2i. A histopathological assessment was used to examine lung architecture (hematoxylin-and-eosin staining; Figure 5C) and collagen distribution (Masson blue trichrome staining; Figure 5C). To determine the effects of MK2i on the expression of activated MK2, we used immunohistochemical staining methods. We observed that MK2i administration, either by nebulizing it locally into the lung or by delivering it systemically via intraperitoneal injection, significantly attenuated MK2 activation levels (Figure 5C and Figure E1B). Bleomycin-injured lungs were severely scarred, whereas inhibiting MK2 resulted in nearly normal-appearing lung parenchyma. Accordingly, an assessment for the transcript expression of collagen-specific genes revealed that the bleomycin-induced up-regulation of *col1a2* (Figure 5D), *col3a1* (Figure 5E), and *lox* (Figure 5F) was significantly abrogated with the inhibition of MK2 activity. Our experiments demonstrate that inhibiting MK2, locally or systemically, effectively arrests the progression of established fibrosis.

MK2i May Ameliorate Established Fibrosis via the Modulation of inhibitory SMADs and IL-1β

To investigate further the mechanisms underlying the MK2i "rescue" of fibrotic lung tissue, a fibrosis-specific 80-gene PCR array was used to analyze mRNA expression in the bleomycin model of established fibrosis (Figure E2). We observed changes in the expression of four major genes related to the TGF-β–inducible pathway, *mothers against decapentaplegics (smads) 3/6/7*, and *serpine1*, in addition to changes in *il-1β* expression. In a comparison of PBS or bleomycin-instilled lungs, we observed significant down-regulation in the expression of *smad6* and *smad7*, which are inhibitory Smads (I-SMADs) implicated as fibroprotective factors in IPF (Figures 6A and 6B). The inhibition of MK2, via both the inhaled and systemic delivery of MK2i, restored I-SMAD expression to levels nearly comparable with those of control mice instilled with PBS rather than bleomycin. Concomitantly, TGF-β–specific intracellular signaling molecules *smad3* and *serpine1* (a TGF-β/Smad-inducible regulator of extracellular matrix production and cell motility) were up-regulated in bleomycin-injured mice (Figures 6C and 6D). Whereas *smad3* was significantly reduced in mice treated with nebulized MK2i, *serpine1* concentrations were lower in mice subjected to local and systemic MK2i treatment. In addition, we observed dramatic changes in another major profibrotic cytokine implicated in IPF pathogenesis, *il-1β* (18). The peptide-mediated inhibition of MK2 significantly suppressed bleomycin-induced transcript levels of *il-1β* (Figure

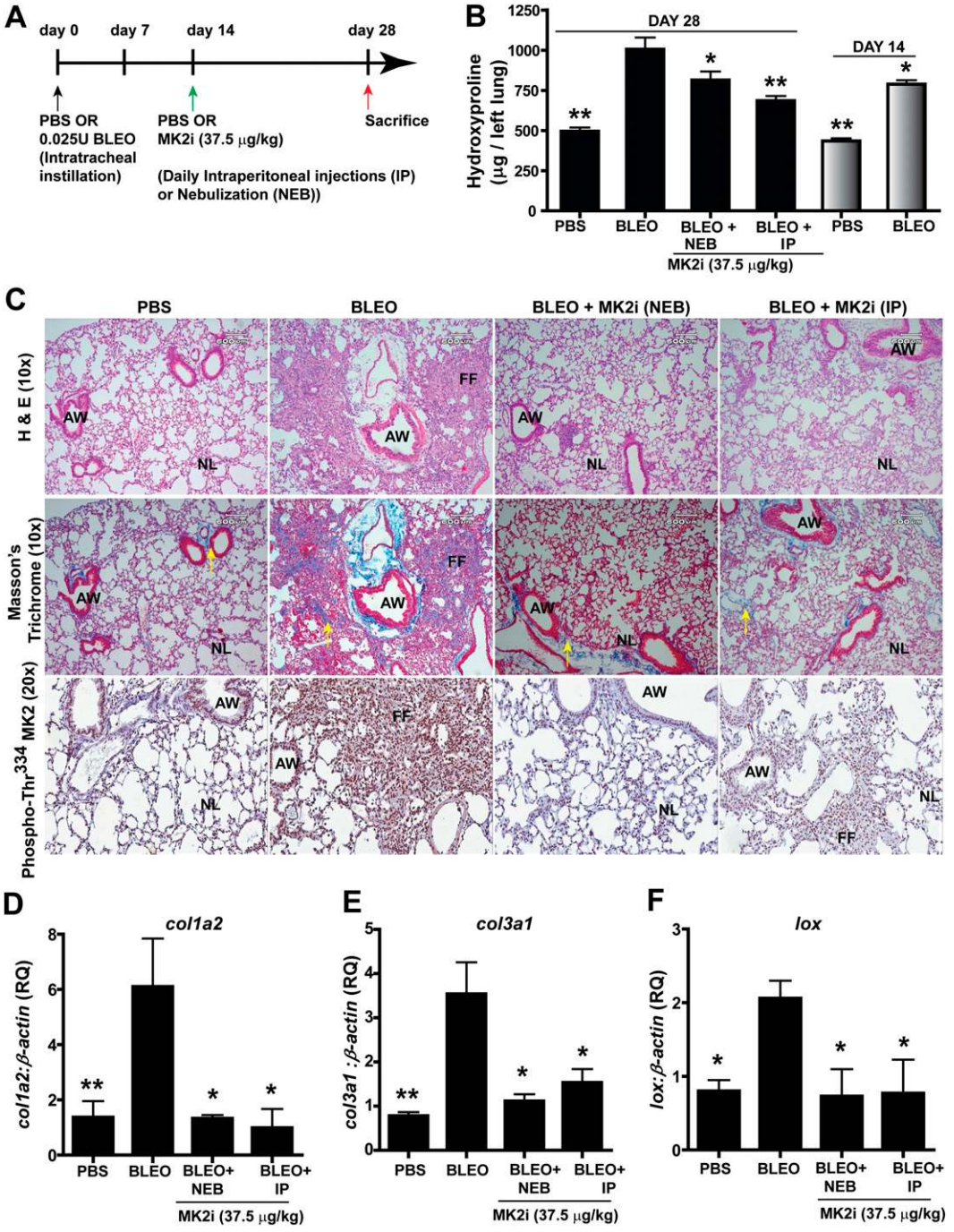

Figure 5. Systemic and nebulized administration of MK2i ameliorates established bleomycin-induced fibrosis in mice (treatment model). C57-BL/6 mice were subjected to bleomycin injury on Day 0. (*A*) Beginning on Day 14, when established fibrosis was already present, and continuing through Day 28, mice were treated with 37.5 μg/kg/day of MK2i daily by intraperitoneal (IP) injection or nebulizer (NEB) delivery. (*B*) MK2i arrested the significant collagen deposition attributable to bleomycin injury. Total collagen (soluble and insoluble) deposition was assessed by analyzing hydroxyproline concentrations in murine lungs on Day 14 or Day 28 after bleomycin injury. Values represent means ± SEMs. PBS = 5 animals per group, BLEO = 4 animals per group, and BLEO + MK2i (NEB and IP groups) = 5 animals per group. *$P < 0.01$ and **$P < 0.001$, one-way ANOVA; *post hoc* test, Bonferroni. (*C*) Hematoxylin-and-eosin staining (*top*) shows lung architecture, which appears scarred in bleomycin-treated animals, but virtually normal in MK2i-treated animals, regardless of the mode of MK2i administration. Masson blue trichrome staining (*middle*) of the same fields reveals extensive collagen deposition (*yellow arrows*) with bleomycin injury. MK2i lungs, similar to normal lungs, are protected, with collagen deposition limited to areas around airways and blood vessels. Images are shown from experimental Day 28 after bleomycin injury. Original magnification, ×10. *Scale bar* = 600 μm. *Below*: Formalin-fixed lung-tissue sections were immunostained with antibodies recognizing phospho-Thr^{334}–MK2. Control staining was applied with biotinylated secondary IgG antibody. Streptavidin-conjugated horseradish peroxidase was used, with 3,3′-diaminobenzidene as substrate (*brown*). Nuclei were counterstained with hematoxylin (*blue*). Original magnifications, ×20. AW, airway; FF, fibrotic foci; NL, normal lung architecture. Results are representative of two independent experiments. RNA was isolated from the right lungs, and cDNA was subjected to real-time PCR reactions, using the Mouse Fibrosis PCR Array (SABiosciences; Qiagen, Valencia, CA). (*D*) *Col1a2:β-actin.* (*E*) *Col3a1:β-actin.* (*F*) *Lox:β-actin.* Relative Quantitation (RQ) values represent means ± SEMs. Numbers of animals per group: PBS = 5, BLEO = 5, BLEO + MK2i (NEB and IP groups) = 5. *$P < 0.05$ and **$P < 0.01$, one-way ANOVA; *post hoc* tests, Newman-Keuls (*D* and *F*) and Bonferroni (*E*).

6E). These data suggest that the inhibition of MK2 activity may lead to an attenuation of key cytokines involved in the pathogenesis of IPF.

MK2i Inhibits Myofibroblast Differentiation and Proinflammatory Cytokine Production

One of the hallmarks of IPF involves the accumulation of myofibroblasts at fibrotic lesions and the expression of abundant α-SMA, a marker for myofibroblast activation. Inhibiting MK2 resulted in decreased α-SMA expression, compared with bleomycin control samples (Figure 7A). Previous studies implicated roles for cytokines such as IL-6 and TNF-α (19, 20) in the pathogenesis of pulmonary fibrosis. In addition, MK2 inhibition has been reported to down-regulate the expression of these cytokines in other non-fibrosis models (4, 7, 21). Therefore, we determined whether inhibiting MK2 would down-regulate the production of IL-6 and TNF-α after MK2 inhibition at the postfibrotic stage. Notably,

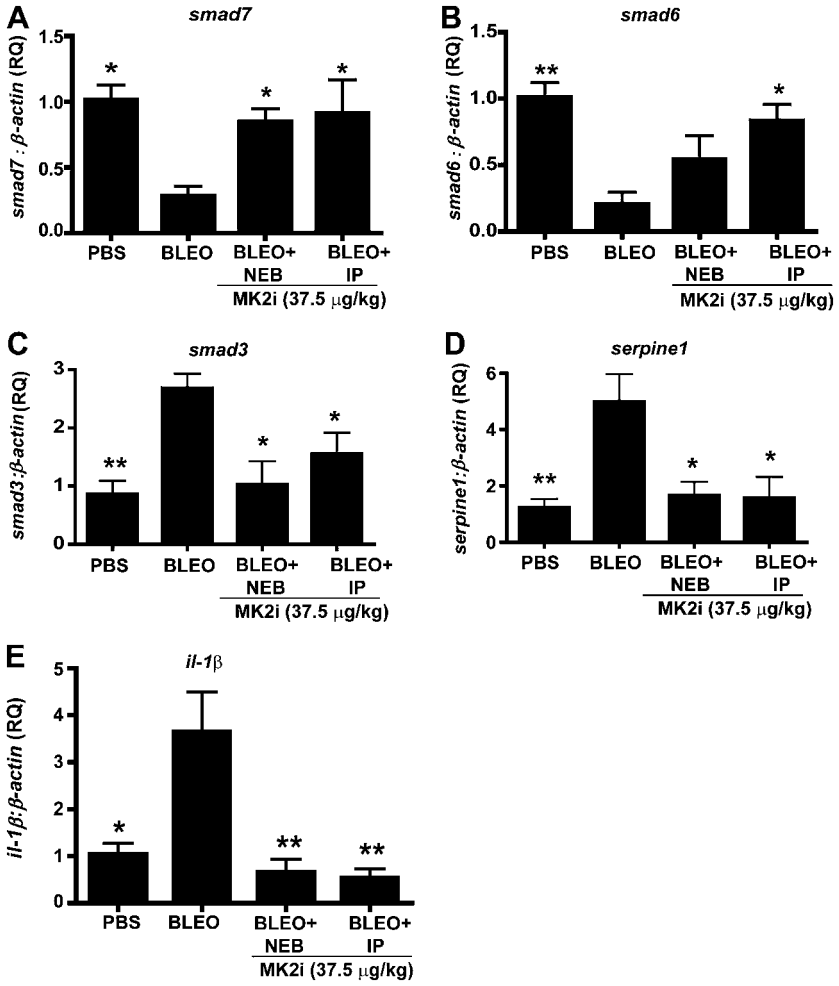

Figure 6. MK2i may ameliorate fibrosis by modulating inhibitory SMADs and IL-1β. C57-BL/6 mice were subjected to bleomycin injury on Day 0. We administered 37.5 μg/kg/day of MK2i daily by intraperitoneal (IP) injection or nebulizer (NEB) from Days 14–28. RNA was isolated from right lungs, and cDNA was subjected to real-time PCR reactions using the Mouse Fibrosis PCR Array. (*A*) *smad7:β-actin*. (*B*) *smad6:β-actin*. (*C*) *smad3:β-actin*. (*D*) *serpine1:β-actin*. (*E*) *il-1β:β-actin*. Values represent means ± SEMs. Numbers of animals per group: PBS = 5, BLEO = 5, BLEO + MK2i (NEB and IP groups) = 5. *$P < 0.05$ and **$P < 0.01$, one-way ANOVA; *post hoc* tests, Bonferroni (*A*, *B*, *D*, and *E*) and Newman-Keuls (*C*).

IL-6 and TNF-α protein concentrations (as measured systemically; Figures 7B and 7C, respectively) were reduced significantly by MK2 inhibition when the MK2 was nebulized. The reduction of TNF-α was not significant when the MKi was administered by the intraperitoneal route. Collectively, these data demonstrate that inhibiting MK2 results in both anti-inflammatory and anti-fibrotic effects.

DISCUSSION

Protein kinases are critical modulators involved in cellular processes such as differentiation (10), proliferation (22), survival (23), and migration (24). MAPK–activated protein kinase–2 (MAPKAPK-2 or MK2), a serine/threonine protein kinase, is a major substrate of p38 MAP kinase (25). In these experiments, the targeted inhibition of MK2, using a specific peptide sequence, MMI-0100, was applied to test proof of concept in a clinically relevant bleomycin model of established fibrosis. These data demonstrate that the effective tissue repair and regeneration of the lung subsequent to chronic injury involves multiple MK2-impacted pathways. Targeting MK2 with this sequence abrogates TGF-β–related signaling molecules and IL-1β, while inducing the negative regulators of TGF-β activity, Smads 6 and 7. MK2i arrests the further progression of fibrosis by inhibiting matrix synthesis (*col1a2*, *col3a1*, *lox*, and fibronectin) and by compromising fibroblast survival (FAK activation with *serpine1*, also known as plasminogen activator inhibitor-1 [PAI-1]). The suppression of the proinflammatory cytokines TNF-α and IL-6 occurs, regardless of the route of administration.

Activated MK2 is up-regulated in IPF versus non-IPF human lung biopsy tissue, suggesting a potential role for MK2 in the pathogenesis of IPF. MK2i treatment down-regulates activated MK2 expression in the murine bleomycin model of pulmonary fibrosis, suggesting that MK2 activity is critical during fibrogenesis. Once a nuclear localization signal in the C-terminus of MK2 is masked by phosphorylation, MK2 has been shown to translocate rapidly into the cytoplasm, to phosphorylate cytosolic targets (26). We report that IPF tissues express significant concentrations of phosphorylated MK2 in several cell types, including fibroblasts and epithelial cells, with both nuclear and cytosolic localization.

Both inflammatory and fibrotic processes have been implicated in the pathogenesis of IPF, with several early studies (11, 14, 17, 19) and more recent reports demonstrating the critical role of TGF-β1 in initiating or exacerbating IPF (27). Our observations *in vitro* suggest that MK2i effectively attenuates the activation of MK2 and the synthesis of total MK2 protein. Interestingly, these observations in clinical tissues, as well as *in vitro* studies, were extended in the bleomycin model, wherein we observed the robust, early activation of MK2 on Day 7 and on Day 14. This signal was not as strong at the two endpoints used in this report (Day 21 and Day 28). With MK2i treatment, the lack of significant differences in MK2 activation on Day 21 (Figure E1) may be attributable to the need for suppressing a significantly higher baseline signal on Day 7 (compared with PBS). Interestingly, significant differences were observed on Day 28 with MK2i treatment, which may likely be explained in terms of the quenching of a relatively lower baseline signal on Day 14 (compared with PBS; Figure E1). Although our early screening of

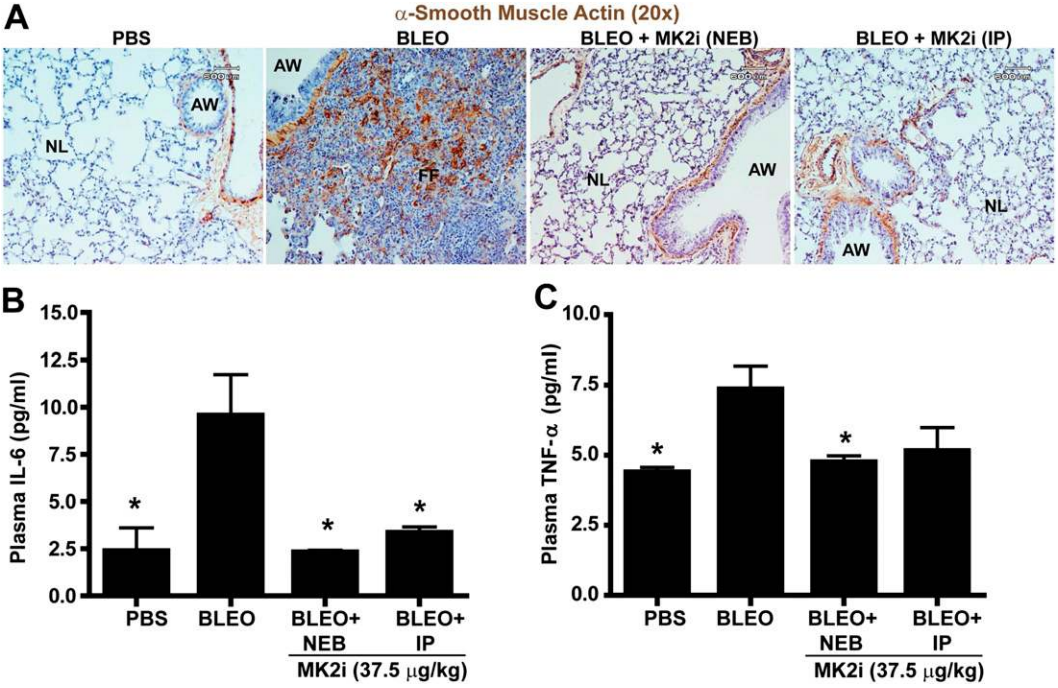

Figure 7. Both the local and systemic delivery of MK2i reduces myofibroblast differentiation and TNF-α and IL-6 concentrations in the murine bleomycin model of established fibrosis. C57-BL/6 mice were subjected to bleomycin injury on Day 0. We administered 37.5 μg/kg/day of MK2i daily by intraperitoneal (IP) injection or nebulizer (NEB) from Days 14–28. (*A*) Formalin-fixed lung-tissue sections were immunostained with antibodies recognizing α–smooth muscle actin. Control staining was applied with biotinylated secondary IgG antibody. Streptavidin-conjugated horseradish peroxidase was used, with 3,3′-diaminobenzidene as substrate (*brown*). Nuclei were counterstained with hematoxylin (*blue*). Original magnifications, ×20. Results are representative of two independent experiments. Plasma, collected when mice were killed, was analyzed for (*B*) TNF-α and (*C*) IL-6 cytokine concentrations. Values represent means ± SEMs. Numbers of animals per group: PBS = 4, BLEO = 5, BLEO + MK2i (NEB and IP groups) = 5. **P* < 0.01, one-way ANOVA; *post hoc* test, Bonferroni. Results are representative of two independent experiments.

the molecule with respect to kinase specificity suggests that the molecule is relatively specific (data not shown), some off-target activity of which we are unaware may have occurred. However, the data presented in this study suggest that the inhibition of MK2 activation, rather than the inhibition of MK2 expression, is a major player in IPF progression.

Sousa and colleagues (5) and Gharaee-Kermani and colleagues (28) demonstrated that MK2 expression regulates myofibroblast differentiation, α-SMA expression, and the exuberant secretion of extracellular matrix, leading to the development of fibrotic foci. Consistent with findings in the literature, the MK2 peptide inhibitor MK2i inhibits myofibroblast differentiation in response to stimulation with TGF-β1 (5, 6). The peptide-mediated inhibition of MK2 abrogated FAK activation, consistent with studies demonstrating that TGF-β–induced FAK activation mediates the stable induction of myofibroblast differentiation (14), and that the blockade of FAK activation is protective against bleomycin-induced pulmonary fibrosis (10). Cumulatively, these data suggest that MK2i may inhibit fibrosis via MK2/FAK/α-SMA signaling, mediating the effects of MK2i on fibroblast differentiation and function.

Fibronectin and collagen secretion are known to be up-regulated in fibrotic tissues (29), including those in in IPF (30), and TGF-β1–stimulated fibronectin expression has been shown to play an essential role in the development of pulmonary fibrosis (31). Increased concentrations of lysyl oxidase (LOX), an amine oxidase critical for the initiation of collagen and elastin cross-linking (32), and increased mRNA concentrations of collagen Types I and III have also been reported in murine models of lung fibrosis (33). In this report, MK2i treatment markedly reduced TGF-β–induced fibronectin and collagen expression. *In vivo*, MK2i significantly reduced collagen deposition and *col1a2*, *col3a1*, and *lox* mRNA expression in the lung. Because matrix remodeling plays a major role in tissue repair, our findings suggest a role for MK2 in regulating key matrix proteins involved in IPF pathogenesis.

Because the TGF-β/Smad2/3 and bonemorphogenic proteins (4/7)/Smad1 signaling pathway has been implicated in clinical IPF (34, 35) and in the murine bleomycin model of IPF (36), the role of MK2 inhibition in Smad regulation was also investigated. TGF-β1 signaling occurs via Type I and II receptor–mediated phosphorylation, whereby activated TGF-β1 receptor I phosphorylates Smad2 and Smad3 (receptor Smads, or R-Smads) at C-termini. R-Smads then complex with other molecules, translocate to the nucleus, and activate extracellular matrix gene transcription, enhancing fibrosis. R-Smads, particularly at the linker region, have also been shown to be phosphorylated by MAPK. R-Smad phosphorylation is antagonized by inhibitory Smad6 (35) and Smad7 (37) overexpression, which down-regulates TGF-β–induced activity and fibrosis. Smad6 and Smad7 are known antagonists of TGF-β signaling (38, 39). To our knowledge, this is the first report demonstrating the up-regulation of the inhibitory Smads via the suppression of MK2 activity. Accordingly, MK2 inhibition may down-regulate the bleomycin-induced TGF-β signaling cascade (40) via the up-regulation of the inhibitory Smads, Smad6 and Smad7. The bleomycin-induced up-regulation of Smad3 and serpine1 mRNA expression was also suppressed with aerosolized MK2i peptide treatment. Interestingly, the presence of serpine1 (also known as TGF-β–regulated protein PAI-1) has been reported to correlate directly with the collagen accumulation subsequent to acute and chronic inflammatory injury (41). Studies also suggest that the TGF-β/PAI-1 signaling axis plays a vital role in myofibroblast survival (42). Notably, in this report, concentrations of TGF-β, Smad2, and Smad4 pulmonary transcripts at the 28-day time point in the bleomycin treatment model were not significantly different. Overall, these data provide additional insights into the mechanism of MK2 inhibition in ameliorating lung fibrosis.

The proinflammatory/profibrotic cytokines IL-6 (43), IL-1β (44), and TNF-α (43) have been reported to be profibrotic, with the expression of IL-6 increased in response to TGF-β stimulation. Interestingly, TNF-α (45) and IL-6 (46) can also induce TGF-β expression in fibroblasts. Several studies showed that the suppression of MK2 activity results in the down-regulation of proinflammatory cytokine expression, including TNF-α, IL-1β,

and IL-6 (4, 7, 21, 44). Regardless of the mode of delivery (local or systemic), the inhibition of MK2 down-regulated IL-6 and TNF-α in the bleomycin model of established fibrosis. Therefore, inhibiting TGF-β induction via the down-regulation of TNF-α and IL-6 may constitute an additional mechanism contributing to the protective effects of MK2 inhibition. Wilson and colleagues reported that bleomycin-induced lung fibrosis may be T cell–dependent, and defined the role of IL-17/TGF-β/IL-1β in the bleomycin model of fibrosis (44). In the present report, bleomycin-induced IL-1β lung transcript levels were also decreased by MK2i peptide treatment. Thus, MK2i suppresses both fibrosis and inflammation, inhibits myofibroblast differentiation and exuberant extracellular matrix synthesis and deposition, up-regulates iSMADs, and reduces proinflammatory/profibrotic cytokine synthesis.

Although the results reported here, demonstrating the efficacy of MK2 inhibition in ameliorating fibrosis, appear to conflict with previous findings within the specific MK2 knockout mice literature, several potential explanations may reconcile this discrepancy. Liu and colleagues reported that the homozygous genetic deletion of MK2 exacerbates bleomycin-induced pulmonary fibrosis (6). In contrast to that study, the present experiments focusing on peptide-mediated MK2 inhibition were performed in wild-type cells and animals. The complete absence of MK2 protein in the knockout animal likely altered the physiologic response of the animal beyond that which is seen with the simple suppression of kinase activity. In addition, MK2 is known to associate in complex with p38 MAPK, protein kinase B, and heat shock protein–27, raising the possibility that a protein complex that includes MK2 is necessary for normal lung repair (47). Finally, the homozygous genetic deletion of MK2 may lead to compensatory effects triggering other mechanisms, substituting for a lack of MK2. Kotlyarov and colleagues reported that eliminating MK2 results in a complex phenotype coupled with lower p38 MAPK concentrations and associated inflammatory cytokines, particularly TNF-α (48). However, the imbalance in the lower p38 MAPK concentrations may result in an activation of the other MAPKs, c-Jun N-terminal kinase (49) and extracellular regulated kinase (50), implicated in IPF pathology.

Despite this, an overwhelming amount of literature supports the contention that the TGF-β/p38 pathway, and specifically MK2, is a critical player in the development of pulmonary fibrosis. The experimental results detailed here provide cumulative evidence that the inhibition of MK2 activity affects both inflammatory and fibrotic processes. We report that the inhibition of MK2 via the cell-permeant peptide inhibitor MMI-0100 may protect against the progression of fibrosis by several mechanisms: up-regulating the I-SMADs Smad6 and Smad7, suppressing IL-1β and the TGF-β–inducible *smad3* and *serpine1*, preventing myofibroblast differentiation and FAK activation (which is implicated in fibroblast differentiation), attenuating the deposition of matrix proteins (specifically fibronectin, collagens, and LOX), and suppressing the secretion of proinflammatory cytokines TNF-α and IL-6. Interestingly, we observed that the peptide-mediated inhibition of MK2 was equally effective via both aerosolized local delivery and systemic administration.

In conclusion, these data suggest that the inhibition of MK2 effectively protects against the progression of established fibrosis via the suppression of inflammatory and fibrotic processes. The peptide-mediated inhibition of MK2 via MMI-0100 may represent a novel therapeutic approach to the treatment of pulmonary fibrosis, for which no successful therapeutic option is currently available.

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