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Macrophage migration inhibitory factor deficiency in chronic obstructive pulmonary disease

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[1]*Section of Pulmonary, Critical Care, and Sleep Medicine, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut;* [2]*Section of Rheumatology, Department of Medicine, Yale University, New Haven, Connecticut; and* [3]*Section of Geriatric Medicine: Claude D. Pepper Older Americans Independent Center, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut*

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Sauler M, Leng L, Trentalange M, Haslip M, Shan P, Piecychna M, Zhang Y, Andrews N, Mannam P, Allore H, Fried T, Bucala R, Lee PJ. Macrophage migration inhibitory factor deficiency in chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol* 306: L487–L496, 2014. First published January 17, 2014; doi:10.1152/ajplung.00284.2013.—The pathogenesis of chronic obstructive pulmonary disease (COPD) remains poorly understood. Cellular senescence and apoptosis contribute to the development of COPD; however, crucial regulators of these underlying mechanisms remain unknown. Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that antagonizes both apoptosis and premature senescence and may be important in the pathogenesis of COPD. This study examines the role of MIF in the pathogenesis of COPD. Mice deficient in MIF ($Mif^{-/-}$) or the MIF receptor CD74 ($Cd74^{-/-}$) and wild-type (WT) controls were aged for 6 mo. Both $Mif^{-/-}$ and $Cd74^{-/-}$ mice developed spontaneous emphysema by 6 mo of age compared with WT mice as measured by lung volume and chord length. This was associated with activation of the senescent pathway markers p53/21 and p16. Following exposure to cigarette smoke, $Mif^{-/-}$ mice were more susceptible to the development of COPD and apoptosis compared with WT mice. MIF plasma concentrations were measured in a cohort of 224 human participants. Within a subgroup of older current and former smokers ($n = 72$), MIF concentrations were significantly lower in those with COPD [8.8, 95%CI (6.7–11.0)] compared with those who did not exhibit COPD [12.7 ng/ml, 95%CI (10.6–14.8)]. Our results suggest that both MIF and the MIF receptor CD74 are required for maintenance of normal alveolar structure in mice and that decreases in MIF are associated with COPD in human subjects.

COPD; emphysema; MIF; CD74; apoptosis; senescence

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is a disease characterized by progressive and nonreversible airflow limitation as well as alveolar wall destruction (14, 61). Age and cigarette smoke (CS) exposure remain the most important identifiable risk factors for the development of COPD (20, 23, 36, 60). However, disease development is far from universal in patients with these risk factors. Studies suggest that heterogeneity in host responses likely explains the incomplete penetrance of this disease in older individuals with extensive smoking histories (9, 18, 36).

Cellular senescence and apoptosis are two processes that occur in COPD (26). Multiple studies have documented evidence of increased apoptosis in the lungs of patients with COPD, and the resultant imbalance between apoptosis and tissue repair is considered to underlie the pathogenesis of emphysema (27, 30, 40, 51, 59, 64). Cellular senescence may limit the response to increased tissue damage, ultimately leading to progressive lung tissue loss. Cellular senescence is a process of irreversible growth arrest as a consequence of repetitive cellular division or environmental stimuli, such as oxidative stress (13, 15). During cellular senescence, the proteins $p16^{INK4a}$, $p19^{ARF}$, and $p21^{CIP1/WAF1/Sdi1}$ antagonize cyclin-dependent kinases, which are required for cell cycle progression; increases in these proteins have been demonstrated in the lungs of patients with COPD (2, 10, 19, 46, 49, 58). Although increased apoptosis is likely countered by regenerative processes in younger patients, an increase in cellular senescence as a result of chronic CS in combination with advancing age may disrupt the homeostatic balance between apoptosis and lung repair.

We previously identified key upstream regulators of innate immunity to have critical roles in the maintenance of lung homeostasis (8). We have shown that Toll-like receptor 4 (TLR4), a canonical innate immune receptor, is required to maintain murine lung integrity and prevent excessive apoptosis in the setting of oxidative stress (1, 48, 65, 66). TLR4 deficiency results in the development of premature emphysema in a murine model, and subsequent studies have highlighted polymorphisms in the TLR4 gene as being associated with the development and/or progression of emphysema (8, 28, 54). This prompted a directed search of other immune molecules that may regulate susceptibility to COPD.

Macrophage migration inhibitory factor (MIF) is a crucial regulator of the innate immune response (6, 11, 12, 50). MIF is preformed within many cell types, including monocytes/macrophages, epithelial, and endothelial cells, and is rapidly se-

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creted in response to varied stimuli including oxidative stress and microbial products (17, 45, 56). Although MIF has been studied in many contexts, including infection, chronic inflammatory diseases, and malignancy, its role in COPD has not been explored.

We studied mouse models with genetic deletion of MIF or the MIF receptor, CD74. We report that both mouse strains develop spontaneous emphysema, a major cause of airflow obstruction in COPD. We then measured circulating MIF levels in a cohort of human subjects with and without CS exposure and spirometric evidence of airflow obstruction and observed reduced MIF concentrations in those with airway impairment. These data suggest a role for MIF-CD74 insufficiency in the pathogenesis of COPD.

METHODS

Animals. Animal protocols were reviewed and approved by the Animal Care and Use Committee at Yale University. Wild-type (WT) 8- to 10-wk-old C57BL/6J mice were purchased from Jackson Laboratories and bred in our facility. *Mif*$^{-/-}$ mice and *Cd74*$^{-/-}$ mice in the C57BL/6J background have been previously described (5, 52). All mice were bred by homozygous mating under specific pathogen-free conditions at the animal facility of Yale University School of Medicine. Lungs from aged mouse experiments detailed in Fig. 1*B* were obtained from the National Institute on Aging (NIA) Aged Rodent Colonies. The NIA colonies are barrier maintained and specific pathogen free. Tissues are fresh frozen and stored at −80°C.

Cigarette smoking exposure. Eight-week-old WT and *Mif*$^{-/-}$ C57BL/6J mice were exposed to room air or the smoke from unfiltered research cigarettes (2R4; University of Kentucky, Lexington, KY) by using the smoking apparatus described by Shapiro and colleagues (25). During the first week, mice received a half cigarette twice a day to allow for acclimation. During the remainder of the exposure, mice received two cigarettes per day. Mice were exposed for a total of 6 mo of CS.

Bronchoalveolar lavage. Mice were euthanized by intraperitoneal ketamine/xylazine injection, and the trachea was cannulated and perfused with two 0.9-ml aliquots of cold saline. The cellular contents and bronchoalveolar lavage (BAL) fluid were separated by centrifugation. Cells were reconstituted in PBS and cell counts were obtained via a Coulter counter (Beckman Coulter). Slides of BAL fluid cells were prepared via cytospin and stained with Hema 3 (Protocol). Cells were differentiated by conventional morphological criteria for macrophages, lymphocytes, and neutrophils. BAL MIF concentrations were measured by sandwich ELISA using specific antibodies as previously described (55).

Assessment of lung volume and morphometric assessment. Lungs were removed from control and experimental mice and inflated slowly at a constant pressure of 25 cm. Lung volume was assessed by volume displacement as previously described (67, 68). The right main bronchus was ligated and the left lung was inflated with 0.5% low-temperature melting agarose at a constant pressure of 25 cm as previously described (24). The lungs were then fixed, paraffin embedded, and stained with hematoxylin and eosin. A minimum of six random fields were evaluated by microscopic projection and the NIH image program; alveolar size was estimated from the mean linear intercept (L_m) of the air space as described previously (65).

Lung immunohistochemistry. Paraffin-embedded lung sections were stained with hematoxylin and eosin and cleaved caspase 3 at the indicated concentrations. Briefly, samples were deparaffinized with xylene, rehydrated gradually with graded alcohol solutions, and then washed with deionized water. For immunofluorescence, sections were incubated with a 1:50 dilution of anti-caspase 3 monoclonal antibody (Cell Signaling) and counterstained with DAPI (Cell Signaling). For immunohistochemistry, sections were incubated with 1:50 of an anti-p16 monoclonal antibody overnight (Abcam). After further washing the sections with PBS, we applied 3,3′-diaminobenzidine tetrachloride/nickel-cobalt substrate as chromogen, yielding a brown/black-colored reaction product, counterstained with hematoxylin. Microscopy was performed with a Nikon Eclipse Ti-S microscope equipped with an Andor Technology camera.

Western blot. Lung tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA, Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche). Western blotting was performed with an SDS-PAGE electrophoresis system (Bio-Rad), and 20 μg of protein sample was electrophoresed on a 4–20% Tris gel (Bio-Rad) in Tris running buffer, blotted to a PVDF membrane (Bio-Rad) and probed with primary antibodies against Ser-15 phosphorylated p53 (Cell Signaling), p53 (Cell Signaling), p21 (Cell Signaling), and caspase-3 (Cell Signaling). After washing in Tris with 0.01% Tween, the blots were incubated with a horseradish peroxidase-conjugated anti-rabbit (Sigma) and signal was detected by autoradiography with ECL Prime Western Blotting Detecting Reagent (GE Healthcare).

mRNA expression. Quantitative Real-Time Polymerase Chain Reaction RNA was isolated and reverse transcribed (33). $p16^{INK4a}$ (5′-CCCAACGCCCCGAACT-3′) (5′-GCAGAAGAGCTGCTACGTGAA-3′); $p19^{ARF}$ (5′-TGAGGCTAGAGAGGATCTTGAGA-3′) (5′-GCAGAAGAGCTGCTACGTGAA-3′).

Human study samples. A cohort of 224 adults without prior testing underwent spirometry to confirm airflow obstruction. In an attempt to determine changes that occur with aging, our inclusion criteria was adults between the ages of 18–45 or older than 65 yr of age. Additionally, recruited persons were required to provide a smoking history and agreed to be contacted for the study. The exclusion criteria were *1*) immunocompromised adults (HIV, malignancy diagnosis in past 5 years, systemic chemotherapy, end-stage renal failure); *2*) adults with other obstructive lung disease (history of asthma, cystic fibrosis, bronchiectasis, bronchiolitis obliterans, or vocal cord dysfunction); *3*) adults who could not give informed consent because of language, cognitive, or other barriers; and *4*) adults who could not perform spirometry because of cognitive, health, or other reasons. Participants completed a questionnaire to assess sociodemographic and self-reported health status, including comorbid conditions and medications, and underwent phlebotomy to obtain plasma samples. COPD was defined by GOLD criteria, which are based on an forced expiratory volume in 1 s obtained by spirometry (61). Because many persons with COPD had quit smoking, participants with COPD include both current smokers, defined as having a greater than 10 pack·year lifetime history and have smoked in the past 11 mo, or former smokers (quit for over 1 year but have a greater than 10 pack·year history). Covariates were age expressed in years, sex, smoking status, and race as self-identified white or self-identified as other than white (Table 1).

We then subdivided the subgroup of adults ≥65 yr of age (an age at which COPD is common) into those who were current or former smokers (n = 72) and by the presence or absence of COPD to determine whether differences in plasma MIF were evident (Table 2).

Statistical analysis. Basic summary measures were calculated: medians, means, and standard errors for continuous variables and counts and percentages for categorical as appropriate. Nonparametric data were compared by a Wilcoxon rank-sum test. Categorical data were compared with a χ^2 statistic. For the human data a multivariable linear regression model was performed to test the association of COPD (present or absent) with plasma MIF levels while adjusting for age, sex, smoking, and race. For all tests, two-sided P values less than 0.05 were considered significant. Statistical analyses were accomplished with SAS v9.3 (SAS Institute, Cary, NC). Graphs and some basic statistical comparisons were performed with GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA.

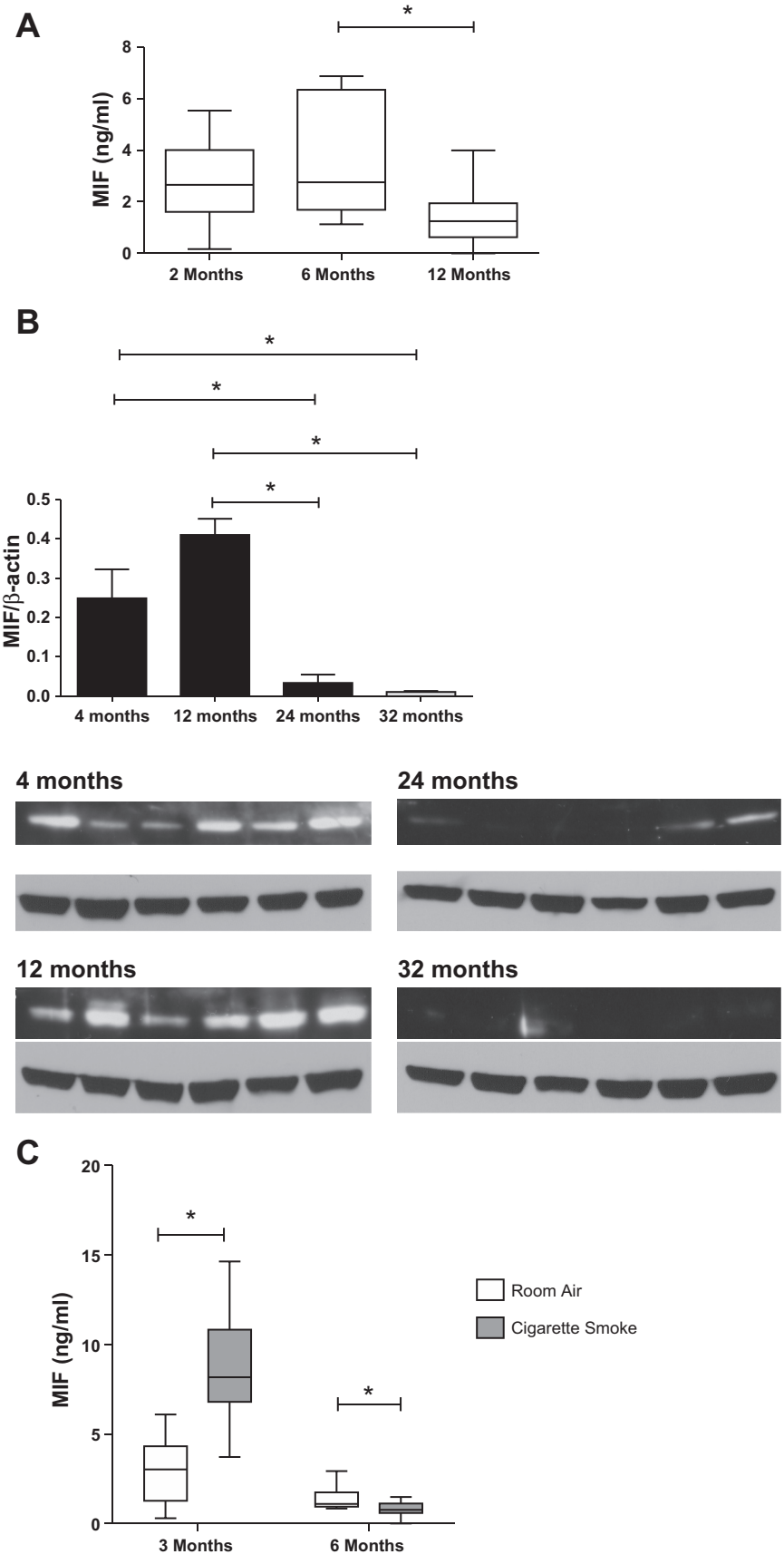

Fig. 1. *A*: box plot with minimum and maximum macrophage migration inhibitory factor (MIF) concentrations (ng/ml) in wild-type (WT) mice at 2 mo, 6 mo, and 12 mo of age; $n = 10$ in 2 mo group, $n = 8$ in 6 mo and 12 mo group. Data represent means ± SE. $^*P < 0.05$ between 12 mo and 6 mo group; Wilcoxon rank-sum. *B*: Western blot of MIF in homogenized lung tissue from WT mice at 4, 12, 24, and 32 mo of age with densitometry analysis. $^*P < 0.05$ between groups; Wilcoxon rank-sum. *C*: box plot with minimum and maximum bronchoalveolar lavage (BAL) MIF concentrations (ng/ml) in WT mice following 3 or 6 mo of cigarette smoke (CS) exposure compared with control; $n = 9–11$ for control group; $n = 8–10$ for CS group. $^*P < 0.0003$ between 3-mo control and 6-mo CS group; Wilcoxon rank-sum. $^*P < 0.05$ between 6-mo control and 6-mo CS group; Wilcoxon rank-sum.

Table 1. *Baseline demographic features of all subjects for which serum MIF levels were obtained*

Variable	Young (n = 80)	Old (n = 100)	P Value
Age, yr	31.5 ± 0.8	74.2 ± 0.6	<0.001
FEV_1/FVC ratio	0.8 ± 0.01	0.7 ± 0.01	<0.001
FEV_1 % predicted	91.3 ± 1.3	81.3 ± 2.5	<0.001
MIF, ng/ml	7.1 ± 0.5	11.5 ± 0.6	<0.001
Sex			0.349
Male	40 (50.0)	43 (43.0)	
Race			0.008
White	56 (70.9)	87 (87.0)	
Smoking status			0.044
Never	30 (37.5)	28 (28.0)	
Former	23 (28.8)	47 (47.0)	
Current	27 (33.8)	25 (25.0)	
FEV_1/FVC ratio			<0.001
FEV_1/FVC <70	3 (4.8)	38 (38.0)	
FEV_1/FVC ≥70	77 (95.2)	62 (62.0)	

Values are means ± SE or n (%). MIF, macrophage migration inhibitory factor; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity. P values were determined by t-test for continuous variables, χ^2 for categorical variables.

RESULTS

Decline of MIF in lungs with age and CS. We determined the changes in lung MIF expression with age and following CS. There was no difference in the MIF concentration in the BAL of 2- and 6-mo-old mice. However, by 12 mo of age, the concentration of MIF in the BAL was significantly decreased compared with 6 mo of age (Fig. 1*A*). Similarly, there was a marked decrease in total MIF in homogenized lung tissue with advanced age in WT mice (Fig. 1*B*). These data are similar to those of recent publications, suggesting that MIF decreases with age both in the lung as well as other organs (38, 42). The BAL of WT mice exposed to CS then was analyzed to determine whether the changes that occurred with age similarly occurred after CS exposure. Acute CS exposure (for 3 mo) led to increased BAL MIF expression, whereas chronic CS exposure (for 6 mo), which coincides with the development of emphysema in mice, led to decreased levels of BAL MIF (Fig. 1*C*).

$Mif^{-/-}$ mice develop spontaneous emphysema. We investigated the role of MIF at baseline by examining the lungs of unchallenged $Mif^{-/-}$ mice. There were no morphometric differences between the lungs of WT and $Mif^{-/-}$ mice at 2 mo of age, which is when mouse lung development is essentially complete. However, at 6 mo of age, $Mif^{-/-}$ mice demonstrated significantly increased lung volumes (Fig. 2*A*). There were no differences between the body weights of WT and $Mif^{-/-}$ mice at 2 and 6 mo of age (data not shown). Histological evaluation revealed enlargement of the air spaces distal to the terminal bronchioles accompanied by loss of normal alveolar architecture in $Mif^{-/-}$ mice, characteristic of emphysema. Morphometric quantitation of the air space enlargement as measured by increased mean linear intercept measurements (L_m) in $Mif^{-/-}$ mice (Fig. 2, *B* and *C*). The BAL of $Mif^{-/-}$ mice exhibited an increase in BAL macrophages (Fig. 2*D*). There was no difference in the expression of inflammatory cytokines among WT and $Mif^{-/-}$ mice (data not shown).

Deficiency of MIF is associated with in vivo evidence of premature cellular senescence activation. As mentioned in the introduction, repetitive cellular replications or cellular stress can lead to the activation of the p19/p53/21 and RB/p16 senescent pathways. We sought to determine whether upregulation of these pathways occur in the aged lungs of $Mif^{-/-}$ mice compared with WT. Compared with WT mice at 6 mo of age, $Mif^{-/-}$ mice demonstrated increased serine 15-phosphorylation of p53 and increased p21 expression (Fig. 3*A*). We then examined mRNA expression of the cell cyclin-dependent kinase inhibitors (CDKIs) $p16^{INK4A}$ and $p19^{ARF}$ expression in the lungs of WT and $Mif^{-/-}$ mice. At 6 mo of age, $Mif^{-/-}$ mice demonstrated upregulation of CDKIs compared with WT mice (Fig. 3*B*). Similar to changes in expression, immunohistochemistry analysis of $Mif^{-/-}$ mice demonstrated increased evidence of p16 staining by immunohistochemistry compared with WT mice (Fig. 3*C*).

Deficiency of the MIF receptor CD74 is associated with age-related, spontaneous emphysema. MIF binds to cell surface CD74 to initiate MIF signaling transduction (35). We investigated whether deletion of the receptor also would result in emphysema. Again, there were no differences between the lungs of WT and $Cd74^{-/-}$ mice at 2 mo of age. However, similar to $MIF^{-/-}$ mice, $Cd74^{-/-}$ mice demonstrated evidence of spontaneous emphysema by 6 mo of age as demonstrated by increased lung volumes and air space enlargement (Fig. 4, *A–C*).

$Mif^{-/-}$ mice are more susceptible to CS exposure. We subsequently exposed $Mif^{-/-}$ mice to 6 mo of CS. Although CS resulted in an increase in the lung volumes of both WT and $Mif^{-/-}$ mice without discernable differences, $Mif^{-/-}$ mice showed evidence of greater air space enlargement compared with WT mice exposed to equal time courses of CS exposure (Figs. 5, *A–C*), suggesting that the internal surface area of the $Mif^{-/-}$ lungs following CS was significantly decreased. Because emphysema can be a heterogeneous disease, we included low-power views of both apical and basolateral regions demonstrating increased air space enlargement throughout the lung (Fig. 5*D*). CS resulted in an increase in BAL macrophages in both WT and $Mif^{-/-}$ mice. Similar to our findings in aging, $Mif^{-/-}$ mice demonstrated an increase in BAL cells after chronic CS exposure compared with WT mice (Fig. 5*E*). Interestingly, this was due to an increase in BAL lymphocytes in both WT and $Mif^{-/-}$ mice (Fig. 5*F*). Following exposure to CS, $Mif^{-/-}$ mice demonstrated evidence of increased apoptosis, as observed by increased cleaved caspase 3 content after immunofluorescent staining or Western blotting of cells (Fig. 5, *G–J*).

Table 2. *Baseline demographic features for subjects ≥65 yr of age (n = 72)*

	Healthy Smokers (No COPD)	COPD	P Value
n (%)	40 (55.6)	32 (44.4)	
Female	19 (47.5)	19 (59.4)	0.560
White	35 (87.5)	25 (78.1)	0.289
Age, yr	73.3 ± 1.0	73.9 ± 0.9	0.646
FEV_1/FVC ratio	0.77 ± 0.01	0.59 ± 0.02	<.001
FEV_1% predicted	85.6 ± 3.7	64.2 ± 4.2	<.001
MIF, ng/ml	13.7 ± 0.9	9.6 ± 0.8	0.002

Values are n (%) or means ± SE. Chronic obstructive pulmonary disease (COPD, by pulmonary function testing) was defined by FEV_1/FVC <0.7. P values were determined by t-test for continuous variables, χ^2 for categorical variables.

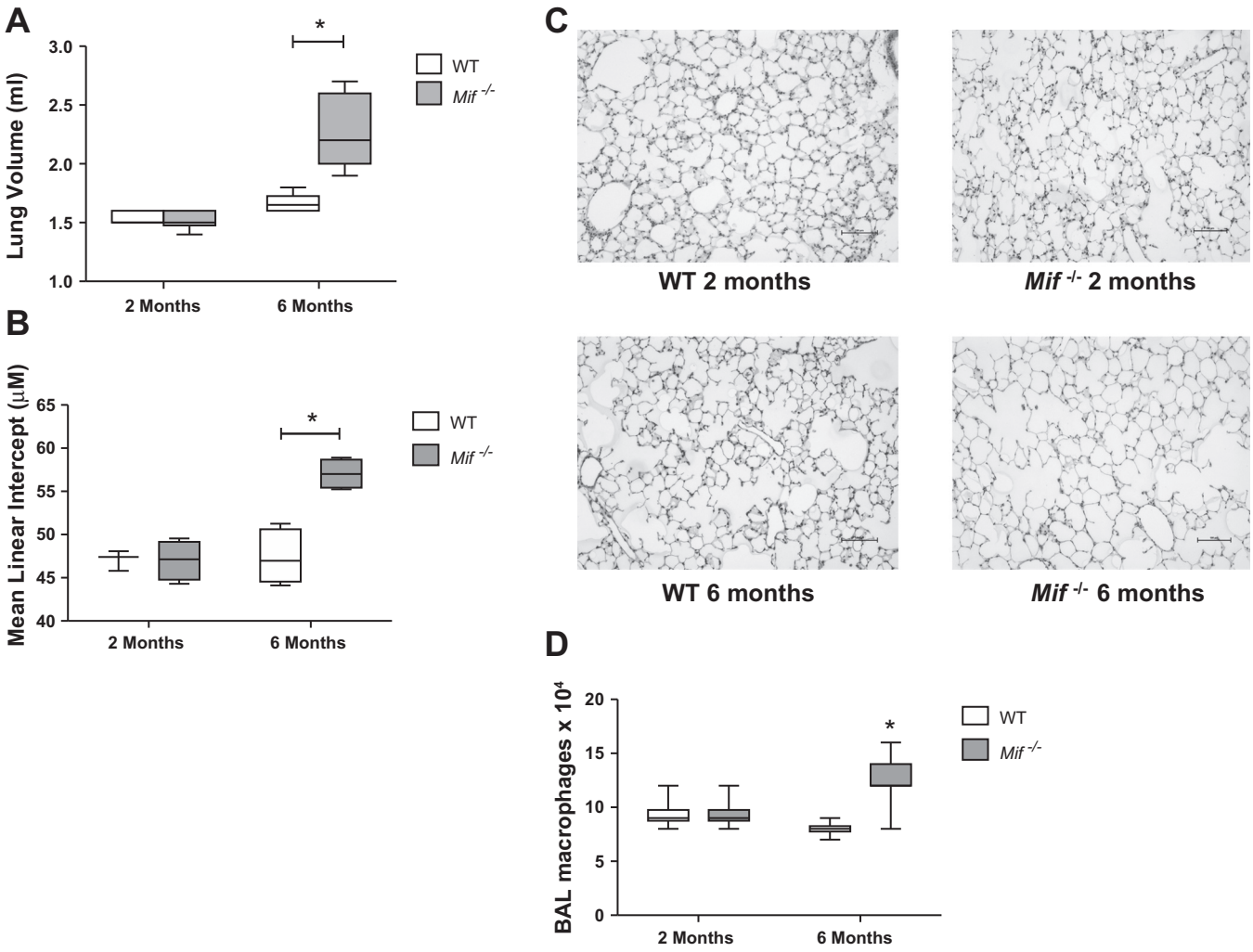

Fig. 2. *A*: box plot with minimum and maximum lung volumes in 2- and 6-mo-old WT and *Mif*$^{-/-}$ mice; $n = 6$ mice in WT groups, 6–9 mice in *Mif*$^{-/-}$ group. *$P < 0.0005$; Wilcoxon rank-sum. *B*: box plot of mean linear intercept in 2- and 6-mo-old WT and *Mif*$^{-/-}$ mice; $n = 3$–5 mice in WT group, 4 in *Mif*$^{-/-}$ group. *$P < 0.05$; Wilcoxon rank-sum. *C*: representative hematoxylin and eosin (H&E) lung histology of WT and *Mif*$^{-/-}$ mice. Original magnification ×10. *D*: box plot with minimum and maximum BAL macrophages in 2- and 6-mo-old WT and *Mif*$^{-/-}$ mice; $n = 6$ mice in WT groups, 6–9 mice in *Mif*$^{-/-}$ groups. *$P <$ 0.05; Wilcoxon rank-sum.

Plasma concentrations of MIF are lower in older smokers with COPD compared with older smokers without COPD. We sought to determine whether MIF expression was altered in COPD. Among older former and current smokers ($n = 72$), decreased plasma MIF was associated with COPD ($P = 0.003$), even after adjusting for age, sex, and race in a multivariable linear regression model. Specifically, least-squared means MIF plasma concentrations were significantly lower in smokers that exhibited COPD [8.8 ng/ml, 95%CI (6.7–11.0)] compared with smokers who did not exhibit COPD [12.72 ng/ml, 95%CI (10.6–14.8)] (Fig. 6).

DISCUSSION

COPD is a devastating disease that remains the third leading cause of death in the United States (47). However, the development of COPD is not universal, even among persons exposed to the major risk factor of cigarette smoking (23). Understanding the determinants of susceptibility to CS is crucial to developing improved preventative and therapeutic strategies. We examined the role of the upstream immunoregulatory cytokine MIF as a possible regulator of COPD susceptibility.

Several findings herein are reported: *1*) *Mif*$^{-/-}$ and MIF receptor *Cd74*$^{-/-}$ mice demonstrate evidence of spontaneous emphysema with age, suggesting a causal role for the observed decreased MIF plasma concentrations among patients with COPD. *2*) Genetic deficiency of MIF results in the expression of premature senescence markers, similar to that observed among individuals with COPD. *3*) Because COPD in Western countries is usually a consequence of CS exposure, we demonstrate that *Mif*$^{-/-}$ mice have increased susceptibility to CS, as assessed by greater alveolar enlargement and increased apoptosis compared with WT and nonexposed *Mif*$^{-/-}$ mice. *4*) Among individuals >65 years of age with a significant smoking history, the presence of COPD is associated with diminished circulating MIF.

Although the demonstration of decreased plasma MIF among at-risk individuals who develop COPD is from a cross-sectional study, the causal role of MIF is suggested by our data

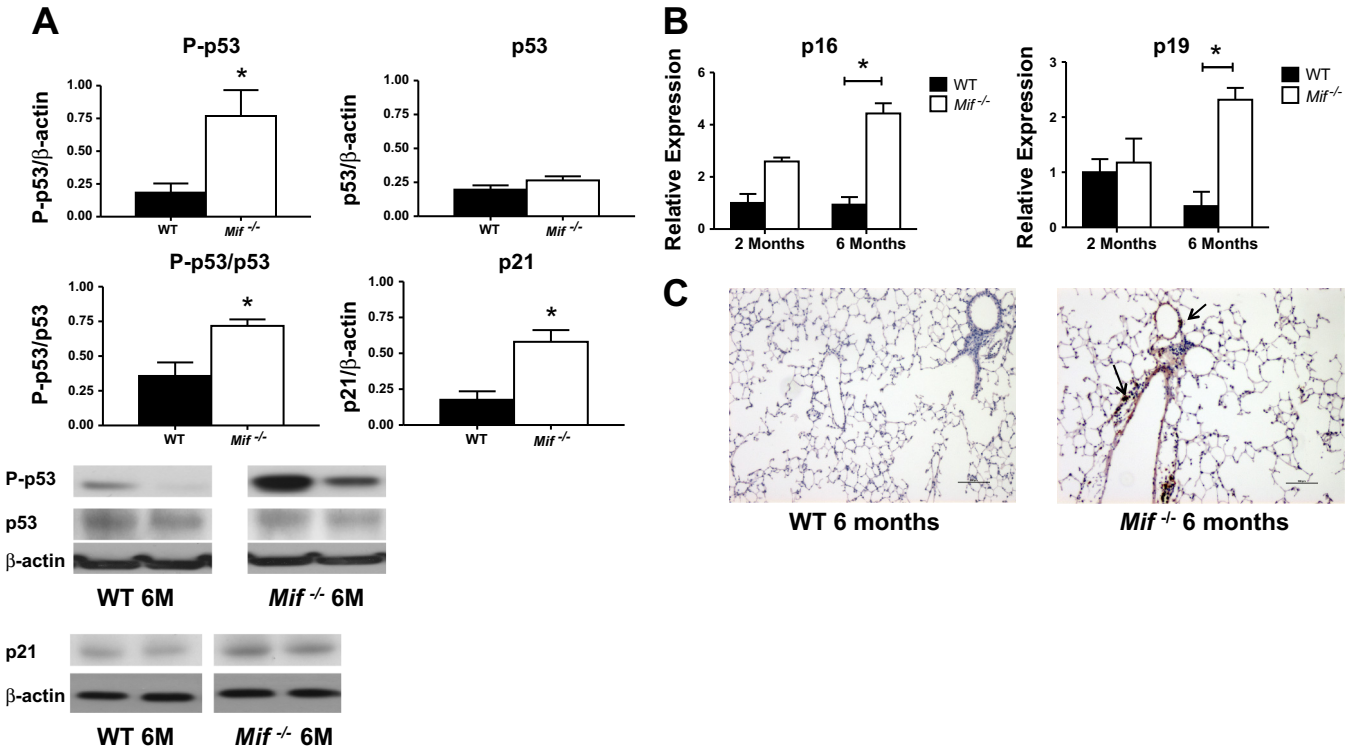

Fig. 3. *A*: densitometry and representative Western blots of p21, phosphorylated-p53 (Ser15), total p53, and β-actin in 6-mo-old WT and *Mif*$^{-/-}$ mice. Data represent means ± SE; $n = 4$ mice/group; Wilcoxon rank-sum. *B*: relative mRNA of p16 and p19 in 2- and 6-mo-old WT and *Mif*$^{-/-}$ lung tissue. Data are means ± SE; $n = 4$–5 in WT group and $n = 5$–6 in *Mif*$^{-/-}$ group. *$P < 0.05$; Wilcoxon rank-sum. *C*: representative immunohistochemistry of p16 in 6-mo-old WT and *Mif*$^{-/-}$ mice. Arrows denote p16-positive staining. Original magnification ×10.

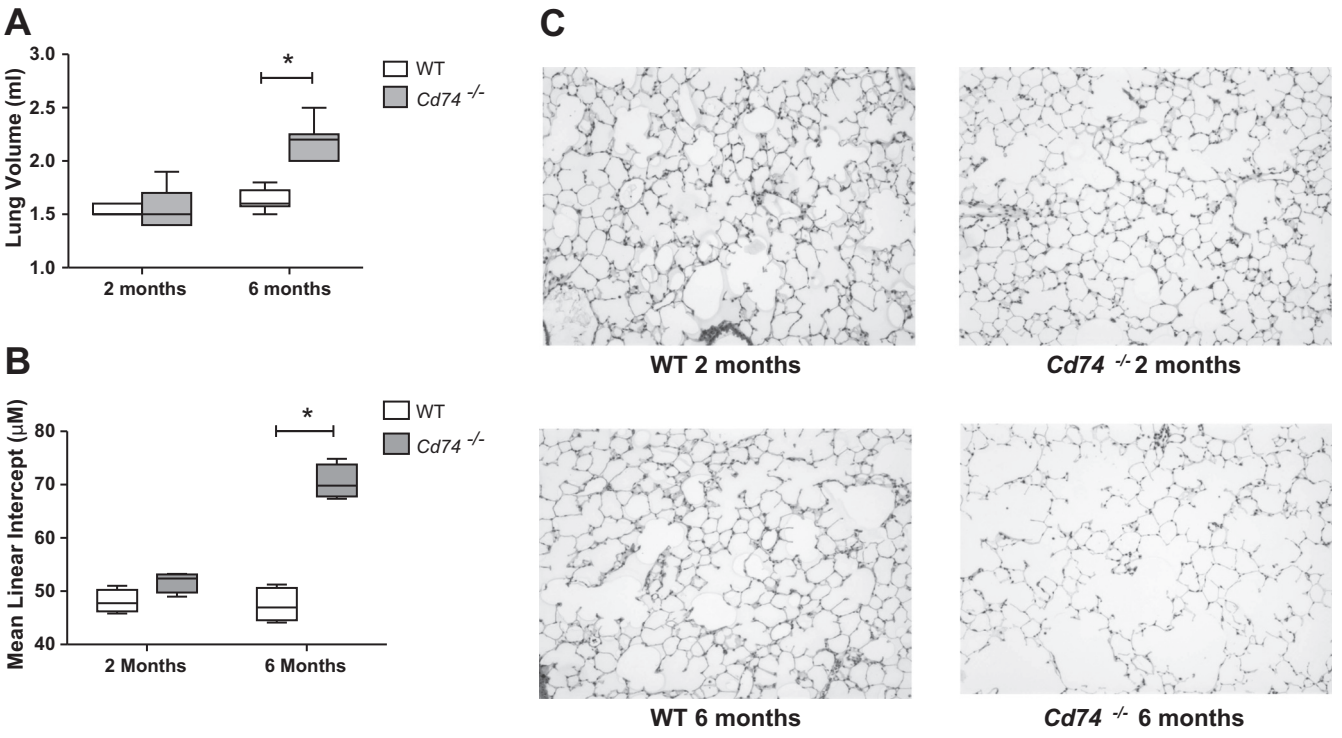

Fig. 4. *A*: box plot with minimum and maximum lung volumes in 2- and 6-mo-old WT and *Cd74*$^{-/-}$ mice; $n = 4$–6 mice in WT groups, 7–9 mice in *Cd74*$^{-/-}$ group. *$P < 0.0001$; Wilcoxon rank-sum. *B*: box plot of mean linear intercept in 2- and 6-mo-old WT and *Cd74*$^{-/-}$ mice; $n = 3$–5 mice in WT group, 4 in *Cd74*$^{-/-}$ group. *$P < 0.05$; Wilcoxon rank-sum. *C*: H&E lung histology of WT and *Cd74*$^{-/-}$ mice. Original magnification ×10.

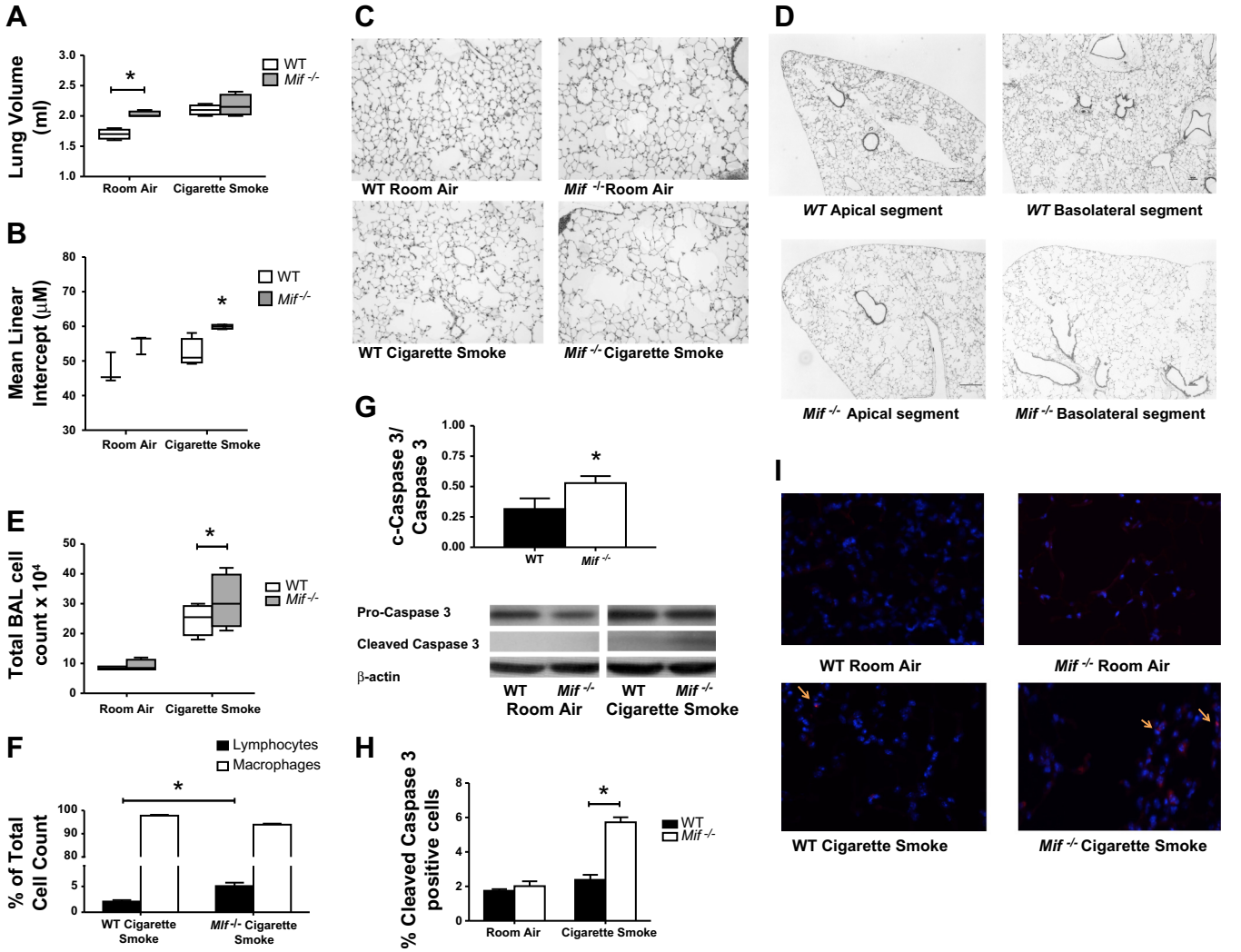

Fig. 5. *A*: box plot with minimum and maximum lung volumes in room air (RA)- and CS-exposed WT and $Mif^{-/-}$ mice; $n = 4$/group. *$P < 0.05$ $Mif^{-/-}$ CS vs. WT CS, $P < 0.05$ WT RA vs. WT CS; Wilcoxon rank-sum. *B*: box plot with minimum and maximum mean linear intercept in RA- and CS-exposed WT and $Mif^{-/-}$ mice; $n = 4$/group. *$P < 0.05$ WT RA vs. $Mif^{-/-}$ RA; *$P < 0.05$ WT CS vs. $Mif^{-/-}$ CS; Wilcoxon rank-sum. *C*: H&E lung histology of WT and $Mif^{-/-}$ mice with and without CS exposure. Original magnification ×10. *D*: H&E lung histology of WT and $Mif^{-/-}$ mice with and without CS exposure, apical and basal regions. Original magnification ×4. *E*: total BAL cell count in RA- and CS-exposed WT and $Mif^{-/-}$ mice; $n = 4$/group. *$P < 0.05$ $Mif^{-/-}$ CS compared with WT RA control; Wilcoxon rank-sum. *F*: BAL cell type distribution in WT and $Mif^{-/-}$ exposed to CS; $n = 4$/group. *$P < 0.05$ $Mif^{-/-}$ mice vs. WT; Wilcoxon rank-sum. *G*: densitometry and representative Western blot of homogenized lung tissue for pro- and cleaved caspase 3 in CS-exposed mice; $n = 4$/group. *$P < 0.05$ $Mif^{-/-}$ mice exposed to CS compared with WT mice exposed to CS; Wilcoxon rank-sum. *H* and *I*: cleaved caspase 3-positive cells as a percentage of total cells with representative immunofluorescence images. Arrows represent cleaved caspase 3-positive cells; $n = 4$/group. *$P < 0.05$ compared with WT CS; Wilcoxon rank-sum).

showing that $Mif^{-/-}$ mice demonstrate spontaneous increases in L_m and in lung volumes at fixed pressures, which is consistent with the presence of emphysema (16, 29, 32). Alternatively, others have suggested that because emphysema is a process of tissue destruction it is only a decrease in internal surface area that is demonstrative of emphysema (L_m adjusted for lung volume represents internal surface area here) (44, 63). This argument would suggest that the increase in L_m in our aged mice is a morphological consequence of an increase in lung volume. However, following CS exposure, WT and $Mif^{-/-}$ mice exhibit equally enlarged lung volumes, but $Mif^{-/-}$ mice have higher L_m, indicating decreased internal surface area compared with CS-exposed WT mice. Because lung volume is a more sensitive marker of emphysema and decreased internal surface area more reflective of disease severity, the emphysema exhibited following aging and CS exposure likely exist along a continuum of disease (16). In the context of evaluating MIF deficiency in human disease, our data support a causative role for decreased MIF in the development of COPD.

Although MIF is considered a proinflammatory cytokine (7), multiple in vitro studies have highlighted MIF's antiapoptotic role and have suggested that MIF may protect against oxidative stress-induced apoptosis as well as the development of premature senescence (22, 43). As a possible mechanism, MIF deficiency has been demonstrated to lead to increased p53 activation in conjunction with upregulation of p21 and p16 (22, 43). Similarly, in our model of COPD, $Mif^{-/-}$ mice show upregulation of markers of cellular senescence and, after CS, demonstrate increased apoptosis, a finding that has been re-

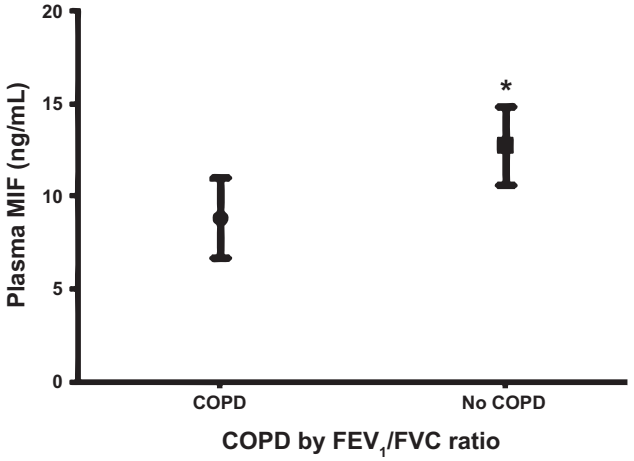

Fig. 6. Current and former smokers, ≥65 yr of age (n = 72) mean and 95% confidence limits. Results adjusted for age (years), sex (M/F), smoking history (former, current, never), and race (white, nonwhite). COPD, chronic obstructive pulmonary disease.

peatedly demonstrated in both murine models of and patients with COPD (2, 57). Cellular senescence is considered to occur as a consequence of the activation of two canonical senescence pathways: Rb-p16 and p53-p19/p21. Although complex interplays between these two canonical cellular senescence pathways have been described, they are thought to act independently in response to stress and/or telomere shortening (34). MIF's regulation of this process remains unclear. A recent study suggests that MIF regulates NRF-2 and, therefore, MIF deficiency may cause increased susceptibility to oxidative stress (42). Alternatively, others have suggested that MIF is a crucial regulator of cell cycle checkpoints and perhaps increased cellular senescence occurs through dysregulation of cell cycle progression (21). Our data would suggest that the mechanism of MIF deficiency causing emphysema occurs via diminished stimulation of the MIF receptor CD74, since both the MIF- and CD74-deficient mice develop spontaneous emphysema.

CD74 is a MIF binding cell surface receptor that signals by recruitment of CD44 (35). CD74 deficiency has been frequently characterized to phenocopy MIF deficiency in mouse models of disease. CD74 engages CD44 to stimulate Src family kinases, which leads downstream to AKT/ERK activation (37, 52). AKT and ERK have a multitude of cytoprotective effects including activation of NRF-2, regulation of cellular metabolism, protection from apoptosis, and, when not in excess, prevention of cellular senescence (31, 39, 41, 62). It is therefore feasible that MIF protects against age- or stress-induced DNA damage via CD74 and thereby defends against cellular senescence and apoptosis. However, further studies are necessary to determine the precise mechanisms of these pathways in the lung.

To assess the relevance of our findings to human disease, we evaluated a cohort of subjects with and without COPD and demonstrated that decreased plasma MIF in our cohort was associated with the presence of COPD among current and former smokers. A multiple linear regression model was used to control for the confounding effects of age, race, smoking status, and sex on our association between MIF and the presence of COPD. Given the susceptibility of *Mif*$^{-/-}$ mice to the development of emphysema, one may postulate that decreased circulating concentration of MIF may reflect a genetic susceptibility to lung disease. MIF is encoded in a functionally polymorphic locus in the human genome, with the number of CATT tetranucleotide repeats at −794 in the promoter correlating with increased *Mif* expression (4). Differences in the distribution of high- and low-expression *Mif* alleles have been related to differing phenotypes in a variety of inflammatory or infectious diseases, and one may hypothesize that the differential expression in the aforementioned cohorts reflects a genetic predisposition to the development of COPD (4). Interestingly, we queried publicly available GEO datasets of differential gene expression between at risk individuals with COPD compared with healthy smokers. In the first GEO-derived cohort (GSE42057), the presence of COPD, as defined by GOLD criteria, was associated with decreased *Mif* expression in peripheral blood mononuclear cells (3). In the second GEO derived cohort (53), for which the primary question was differences in sputum gene expression among GOLD stage COPD II–IV, *Mif* expression was decreased among the individuals with more severe disease. This supports our findings that decreased MIF, in circulation or lungs, is associated with COPD (53). Alternatively, differences in MIF gene expression or plasma concentration may simply be a consequence of COPD or represent variations in the cell populations of these samples.

Additionally, our MIF expression in humans has notable similarities to our mouse model. Our multiple linear regression model for plasma MIF in older adults showed that circulating MIF increased with age. In mice, there was a trend toward increased MIF in the BAL and lungs of mice with shorter time courses of aging and smoking, although we have not yet tested circulating MIF in mice. With very advanced age or chronic CS exposure MIF levels decreased in lungs of mice. Similarly, amongst human subjects with COPD, circulating MIF levels were decreased. Further studies are necessary to determine the relationship between MIF and "healthy" aging.

We recognize that the development of emphysema in a mouse does not necessarily reflect lung pathology in humans. Further studies are required to elucidate the precise role of MIF in clinical lung disease. Additionally, the cell type(s) involved in MIF-mediated lung protection against the development of emphysema remains unknown. As an example, we observed an increase in BAL lymphocytes following CS smoke that was exaggerated in MIF deficiency, as the *Mif*$^{-/-}$ mice demonstrated a marked increase in lymphocytes compared with CS-exposed WT controls. It remains unclear whether MIF deficiency caused the increase in lymphocytes or this difference in lymphocytes is a consequence of increased disease severity.

We detected decreased circulating MIF in smokers (former and current) with COPD, compared with those without COPD, and linked genetic deficiencies in MIF and its receptor, CD74, to emphysema in murine models. Therefore, MIF may be a suitable biomarker of disease or MIF-CD74 signaling may represent a potential therapeutic target in individuals with COPD.

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DISCLOSURES

Dr. Bucala is listed as coinventor on a Yale University patent application describing the potential utility of MIF agonists.

AUTHOR CONTRIBUTIONS

M.S., M.T., H.A., T.F., R.B., and P.J.L. conception and design of research; M.S., L.L., M.T., H.A., and P.J.L. analyzed data; M.S., M.T., H.A., R.B., and P.J.L. interpreted results of experiments; M.S., M.T., H.A., R.B., and P.J.L. edited and revised manuscript; M.S., R.B., and P.J.L. approved final version of manuscript; L.L., M.H., P.S., M.P., Y.Z., N.A., and P.M. performed experiments; M.S. and M.T. prepared figures M.S. drafted manuscript.

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