Macrophage Migration Inhibitory Factor Enzymatic Activity, Lung Inflammation, and Cystic Fibrosis

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***Rationale*: Macrophage migration inhibitory factor (MIF) is a proinflammatory mediator with unique tautomerase enzymatic activity; the precise function has not been clearly defined. We previously demonstrated that individual patients with cystic fibrosis (CF) who are genetically predisposed to be high MIF producers develop accelerated end-organ injury.**

***Objectives*: To characterize the effects of the MIF-CATT polymorphism in patients with CF *ex vivo*. To investigate the role of MIF's tautomerase activity in a murine model of *Pseudomonas aeruginosa* infection.**

***Methods*: MIF and tumor necrosis factor (TNF)-α protein levels were assessed in plasma or peripheral blood mononuclear cell (PBMC) supernatants by ELISA. A murine pulmonary model of chronic *Pseudomonas* infection was used in MIF wild-type mice ($mif^{+/+}$) and in tautomerase-null, *MIF* gene knockin mice ($mif^{P1G/P1G}$).**

***Measurements and Main Results*: MIF protein was measured in plasma and PBMCs from 5- and 6-CATT patients with CF; LPS-induced TNF-α production from PBMCs was also assessed. The effect of a specific inhibitor of MIF-tautomerase activity, ISO-1, was investigated in PBMCs. In the murine infection model, total weight loss, differential cell counts, bacterial load, and intraacinar airspace/tissue volume were measured. MIF and TNF-α levels were increased in 6-CATT compared with 5-CATT patients with CF. LPS-induced TNF-α production from PBMCs was attenuated in the presence of ISO-1. In a murine model of *Pseudomonas* infection, significantly less pulmonary inflammation and bacterial load was observed in $mif^{P1G/P1G}$ compared with $mif^{+/+}$ mice.**

***Conclusions*: MIF-tautomerase activity may provide a novel therapeutic target in patients with chronic inflammatory diseases such as CF, particularly those patients who are genetically predisposed to produce increased levels of this cytokine.**

Keywords: cytokines; macrophage migration inhibitory factor (MIF); infection; inflammation

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Macrophage migration inhibitory factor (MIF) is a major proinflammatory mediator with unique tautomerase enzymatic activity. The role of this tautomerase activity in pulmonary inflammation has not been established and represents a potential candidate therapeutic target in chronic inflammatory diseases, such as cystic fibrosis (CF).

What This Study Adds to the Field

Inhibition of MIF's tautomerase activity in *in vitro* and *in vivo* models of inflammation and infection significantly attenuates an exaggerated injurious inflammatory response.

Macrophage migration inhibitory factor (MIF) is a key proinflammatory cytokine that has been implicated in the pathogenesis of acute and chronic inflammatory diseases (1–4). It mediates its proinflammatory effects directly by inducing a number of cytokines, including tumor necrosis factor (TNF)-α (5, 6). Up-regulation of toll-like receptor 4 (TLR4) expression by MIF facilitates its ability to potentiate the effects of LPS in gram-negative sepsis (1). It also has the capacity to exacerbate inflammatory responses indirectly by overriding the effects of glucocorticoids (7). MIF can also promote cell growth and tumorigenesis via sustained activation of the p44/p42 (ERK-1/2) MAP kinase pathway (8) and inhibition of p53-dependent apoptosis (9, 10).

Previously, it has been shown that deletion of MIF attenuates lethality, neutrophilia, and TNF-α levels in an acute model of *Pseudomonas aeruginosa* infection (11). Furthermore, administration of an anti-MIF antibody up to 8 hours post insult attenuates mortality in murine sepsis models (6). Therefore, MIF represents a candidate therapeutic target in the treatment of inflammatory conditions, with its capacity to exacerbate inflammation being partly dependent on the induction of TNF-α.

MIF possesses a unique tautomerase activity that resides within an N-terminal proline (Pro1) and surrounding hydrophobic pocket of MIF (12, 13). The specific role of this tautomerase activity is not fully defined, and no physiological substrate has been identified (14). Inhibition of MIF-tautomerase activity in a murine sepsis model using the compound ISO-1 decreases lethality and TNF-α levels in mice (15). More recently, a novel tautomerase-null *MIF* gene knockin mouse was generated, in which a Pro1 to a Gly1 substitution was made ($mif^{P1G/P1G}$ mice) (16). These authors reported that MIF-tautomerase activity was necessary for optimizing cell growth and tumorigenesis, but they did not investigate its role in an inflammatory disease model.

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P. aeruginosa represents the archetypical opportunistic gram-negative organism and is implicated in a variety of chronic suppurative lung conditions, including cystic fibrosis (CF). CF is the most common fatal inherited disease in whites internationally (17) and has an incidence of 1 in 1,461 live births in Ireland (18). We previously identified a novel CATT-tetranucleotide repeat polymorphism at -794 of the human MIF promoter, which has been shown to modulate MIF activity *in vitro* in gene reporter assays (19). Furthermore, we have shown that patients with CF with six to eight copies of the CATT repeat have earlier *P. aeruginosa* colonization and more severe pancreatitis compared with patients with five CATT (5-CATT) repeats (20). This is associated with adverse prognosis in CF. In this study, we investigated the role of the MIF-CATT polymorphism *ex vivo* in patients with CF. We also used $mif^{P1G/P1G}$ tautomerase-null mice in a chronic model of *P. aeruginosa* lung infection to investigate the therapeutic potential of an antitautomerase strategy in CF.

METHODS

Study Population

Patients attending the National Adult Referral Centre for Cystic Fibrosis at St Vincent's University Hospital (SVUH), Dublin, Ireland who had a proven diagnosis of CF (CFTR genotyping, sweat testing, and clinical phenotype) were recruited. Healthy control subjects consisted of age- and sex-matched adults. This study was approved by the Medical Ethics Committee of SVUH.

Longitudinal Study of the Effect of the MIF CATT-Repeat Polymorphism on Lung Function in Patients with CF

We conducted a retrospective longitudinal genetic association study of patients with CF attending the National Adult Referral Centre for Cystic Fibrosis (SVUH, Dublin). Patients who participated in the original study of MIF genotype and CF lung disease severity (20) were identified. Clinical data including age, sex, serial lung function testing, measures of nutrition, and vital status (alive/dead or transplanted) were abstracted. Clinical data were linked to previous MIF genotyping results. Generalized estimating equations with an exchangeable working model were used to test for association between outcome variables and MIF genotype (*see* online supplement).

Analysis of MIF and TNF-α Protein Levels

Healthy control subjects, patients with CF, and patients with CF who were homozygous for the 5-CATT (low MIF producers; n = 6) compared with the 6-CATT repeat polymorphism (high MIF producers; n = 18) were investigated for levels of MIF or TNF-α protein, respectively, in their plasma or peripheral blood mononuclear cell (PBMC) supernatants using commercial human MIF or TNF-α ELISAs (DuoSet ELISA Development System; R&D Systems, Abingdon, UK) according to manufacturer's instructions (*see* online supplement).

Quantification of TNF-α mRNA Expression by Quantitative Real-Time Polymerase Chain Reaction

Two milliliters of RPMI with 2×10^6 cells was added to polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ). Cells were subsequently treated with medium only or LPS (100 ng/ml) for 6 hours at 37°C before harvesting by centrifugation. TRI REAGENT (Sigma, Dorset, UK), 1 ml, was added per sample, total RNA extracted, and first-strand cDNA synthesized, and subsequently quantitative real-time polymerase chain reaction for TNF-α mRNA expression was performed (Stratagene MX3000P Real-Time PCR System; Stratagene, Santa Clara, CA) as described previously (21).

Tautomerase-Null MIF Gene Knockin Mice

We used tautomerase-null *MIF* gene knockin mice in which the enzymatically-active Pro1 was substituted for a Gly1 MIF protein ($mif^{P1G/P1G}$ mice; 6–9 wk old, C57BL/6 background) (16). Protocols were approved by Animal Research Ethics Committee, University College Dublin and conducted under license from the Department of Health, Ireland.

Chronic Airway Infection Model

Chronic pulmonary infection with *P. aeruginosa* was produced in $mif^{+/+}$ and $mif^{P1G/P1G}$ mice using previous methodology (22). Adult male specific pathogen-free mice were anesthetized and inoculated intratracheally with mucoid *P. aeruginosa* incorporated into agar beads and allowed to recover from anesthesia. Total weight loss, differential cell counts, bacterial load, intraacinar airspace/tissue volume, and histological analyses were performed as described previously (22–25) (*see* online supplement).

Statistical Analysis

The Student *t* test (parametric) or Mann-Whitney test (nonparametric) were used using GraphPad InStat 3.00 (GraphPad Software, La Jolla, CA). One-way analysis of variance with the Tukey-Kramer multiple comparisons *post hoc* test (parametric) or the Kruskal-Wallis test with the Dunn multiple comparisons posttest (nonparametric) were used to test for statistical significance of differences between more than two experimental groups. Statistical significance was recorded at $P < 0.05$.

RESULTS

Patients with CF with at Least One 5-CATT Repeat Allele Who Are Low-MIF Expressers Have a Significantly Slower Rate of Decline in FEV_1 Compared with Patients with CF with No 5-CATT Repeat Allele Who Are High-MIF Expressers

One hundred forty-three patients with CF were identified who had clinical data (Table 1) and were genotyped historically for the MIF 5-CATT repeat polymorphism (Table 2). We then tested for an association between the annual rate of change in FEV_1 (% predicted) and MIF genotype. Subjects were followed up for an average of 3.13 years and had a median of seven measures of lung function (interquartile range, 3–11) each. Thirty-five percent of patients with CF had a least one 5-CATT repeat allele. We determined that there was a significant nonlinear association between MIF genotype and annual rate of change in FEV_1 (% predicted) (regression coefficient, 1.25 [95% confidence interval, 0.03–2.5] ml/yr; $P = 0.45$; −0.21 [95% confidence interval, −0.05 to −0.5] ml/yr^2; $P = 0.013$) after adjusting for age and sex (Figure 1A). This result indicated a protective effect of the 5, X-CATT genotype on lung function. There was no association between MIF genotype and rate of change in FVC (Figure 1B) or any of the other secondary outcome measures (*see* online supplement). Furthermore, there was no significant difference in absolute FEV_1 or FVC across MIF genotypes at the time of entry into the study.

MIF Is Significantly Increased *In Vivo* and in *Ex Vivo* PBMC Cultures from 6, 6-CATT Patients with CF with High-MIF Expression Alleles Compared with 5, 5-CATT Low-MIF Expressers

We recently demonstrated that patients with CF with six to eight copies of the MIF CATT-repeat polymorphism have earlier *P. aeruginosa* colonization and evidence of earlier extrapulmonary end-organ injury (20). In this study, we investigated the role of the MIF-CATT polymorphism *in vivo* and in *ex vivo* PBMCs derived from patients with CF. First, we analyzed the circulating levels of MIF in a cohort of Irish patients with CF (n = 70) compared with healthy control subjects (n = 72). MIF protein

TABLE 1. CLINICAL CHARACTERISTICS OF SUBJECTS WITH CYSTIC FIBROSIS

	Total Population
No.	143
Sex, % male	52
Age, yr	28 ± 7
FEV_1, % predicted	56 ± 25
FVC, % predicted	76 ± 23
Height, cm	165 ± 11
Weight, kg	60 ± 28

levels were significantly elevated in plasma from patients with CF (1.6 ± 0.1 fold increase; $P < 0.001$; Figure 2A) compared with control subjects. This result confirms that of a previous study that demonstrated increased plasma-MIF in Swiss patients with CF (n = 6) (26). To examine the specific effect of the CATT repeat polymorphism, we quantitated MIF levels in the plasma from 5, 5-CATT compared with 6, 6-CATT patients with CF and observed a significant increase in MIF in the plasma from 6, 6-CATT- (1.9 ± 0.4 fold increase; $P < 0.05$; Figure 2B) compared with 5, 5 CATT-patients with CF. Furthermore, PBMCs, which were isolated from whole blood from 6, 6-CATT-patients with CF, produced significantly more MIF, constitutively (4.97 ± 1.8 fold increase; $P < 0.05$; Figure 2C), compared with 5, 5-CATT patients with CF (1.0 ± 0.3 fold increase; Figure 2C). We have demonstrated elevated MIF protein in those patients with CF genetically primed to be high MIF producers.

TNF-α mRNA Expression and Protein Production Is Increased in PBMCs from 6, 6-CATT Compared with 5, 5-CATT Patients with CF after LPS Treatment

Increased levels of TNF-α have been detected in sputum (27, 28) and bronchoalveolar lavage fluid (27, 29) of patients with CF by other authors. In addition, the ability of MIF to promote TNF-α production is well described (5, 6). Furthermore, various anti-MIF strategies have been shown to reduce levels of TNF-α *in vivo* in murine models of gram-negative sepsis (11, 15). In this study, we investigated if the increased levels of MIF produced constitutively by PBMCs from 6, 6-CATT compared with 5, 5-CATT patients with CF had any functional consequences on TNF-α production in these patients. For this purpose, we investigated TNF-α mRNA expression (Figure 3A) and protein production (Figure 3B) from PBMCs from 6, 6-CATT and 5, 5-CATT patients with CF after treatment with LPS. LPS treatment induced an 11.9-fold increase in TNF-α production from PBMCs from 6, 6-CATT patients compared with a 3.5-fold increase from cells from 5, 5-CATT patients after 24 hours of treatment (Figure 3B). These results demonstrate that PBMCs from 6, 6-CATT patients produce increased levels of MIF and have a greater capacity to produce TNF-α in response to LPS treatment compared with PBMCs from 5, 5-CATT patients with CF.

TABLE 2. MACROPHAGE MIGRATION INHIBITORY FACTOR CATT REPEAT POLYMORPHISM FREQUENCY

MIF CATT Genotype	Frequency (%)
5/5	6
5/6	24
5/7	5
6/6	52
6/7	12
7/7	1

Definition of abbreviation: MIF = macrophage migration inhibitory factor.

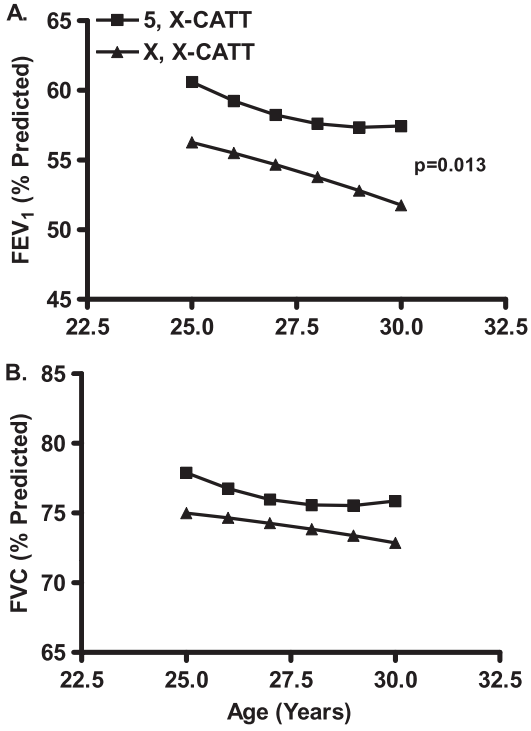

Figure 1. Patients with cystic fibrosis (CF) with 5, X-CATT low–macrophage migration inhibitory factor (MIF) expression alleles have a significantly lower rate of decline in FEV_1 (% predicted) compared with patients with CF with X, X-CATT high-MIF expression alleles over time. (*A*) Average rate of decline in FEV_1 (% predicted) is significantly less ($P = 0.013$; nonlinear association) in 5, X-CATT (n = 50) compared with X, X-CATT patients with CF (n = 93), who had an average follow-up period of 3.13 years and were aged 25 to 30 years. A median of seven measures of lung function (interquartile range, 3–11) were recorded per patient. Results were adjusted for age and sex. (*B*) There was no significant association between rate of change in FVC and MIF genotype over time. (*A, B*) There was no significant difference in FEV_1 and FVC across MIF genotype groups at time of study entry.

LPS-Induced TNF-α Production from PBMCs from Healthy Donors and 5, 5-CATT and 6, 6-CATT Patients with CF Is Attenuated after Pretreatment with ISO-1, a Small Molecular Weight Inhibitor of MIF-Enzymatic Activity

MIF possesses a unique tautomerase activity, the function of which is not clearly defined. It was previously reported that a specific inhibitor of MIF-tautomerase activity, ISO-1, reduces lethality and decreases TNF-α levels in a murine model of sepsis (15). In this study, we first investigated the effects ISO-1 (100 μM) on LPS-induced TNF-α from PBMCs from healthy donors (n = 3; Figure 4A). We demonstrated that LPS-induced TNF-α production was significantly reduced after pretreatment (30 min) with 100 μM ISO-1 (117.7 ± 11 pg/ml; $P < 0.01$; Figure 4A) compared with LPS-treated PBMCs only (1,925 ± 65.3 pg/ml; Figure 4). Subsequently, we investigated the effects of ISO-1 (range, 10–100 μM) in PBMCs from 5, 5-CATT (n = 4; Figure 4A) and 6, 6-CATT patients with CF (n = 4; Figure 4B). ISO-1 significantly reduced LPS-induced TNF-α production, in a dose-dependent manner, in patients with CF from both genotype groups (Figures 4B and 4C). However, the ability of ISO-1 at 100 μM to decrease LPS-induced TNF-α production was higher in healthy donors (16.3-fold decrease) and 5-CATT patients with CF (8.6-fold decrease) compared with 6-CATT patients with CF (4.2-fold decrease). This result may reflect the increased ability of 6, 6-CATT patients with CF to constitutively produce MIF compared with 5, 5-CATT patients with CF. These results

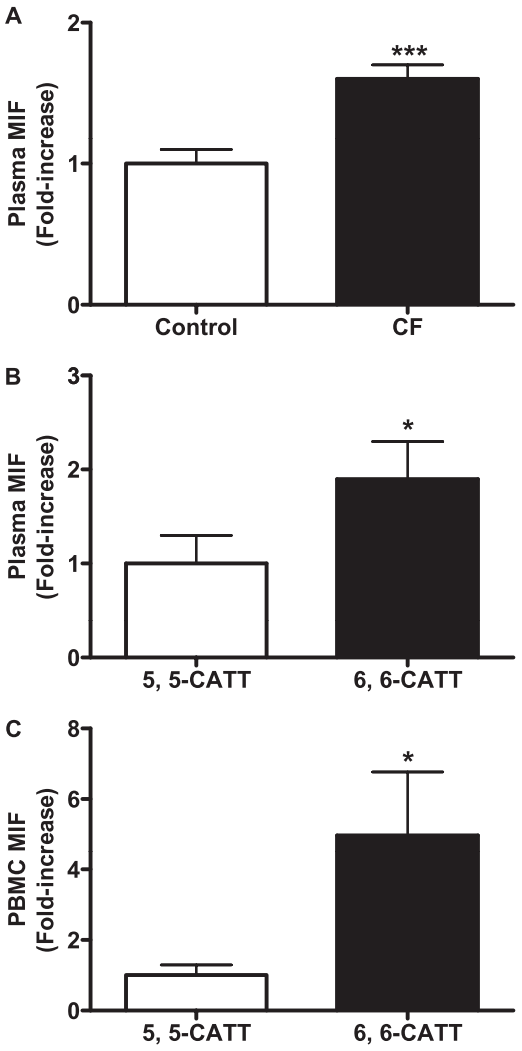

Figure 2. Macrophage migration inhibitory factor (MIF) is significantly increased *in vivo* and in ex vivo peripheral blood mononuclear cell (PBMC) cultures from 6, 6-CATT patients with cystic fibrosis (CF) with high-MIF expression alleles compared with 5, 5-CATT low-MIF expressers. (*A*) MIF is significantly increased in the plasma of patients with CF (n = 70) compared with control subjects (n = 72). ***$P < 0.001$, plasma from control versus CF. (*B*) MIF is significantly increased in the plasma of patients with CF with 6, 6-CATT alleles (high-MIF expressers; n = 18) compared with patients with 5, 5-CATT alleles (low-MIF expressers; n = 6). *$P < 0.05$, plasma from 6, 6-CATT- versus 5, 5-CATT-patients with CF. (*C*) PBMCs derived from patients with CF with 6, 6-CATT alleles (n = 6) secrete increased MIF compared with patients with 5, 5-CATT alleles (n = 6). *$P < 0.05$, PBMCs from 6, 6-CATT- versus 5, 5-CATT-patients with CF. MIF protein concentrations were quantitated by ELISA. Values represent mean ± SEM of MIF protein (pg/ml) and are expressed as fold increase over control subjects.

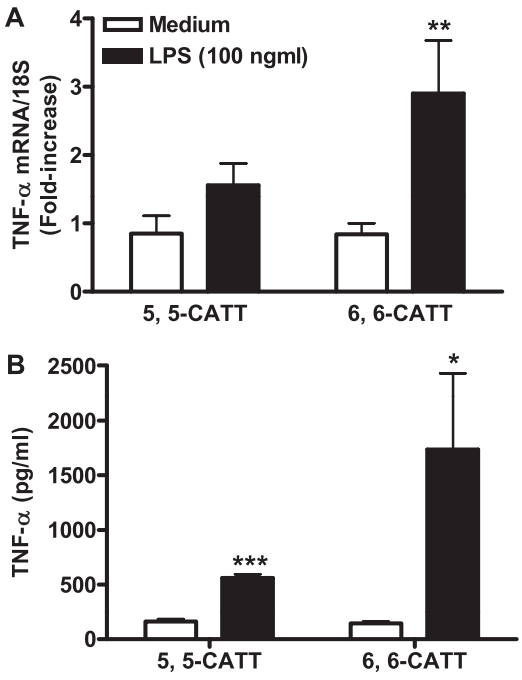

Figure 3. Tumor necrosis factor (TNF)-α mRNA expression and protein production are increased in peripheral blood mononuclear cells (PBMCs) from 6, 6-CATT compared with 5, 5-CATT patients with cystic fibrosis (CF) after LPS treatment. (*A*) TNF-α mRNA expression is significantly increased in PBMCs from patients with CF with 6,6-CATT alleles (high–macrophage migration inhibitory factor [MIF] expressers; n = 6) compared with patients with 5,5-CATT alleles (low-MIF expressers; n = 6) after LPS treatment (100 ng/ml) for 6 hours. (*B*) TNF-α protein production is increased from PBMCs from patients with CF with 6, 6-CATT alleles (high-MIF expressers; n = 6) compared with patients with 5, 5-CATT alleles (low-MIF expressers; n = 6) after LPS treatment (100 ng/ml) for 24 hours. TNF-α mRNA expression was quantitated by quantitative real-time polymerase chain reaction. Values represent mean ± SEM for TNF-α mRNA and are expressed per unit of 18S ribosomal mRNA and are expressed as fold increase over medium only control. TNF-α protein concentrations were quantitated by ELISA. Values represent mean ± SEM of TNF-α protein (pg/ml). **$P < 0.01$, *$P < 0.05$: LPS versus medium only in PBMCs from 6, 6-CATT patients with CF; ***$P < 0.001$: LPS versus medium only in PBMCs from 5, 5-CATT-patients with CF.

also demonstrate a role for MIF's enzymatic activity in augmenting TNF-α production from LPS-treated human PBMCs.

Mice Devoid of MIF-Enzymatic Activity Reveal Less Weight Loss Compared with Wild-Type Mice after *P. aeruginosa* Infection

Recently, a novel tautomerase-null *MIF* gene knockin mouse was generated, in which a Pro1 to a Gly1 substitution was made ($mif^{P1G/P1G}$ mice) (16). These authors reported that MIF-tautomerase activity was required for optimum cell growth and enhanced tumorigenic properties in a murine skin cancer model. In this study, we investigate the role of MIF-tautomerase activity in a murine pulmonary model of chronic *P. aeruginosa* infection using MIF wild-type ($mif^{+/+}$ mice) and tautomerase-null mice $mif^{P1G/P1G}$, using methodology as described previously (22). At Day 3 postinfection, we observed that $mif^{P1G/P1G}$ mice had undergone a reduced percentage of weight loss (5.15 ± 0.81%; $P < 0.01$; Figure 5) compared with wild-type mice (12.38 ± 1.59%; Figure 5). At Day 14 postinfection, $mif^{P1G/P1G}$ mice also experienced less weight loss (5.04 ± 1.84%; $P = 0.063$; Figure 4) compared with $mif^{+/+}$ mice (9.13 ± 0.87%; Figure 5).

Tautomerase-Null Mice Experience Reduced Pulmonary Neutrophil Infiltration, TNF-α mRNA Expression, TNF-α Protein Levels, and Bacterial Load Compared with MIF Wild-Type Mice after *P. aeruginosa* Infection

To investigate the mechanisms underlying the reduced weight loss in $mif^{P1G/P1G}$ mice compared with wild-type mice, we quantitated levels of neutrophil infiltration and TNF-α mRNA expression in lungs from these mice. We determined that the

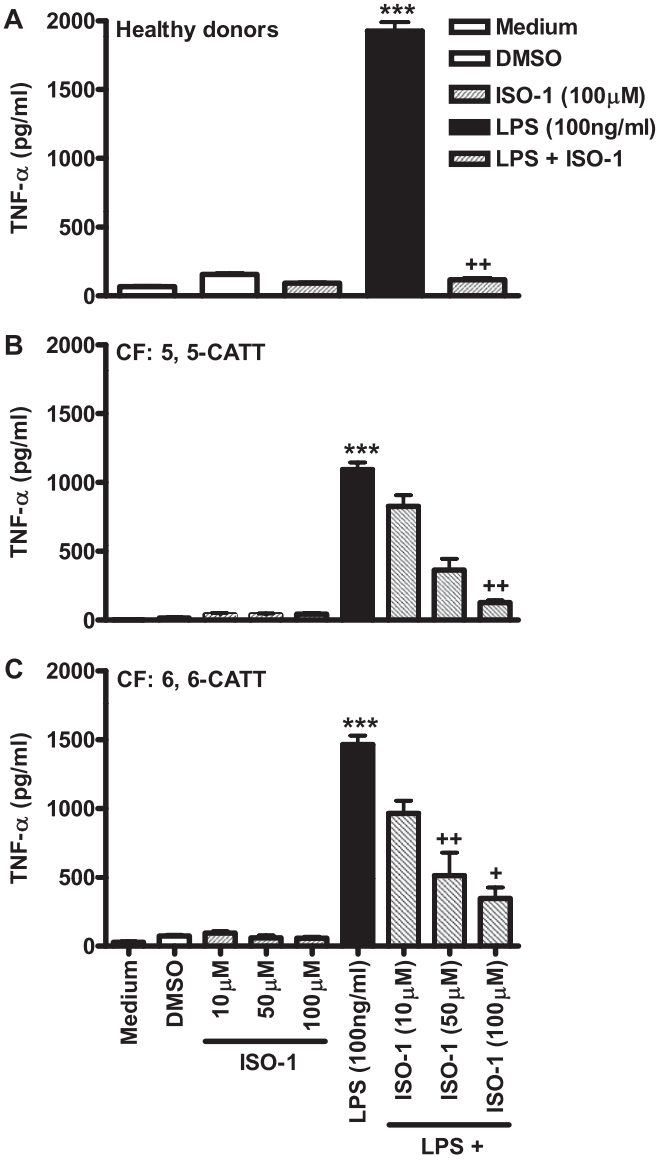

Figure 4. LPS-induced tumor necrosis factor (TNF)-α production from peripheral blood mononuclear cells (PBMCs) from healthy donors, and 5, 5-CATT and 6, 6-CATT patients with cystic fibrosis (CF), is attenuated after pretreatment with ISO-1, a small molecular weight inhibitor of macrophage migration inhibitory factor (MIF) enzymatic activity. A significant increase in TNF-α production was observed in PBMCs from (*A*) healthy donors, (*B*) 5, 5-CATT, and (*C*) 6, 6-CATT patients with CF (*A–C*, $P < 0.001$) after 24 hours of treatment with LPS (100 ng/ml) compared with medium-only cells. After a 30-minute pretreatment with 10 to 100 μM ISO-1, a specific inhibitor of MIF-tautomerase activity, a significant decrease in LPS-induced TNF-α was observed in PBMCs from (*A*) healthy donors (100 μM ISO-1; $P < 0.01$), (*B*) 5, 5-CATT patients with CF (100 μM; $P < 0.01$), and (*C*) 6, 6-CATT patients with CF (10 μM, $P < 0.01$; 100 μM, $P < 0.05$). The fold decrease in LPS-induced TNF-α production using 100 μM pretreatment with ISO-1 was (*A*) 16.3-fold in healthy donors, (*B*) 8.6-fold in 5, 5-CATT-patients with CF, and (*C*) 4.2-fold in 6, 6-CATT patients with CF. TNF-α protein concentrations were quantitated by ELISA. Values represent mean ± SEM of TNF-α protein (pg/ml). ***$P < 0.001$, LPS versus medium only; **$P < 0.01$, LPS versus pretreatment with ISO-1 before LPS. Results are representative of a minimum of four replicate samples from n = 3 healthy donors and n = 4 patients with CF (both genotypes). DMSO = dimethyl sulfoxide.

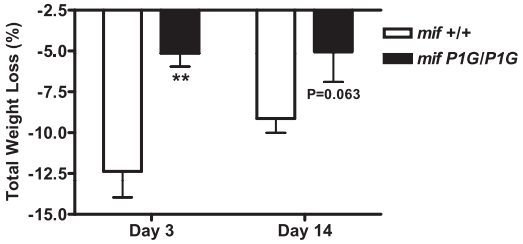

Figure 5. Mice devoid of macrophage migration inhibitory factor (MIF) enzymatic activity reveal less weight loss compared with wild-type mice after *Pseudomonas aeruginosa* infection. Enzymatically devoid mice ($mif^{P1G/P1G}$) experience less weight loss after infection compared with wild-type mice at Day 3 and Day 14 postinfection (Day 3: n = 8/group; Day 14: n = 10/group). Change in mean body weight is expressed as a percentage ± SEM. **$P < 0.01$, $mif^{P1G/P1G}$ enzymatic-knockout (n = 8) versus $mif^{+/+}$ wild-type mice at Day 3 postinfection.

mean percentage of neutrophils in *Pseudomonas*-infected lungs was significantly less in $mif^{P1G/P1G}$ mice (9.68 ± 1.80%; $P < 0.01$; Figure 6A) compared with wild-type mice (34.87 ± 5.06%; Figure 6A) at Day 3 postinfection. In addition, we also investigated the expression of TNF-α mRNA in lung tissue from $mif^{P1G/P1G}$ mice compared with wild-type mice and found a significant decrease in TNF-α mRNA at Day 3 and Day 14 postinfection with *P. aeruginosa* in $mif^{P1G/P1G}$ mice (Day 3: 2.79 ± 0.22 fold-change; $P < 0.01$, and Day 14: 1.1 ± 0.16 fold-change; $P < 0.001$; Figure 6B) compared with wild-type mice (Day 3: 11.25 ± 4.37 fold-change, and Day 14: 2.30 ± 0.22 fold-change; Figure 6B). Histological analysis also revealed decrease in TNF-α protein levels in $mif^{P1G/P1G}$ mice (Figure 6E) compared with wild-type mice (Figure 6D) at 3 days post *P. aeruginosa* infection. Furthermore, we also demonstrated a reduction in *P. aeruginosa* load (Figure 6C; 6.27-fold decrease; $P < 0.05$) in $mif^{P1G/P1G}$ mice compared with wild-type mice at 3 days postinfection. These data collectively demonstrate that MIF-tautomerase activity drives pulmonary TNF-α production, neutrophilia, weight loss, and bacterial load in mice after *P. aeruginosa* infection.

Tautomerase-Null Mice Reveal Reduced Pulmonary Intraacinar Tissue Volume and Reduced Pathology Compared with $mif^{+/+}$ Mice after Chronic *P. aeruginosa* Infection

Here, we investigated the effects of MIF-tautomerase activity on intraacinar airspace and tissue damage in response to chronic *P. aeruginosa* infection. We used increased alveolar wall volume as a quantitative index of pathology, which was caused by inflammatory cell infiltration and edema. First, histological analysis of lung tissue revealed extensive regions of chronic inflammation, thickening of the alveolar walls, and inflammatory cell infiltration in wild-type mice after *Pseudomonas* infection (Figures 7A–7C) compared with $mif^{P1G/P1G}$ mice (Figures 7D–7F). In contrast, infected $mif^{P1G/P1G}$ mice (Figures 7D–7F) had normal alveolar structure and minimal evidence of inflammation compared with $mif^{+/+}$ mice. We subsequently determined quantitatively that the ratio of intraacinar airspace/tissue volume is significantly increased in $mif^{P1G/P1G}$ mice, as demonstrated by a significant reduction in tissue volume in $mif^{P1G/P1G}$ mice at Day 3 (0.16 ± 0.01 ml; $P < 0.01$; Figure 8A) and Day 14 postinfection (0.17 ± 0.01 ml; $P < 0.001$; Figure 8B) compared with $mif^{+/+}$ mice (Day 3: 0.14 ± 0.01 ml; Figure 8A, and Day 14: 0.12 ± 0.01 ml; Figure 8B), respectively. These results demonstrate that tautomerase-null mice undergo reduced pulmonary

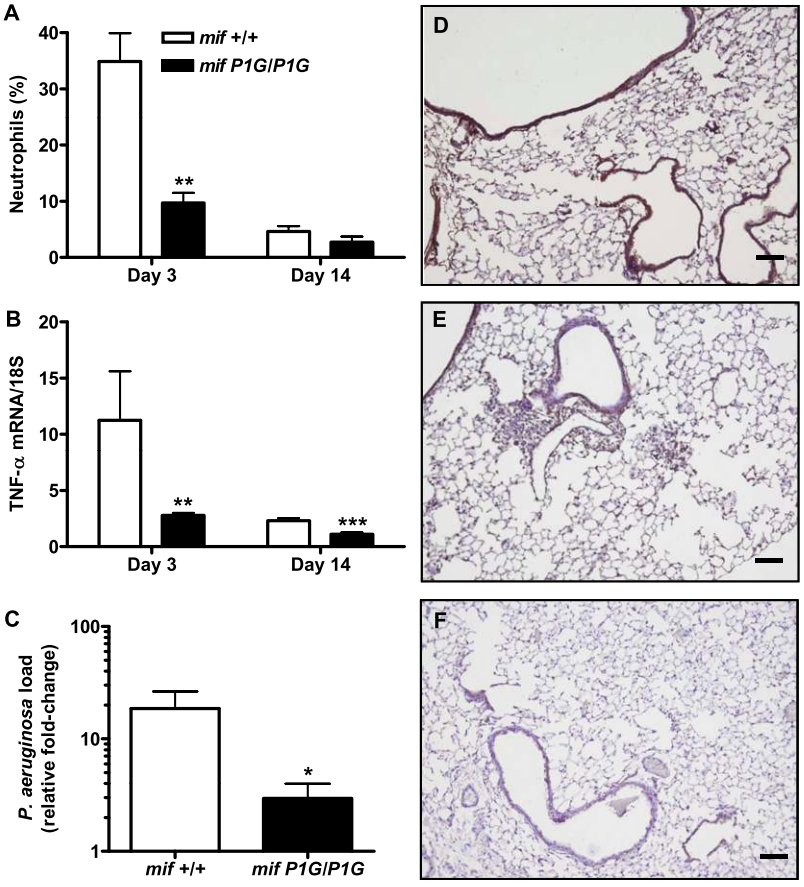

Figure 6. Tautomerase-null mice experience reduced pulmonary neutrophil infiltration, tumor necrosis factor (TNF)-α mRNA expression, TNF-α protein levels, and bacterial load compared with macrophage migration inhibitory factor (MIF) wild-type mice after *Pseudomonas aeruginosa* infection. (*A*) Differential cell counts revealed a significant decrease in the percentage of neutrophils at Day 3 postinfection in *Pseudomonas*-infected lungs from $mif^{P1G/P1G}$ mice versus wild-type mice (Day 3: n = 8/group; Day 14: n = 10/group). Neutrophil numbers were assessed by differential cell count using Diff-Quick and are expressed as a percentage ± SEM of total cell count. (*B*) A significant decrease in pulmonary TNF-α mRNA expression was observed at Days 3 and 14 in lungs from tautomerase-null mice compared with wild-type mice after *P. aeruginosa* infection. TNF-α mRNA expression was quantitated by quantitative real-time polymerase chain reaction. Values represent mean ± SEM for TNF-α mRNA and are expressed per unit of 18S ribosomal mRNA and are expressed as fold increase over medium only control. $^{**}P < 0.01$, $^{***}P < 0.001$; $mif^{P1G/P1G}$ enzymatic-knockout versus wild-type mice. (*C*) A significant decrease in *P. aeruginosa* load was observed in $mif^{P1G/P1G}$ mice compared with wild-type mice at 3 days postinfection (n = 8 per group; $P < 0.05$). Bacterial load was quantitated by TaqMan assay for *P. aeruginosa* detection (Applied Biosystems, Foster City, CA) (24, 25), using genomic DNA, which was extracted from formalin-fixed and paraffin-embedded, murine lung sections at 3 days post *P. aeruginosa* infection (23) (*see* online supplement). A 10^3 to 10^8 serial dilution of an overnight culture of *P. aeruginosa* (1.27×10^9 cfu/ml) was used to generate a standard curve for analyses (24, 25). Results are expressed in arbitrary units and show the relative fold-change in *P. aeruginosa* load in $mif^{+/+}$ mice compared with $mif^{P1G/P1G}$ mice at 3 days postinfection. (*D–F*) Using immunohistochemistry, a trend toward a decrease in TNF-α protein levels was observed in lungs using from tautomerase-null mice (*E*) compared with wild-type mice (*D*) at 3 days post *P. aeruginosa* infection (*see* online supplement). No staining was observed with irrelevant rabbit polyclonal IgG (*F*). ×10 Objective, scale bar = 100 μm.

inflammation and pathology compared with $mif^{+/+}$ mice in response to chronic *Pseudomonas* infection.

DISCUSSION

In this study, we investigated the role of MIF and its enzymatic activity in CF and, in addition, in a chronic model of murine pulmonary infection using *P. aeruginosa* challenge. Chronic pulmonary infection with *P. aeruginosa* is characteristically found in patients with CF, and over time, strains become increasingly resistant to antimicrobial agents and effective therapy becomes progressively more difficult. We have previously demonstrated a functional genetic polymorphism for MIF and showed evidence at the promoter level of enhanced MIF activity *in vitro*

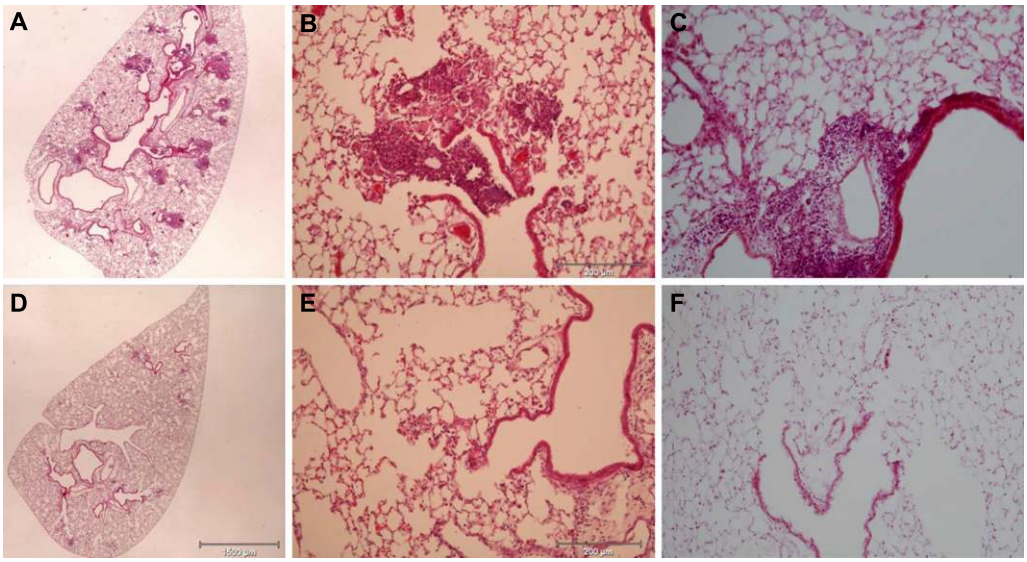

Figure 7. Tautomerase-null mice reveal less inflammation, alveolar wall thickening, and inflammatory cell infiltrate compared with $mif^{+/+}$ mice after chronic *Pseudomonas aeruginosa* infection. Histological analysis was performed on lung sections from (*A–C*) $mif^{+/+}$ and (*D–F*) tautomerase-null mice, respectively (hematoxylin and eosin stain; ×1.25 or ×10 objective). (*A, D*) Day 3 postinfection, ×1.25 objective; (*B, E*) Day 3 postinfection, ×10 objective; (*C, F*) Day 14 postinfection, ×10 objective. (*B, E*) Scale bar = 200 μm and (*D*) 1,500 μm.

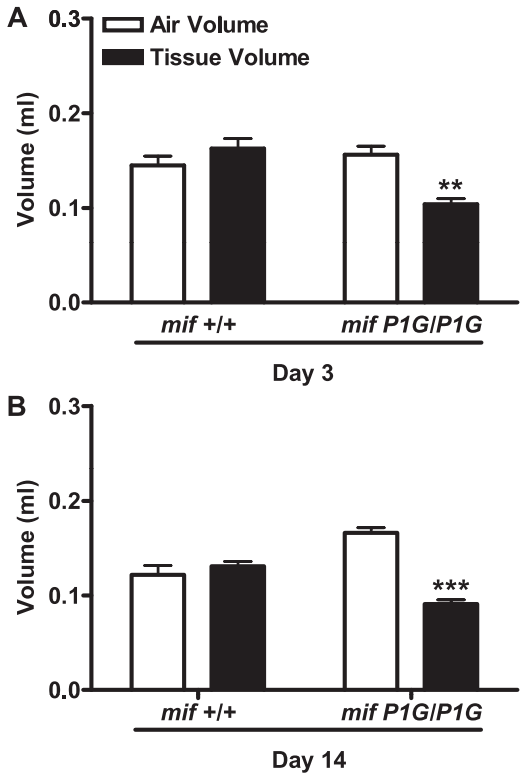

Figure 8. Tautomerase-null mice reveal reduced pulmonary intraacinar tissue volume and reduced pathology compared with $mif^{+/+}$ mice after chronic *Pseudomonas aeruginosa* infection. (*A, B*) Stereological examination reveals that $mif^{P1G/P1G}$ mice have decreased intraacinar tissue volume, or an increased air/tissue ratio, compared with wild-type mice at (*A*) Day 3 and (*B*) Day 14 postinfection (Day 3: n = 8/group; Day 14: n = 10/group). Volumes (ml) of intraacinar airspace (air volume) and intraacinar tissue (tissue volume) of left lungs are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$: intraacinar tissue volumes of left lungs from $mif^{P1G/P1G}$ versus $mif^{+/+}$ wild-type mice at Day 3 postinfection.

in 6-, 7-, and 8-CATT luciferase constructs (20). Furthermore, patients with CF with high-MIF expression alleles demonstrated more severe end-organ injury and earlier *P. aeruginosa* colonization (20). We build on this initial work to show in this paper evidence of enhanced systemic MIF protein levels in patients with CF who are homozygous for the 6, 6-CATT polymorphism (compared with 5, 5-CATT patients). Similar findings are found in PBMCs from *in vitro* cultures derived from both genetic patient populations. This study demonstrates for the first time that the -794 CATT repeat polymorphism is functionally active *in vivo* and specifically in patients with CF.

FEV_1 decline has been shown to be one of the most important independent predictors of mortality in CF (30, 31). In this paper, we report follow-up analysis of the original cohort of patients with CF in whom we initially found a significant association between patients genetically primed to be high-MIF producers and earlier *Pseudomonas* colonization and earlier onset of pancreatic injury (20). We have now analyzed 1,156 subsequent pulmonary function readings of these patients over time. We have found a significant and profound association between patients who are genetically primed to be high-MIF producers (X, X-CATT patients with CF) and accelerated decline in FEV_1 over time. No significant association was found with decline in FVC, suggesting that this is not simply a lung maturation observation but a specific observation supporting the hypothesis that the increased levels of MIF in X, X-CATT patients with CF significantly contributes to chronic inflammation.

A classic example of where "bench to bedside" translational medicine has delivered is in the recent advances in biological therapies. TNF-α represents the archetypical cytokine target in which selected novel therapies have been developed for a variety of chronic inflammatory diseases (32). MIF has previously been shown to be an important promoter of TNF-α production (5, 6). In this paper, we present data demonstrating first that human PBMCs, genetically predisposed to be high-MIF secretors (6, 6-CATT patients with CF), produced significantly enhanced TNF-α after LPS stimulation. We subsequently demonstrated that pretreatment of PBMCs prepared from healthy donors, as well as 5, 5-CATT and 6, 6-CATT patients with CF, with an inhibitor of MIF's tautomerase activity (ISO-1) could significantly attenuate the LPS-driven TNF-α production from these cells. ISO-1 has previously been shown to exert its anti-inflammatory effects by attenuating the nuclear translocation of nuclear factor (NF)-κB in LPS-treated cells (15). We have also shown in this study that the effects of ISO-1 are NF-κB–specific, as ISO-1 was unable to inhibit LPS-induced rantes (an IRF3-dependent gene) production from PBMCs from patients with CF (data not shown).

To define the role of MIF's tautomerase enzymatic activity in chronic pulmonary inflammation, we used a murine model of chronic lung infection (14 d) using *P. aeruginosa* challenge. In addition, a novel mouse, which was devoid of MIF-tautomerase activity ($mif^{P1G/P1G}$), was used, which was generated previously (16). In this study, we demonstrated that $mif^{P1G/P1G}$ tautomerase-null mice have significantly less pulmonary inflammation and bacterial load after chronic bacterial challenge. These mice exhibited significantly less weight loss, significantly fewer pulmonary neutrophils within the alveolar airspace, and significantly lower TNF-α mRNA and protein levels within lung tissue. Further evidence of significantly less pulmonary inflammation was found on stereological analyses, which showed markedly enhanced airspace/tissue volume ratios in our transgenic mice lacking enzymatic activity. Histological analysis of lung sections from $mif^{P1G/P1G}$ mice also demonstrated less inflammation and pathological tissue damage after infection compared with $mif^{+/+}$ mice. It has previously been demonstrated that $MIF^{-/-}$ mice clear *P. aeruginosa* infection more quickly than wild-type mice (11). Here, we confirm and expand on this finding by demonstrating that *P. aeruginosa* load is attenuated in the absence of MIF-tautomerase activity in mice. This effect was accompanied by a decrease in TNF-α mRNA and protein levels. These results suggest that MIF, or its tautomerase activity, may have a direct effect on *P. aeruginosa* growth. Future studies should help elucidate the mechanism by which this may occur. In support of this hypothesis, Gadjeva and colleagues recently showed that corneas from $MIF^{-/-}$ mice experience less bacterial burden compared with $MIF^{+/+}$ mice in a *P. aeruginosa*–induced keratitis model (33). This reduced bacterial load was also accompanied by a reduction in corneal pathology scores, neutrophil influx, and TNF-α levels. One mechanism by which increased *P. aeruginosa* growth may lead to enhanced inflammation is via MIF-induced TLR4 expression (1). Roger and colleagues previously demonstrated that MIF could potentiate host immune responses to gram-negative bacteria, including *P. aeruginosa*, through its ability to up-regulate TLR4 expression and to subsequently increase TNF-α production (1).

Considerable clinical attention is being focused on targeting therapy based on an individual patient's genetic background. Currently, in the clinical domain, gene expression profiling is being used to predict response to chemotherapy in leukemia and guide therapy in cancer (34, 35). Data presented in this

paper show enhanced circulating MIF in stable patients with CF and, furthermore, that there is enhanced MIF expression in those patients with CF carrying the high-expressing 6, 6-CATT MIF allele (19). These data, combined with the proinflammatory role of MIF-tautomerase activity *in vivo* and *in vitro*, which we have demonstrated, highlight the potential benefits of targeting individuals who are genetically predisposed to develop a heightened MIF-induced exaggerated inflammatory response with focused therapeutic strategy targeting MIF's tautomerase enzymatic activity.

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

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