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## Role of macrophage migration inhibitory factor in granulomatosis with polyangiitis

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## Abstract

**Objective:** This study examined the association between *Macrophage Migration Inhibitory Factor (MIF)* promoter polymorphisms and granulomatosis with polyangiitis (GPA), and MIF's role in a murine model of granulomatous vasculitis.

**Methods:** The human study involved 1077 patients with GPA and controls. Genotyping for the *MIF*-794 CATT<sub>5-8</sub> promoter microsatellite (*rs5844572*) was performed by capillary electrophoresis. *MIF* promoter CATT-length dependent expression in response to  $\beta$ -glucan was assessed by gene reporter assays. Granulomatous disease was induced in wild type (WT), *Mif*<sup>-/-</sup> (*Mif*-KO), and *Mif*lung-transgenic (*Mif*lung-Tg2.1) C57BL/6 mice by injection of *Candida albicans*  $\beta$ -glucan (CAG). Mice were treated with a neutralizing anti-MIF and analyzed for lethality, pulmonary granulomas and inflammatory chemokine expression.

**Results:** The percentage of individuals carrying >5 CATT repeats in each *MIF* allele (high *MIF* expressers) was 60.9% in patients with GPA and 53.7% in controls (adjusted  $p=0.049$ ). Human *MIF* gene expression increased proportionally by CATT-length in response to granulomatous stimulation. *Mif*lung-Tg2.1 mice exhibited more pulmonary granulomas than WT, which in turn showed more granulomas than *Mif*-KO. A significantly higher percentage of *Mif*lung-Tg2.1 mice died when injected with CAG when compared to *Mif*-KO or WT, and anti-MIF protected against lethal disease. MIF-dependent neutrophil/macrophage chemokines were elevated in the bronchoalveolar lavage or plasma of *Mif*lung-Tg2.1 mice.

**Conclusion:** Patients with GPA have an increased frequency of high-expression *MIF*CATT alleles. Higher MIF expression increased mortality and pulmonary granulomas while anti-MIF protected mice from lethal disease. MIF blockade in high genotypic *MIF* expressers may offer a selective pharmacologic therapy for GPA.

## Keywords

CD74; macrophage migration inhibitory factor; granulomas; granulomatosis with polyangiitis; vasculitis

## INTRODUCTION

Granulomatosis with polyangiitis (GPA; Wegener's) is an ANCA-associated vasculitis (AAV) characterized by the presence of anti-neutrophil cytoplasmic antibodies (ANCA) (1). A major pathologic feature of GPA includes the presence of granulomatous inflammation in addition to vasculitis (2). The fundamental immunopathogenesis of granulomatous inflammation in GPA remains poorly understood.

The cytokine macrophage migration inhibitory factor (MIF) plays a central regulatory role in innate immunity by inhibiting the activation-induced apoptosis of macrophages and upregulating the expression of pattern recognition receptors. The protein is produced by monocytes/macrophages and barrier epithelial cells to promote autocrine/paracrine

activation and survival signals (3, 4). MIF is expressed within granulomas in hypersensitivity pneumonitis (5), sarcoidosis (6), and tuberculosis (7), and circulating MIF levels are elevated in patients with AAV and associated with disease activity (8). Both MIF and its receptor CD74 are overexpressed in renal mesangial and parietal epithelial cells in proliferative glomerulonephritis (9), and in inflammatory lung disease (10, 11); both processes are common in AAV.

Human genetic studies have identified a four-nucleotide microsatellite in the *MIF* promoter,  $-794$  CATT<sub>5-8</sub> (*rs5844572*) that functionally regulates *MIF* transcription (12) (Figure 1). Microsatellite repeat number is associated with constitutive and inducible *MIF* expression such that the CATT<sub>5</sub> repeat is a low expression allele and the CATT<sub>6-8</sub> repeats are progressively higher expression alleles (13, 14). These *MIF* promoter variants occur commonly in the population (minor allele frequency >5%) and higher CATT number has been linked to the susceptibility or the clinical severity of rheumatoid and juvenile idiopathic arthritis (12, 15), sarcoidosis (6), systemic sclerosis (14), and systemic lupus erythematosus (13). A single nucleotide polymorphism (SNP) in the *MIF* promoter region,  $-173$  G/C SNP (*rs755662*) also has been shown to be associated with autoimmune diseases likely because of linkage disequilibrium with the functional high expression CATT<sub>7</sub> (13, 14).

In the present study, we sought to determine potential relationships between *MIF* gene polymorphisms and GPA by conducting a candidate gene study using samples from a multicenter observational study of 1077 vasculitis and control patients in the United States. We analyzed *MIF* genotype and MIF levels in relation to disease incidence for their correlation with two *MIF* promoter polymorphisms: the  $-794$  CATT<sub>5-8</sub> microsatellite and  $-173$  G/C SNP. We additionally studied an experimentally-induced model of granulomatous disease using genetically engineered mice that overexpress MIF in the lung in order to mimic the elevation in MIF expression in target organs that may be evident in human high genotypic *MIF* expressers.

## MATERIALS AND METHODS

### Patients:

Our study cohort consisted of 501 patients with GPA and 576 healthy controls recruited from the Wegener's Granulomatosis Genetic Repository (WGGER) conducted at 9 US academic centers between 2001 and 2005 Group (16). Plasma samples from patients with GPA were obtained from the Vasculitis Clinical Research Consortium (VCRC), a large, multicenter, North American observational cohort. All patients were 18 years old and fulfilled the modified American College of Rheumatology (ACR) criteria for GPA (16, 17). Ethnicity was determined by self-report and given the rarity of GPA in minority groups, the study focused on Caucasians. Disease activity was measured by the Birmingham Vasculitis Activity Score (BVAS). All relevant Institutional Review Boards approved the study.

### Genotyping:

Genomic DNA was isolated from serum using the easy-DNA kit (Invitrogen, Carlsbad, CA, USA) and the two *MIF* promoter polymorphisms (*rs5844572*, *rs755662*) were analyzed

following methodologies previously described (14). *MIF*-794 CATT<sub>5-8</sub> genotyping was performed by PCR using a forward primer (5'-TGCAGGAACCAATACCCATAGG-3') and a TET fluorescent reverse primer (TET lab5'-AATGGTAAACTCGGGGAC-3'). The quality of the PCR product was assessed by agarose gel electrophoresis. Automated capillary electrophoresis on a DNA sequencer was performed on each sample and the CATT<sub>5-8</sub> repeat length was identified using Genotyper 3.7 software (Applied Biosystems). *MIF*-173 G/C genotyping was performed using a pre-developed TaqMan assay for allelic discrimination (Applied Biosystems, Foster City, CA, USA). After real-time PCR, the genotype of each sample was automatically attributed using fluorescence detection in an ABI Prism 7900HT instrument (Applied Biosystems). Allelic discrimination was analyzed with SDS 2.1.1 software (Applied Biosystems). Although at least 8 additional polymorphisms have been identified within the human *MIF* locus, these additional variants (all SNPs) are rare and have a low likelihood of functionality given their location in introns or within the 3'-untranslated region (18).

#### Plasma MIF:

Plasma samples were obtained at the time of study entry. Circulating MIF levels were measured by enzyme-linked immunosorbant assay (ELISA) using specific antibodies (19). A native sequence and fully active recombinant human MIF was used as a standard (20). Plasma MIF levels were compared and culled from patients with GPA identified as having the high expression, -794 CATT<sub>6-8</sub> and -173 C containing genotypes or the low expression, -794 CATT<sub>5</sub> and -173 G/G genotypes.

#### Granulomatous Vasculitis Mouse Model:

A murine model of granulomatous vasculitis induced by the injection of *Candida albicans*  $\beta$ -glucan (Sigma-Aldrich, St Louis, MO, USA) into mice (C57BL/6, female, aged 8–12 weeks) was used for the experimental studies (21, 22). Discrete and multiple granulomatous lesions appear in lungs within 48 hours after the intravenous injection of  $\beta$ -glucan. All procedures were performed in accordance with the Institutional Animal Care and Use Committee protocols of Yale University.

We developed tissue conditional transgenic mouse lines that overexpress MIF in lung epithelium and selected a founder line that showed a two-fold increase in *Mif* mRNA expression (designated *Mif*lung-Tg2.1) (23). Briefly, a 0.4 Kb DNA fragment containing mouse *Mif* cDNA was inserted into an expression plasmid under the control of the pulmonary Type II epithelial-cell specific CC10 promoter and transfected into Bruce4 (C57BL/6) ES cells for the establishment of founder mice in the pure C57BL/6 background. Quantitative PCR (qPCR) confirmed a two-fold increase in *Mif* mRNA expression in lung and corresponding increases in MIF protein in bronchoalveolar lavage fluid (BAL) and alveolar lung epithelium (Figure 2). A *Mif*-KO mouse strain created previously in the pure C57/BL6 background was used for comparison studies (24).

Following the injection of  $\beta$ -glucan into tail veins, lungs were resected from dead or sacrificed mice (48 hours post-injection) and examined histologically for pulmonary pathology. Additional cohorts of mice were observed for mortality and sacrificed when

judged to be agonal. After initial dose-ranging experiments, responses to  $\beta$ -glucan were assessed in a blinded fashion. The granuloma density (number of granulomas per  $\text{mm}^2$ ) of at least ten random lung sections was calculated and compared between experimental groups by an observer blinded to the experimental groups. For lung immunohistochemistry, formalin-fixed and paraffin-embedded sections were stained with polyclonal anti-MIF (11b, 1:1500 dilution) or non-immune serum and developed with immunoperoxidase. Bronchoalveolar lavage (BAL) was sampled (25) and plasma cytokines of (IL-1, IL-6, IL-10, IL-17, TNF, KC, MCP-1, VEGF and MIP-2) were measured using a cytokine multiplex panel (Invitrogen, Carlsbad, CA, USA) and levels expressed as mean  $\pm$  SD (pg/ml). Selected mice also received treatment with a neutralizing anti-MIF mAb (clone NIH.IIID9) (26) or an anti-IgG1 isotype control (20 mg/kg, i.p.).

The ability of  $\beta$ -glucan to stimulate *MIF* expression in a  $-794 \text{ CATT}_{0-8}$  length-dependent manner was assessed in human pulmonary epithelial A549 cells co-transfected with expression plasmids engineered to contain the  $-794 \text{ CATT}_{0,5,6,7,8}$  *MIF* promoter sequences ligated to a luciferase gene together with a  $\beta$ -actin promoter luciferase control. These studies utilized the high efficiency Amaxa nucleofactor platform (Lonza, Ltd, Switzerland) with 1  $\mu\text{g}$  of each plasmid per transfection reaction (12). The -173 SNP status of these *MIF* constructs was in accordance with the most commonly occurring genotypes: e.g., 5G, 6G, 7C, and 8G (13). The transfected cells were incubated at  $37^\circ\text{C}$  for 24 hours followed by stimulation with 100 ng/ml of  $\beta$ -glucan before luminescence measurement.

### Statistical analysis:

The Pearson chi-square test was used to analyze Hardy-Weinberg equilibrium. Genotypic frequencies were compared using the chi-square test and (when appropriate) Fisher's exact test. We compared high *MIF* expression  $> -794 \text{ CATT}_5$  containing genotypes ( $-794 \text{ CATT}_{6/6}$ ,  $-794 \text{ CATT}_{6/7}$ ,  $-794 \text{ CATT}_{7/7}$ ) with low *MIF* expression  $-794 \text{ CATT}_5$  containing genotypes ( $-794 \text{ CATT}_{5/5}$ ,  $-794 \text{ CATT}_{5/6}$ ,  $-794 \text{ CATT}_{5/7}$ ) and  $-173 \text{ C}$  containing genotypes ( $-173 \text{ C/C}$ ,  $-173 \text{ G/C}$ ) with  $-173 \text{ G/G}$  genotypes between patients and healthy controls. The  $-794 \text{ CATT}_8$  allele was found rarely ( $<1\%$ ) (27) and was excluded from analysis. The difference between the 2 groups was adjusted for age and sex.

To account for population stratification and its effect on genotype differences, we used formulas established by Lee *et al.* to gauge the impact of population stratification bias (28). The population stratification bias can be quantified by the Confounding Rate Ratio (CRR), which equals the biased relative rate divided by the true relative rate. Lee and Wang showed that CRR is always bound above by "U" and below by "1/U", where "U" is the highest odds ratio that can be attributed to the population stratification bias and "1/U" is the lowest odds ratio. "U" takes into account the highest and lowest reported prevalence of a disease in a population affected by stratification and the highest and lowest frequency of the genotype in question reported in the same population. If the observed odds ratio is above the highest calculated odds ratio "U" or lower than the lowest calculated odds ratio "1/U", then these observed odds ratio cannot just be explained by population stratification.

Differences in circulating plasma MIF levels between groups were compared using the Student's t test. Similar *MIF* expression categories were used: High *MIF* expression,  $> -794$

CATT<sub>5</sub> and -173 C containing genotypes versus low *MIF* expression, -794 CATT<sub>5</sub> containing and -173 G/G genotypes. Genetic statistical analyses were performed using STATA version 13. All significance levels reported are 2-tailed. P values < 0.05 were considered statistically significant.

Mice survival curves were compared using Log-rank (Mantel-Cox) test. Lung granuloma densities and cytokine levels were compared using t test. Statistical analyses for the animal studies were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). P values < 0.05 were considered statistically significant.

## RESULTS

### MIF gene polymorphisms.

A total of 1077 subjects were studied (501 patients and 576 controls). Table 1 describes the baseline demographics of the participants and the frequencies of the -794 CATT<sub>5-8</sub> and -173 G/C *MIF* genotypes. The studied populations did not deviate from the Hardy-Weinberg equilibrium and the allelic distributions are similar to those previously reported in the general population (14, 15).

The percentage of patients carrying > -794 CATT<sub>5</sub> repeats in their genotypes was 60.2% in patients with GPA and 53.9% in controls (odds ratio: 1.38 (1.08–1.76), unadjusted p=0.030 and p=0.049 adjusted for sex and age). There was no significant difference in the -173 C containing genotypes between the GPA and healthy control groups (34.2% in patients versus 40.2% in controls, unadjusted p=0.050 and p=0.210 adjusted for age and sex).

The prevalence of GPA in the United States is 26 to 90 per 100,000 (29), and the frequency of the > -794 CATT<sub>5</sub> containing genotypes in US Caucasians is reported to be 49.7–57.1% (14, 15), which is similar to our reported frequencies. Based on the prevalence of GPA and the frequency of > -794 CATT<sub>5</sub> containing genotypes, we calculated the highest odds ratio “U” and the lowest “1/U” that can be attributed to population stratification. The highest odds ratio was “U”=1.10 and the lowest was “1/U”=0.91. Our measured odds ratio of 1.38 is higher than the highest calculated odds ratios, indicating that the observed association between GPA and > -794 CATT<sub>5</sub> genotype cannot be attributed to population stratification.

### MIF plasma levels.

We measured MIF levels in plasma obtained at the time of study entry in a subset of patients with GPA, and in age- and sex-matched healthy controls. The mean plasma MIF level in patients was significantly higher than in disease-free controls (15.9 ± 10.4 ng/ml in patients [n=73] versus 6.7 ± 5.0 ng/ml in controls [n=45]; mean ± SD, p<0.001) (Figure 3). There were no differences in the mean MIF plasma levels between female and male patients (15.1 ± 11.1 ng/ml for females [n=45] vs. 17.2 ± 9.2 ng/ml for males [n= 28], p=0.405). Also, there was no correlation between disease activity as measured by the BVAS and plasma MIF levels.

To determine whether there was a relationship between *MIF* polymorphisms and MIF levels in patients with GPA, we compared plasma MIF levels in high genotypic (>-794 CATT<sub>5</sub>)



and low genotypic ( $-794$  CATT<sub>5</sub>) *MIF* expressers (Figure 3). The mean MIF plasma levels were higher in GPA patients with high *MIF* expression  $-794$  CATT<sub>6-8</sub> containing genotypes ( $17.7 \pm 11.7$  ng/ml, n=35) than in those with low *MIF* expression  $-794$  CATT<sub>5</sub> containing genotypes ( $13.5 \pm 8.8$  ng/ml, n=38; mean  $\pm$  SD, p=0.040). A similar elevation in MIF plasma levels was observed in patients with  $-173$  C containing genotypes ( $17.7 \pm 2.1$  ng/ml, n=23) when compared with patients with  $-173$  G genotypes ( $7.8 \pm 1.2$  ng/ml, n=50; mean  $\pm$  SD, p<0.001) (Figure 3).

### Granulomatous Disease Model Studies.

To examine potential roles for MIF in GPA, we examined the impact of MIF expression in a mouse model of granulomatous vasculitis. MIF is known to be expressed constitutively in lung epithelium, and both mRNA and protein expression increase as a consequence of inflammatory stimulation (10, 30). While the precise stimuli underlying granulomatous inflammation in GPA is unknown, we confirmed the ability of  $\beta$ -glucan to stimulate *MIF* expression in a  $-794$  CATT length-dependent manner by measuring transcriptional activation in human lung epithelial cells transfected with *MIF*- $-794$  CATT<sub>0-8</sub> promoter-luciferase reporter plasmids. As shown in Figure 2,  $\beta$ -glucan stimulation increased *MIF* expression, with  $-794$  CATT<sub>5</sub> showing the lowest gene transcription and  $-794$  CATT<sub>8</sub> showing the highest transcription.

We studied C57BL/6 mice that were wild type (WT), *MIF* gene deficient (*Mif*-KO) (24), or engineered to transgenically express a 2-fold increase in MIF mRNA in lung alveolar epithelium (*Mif*lung-Tg2.1) in order to mimic the  $>$  CATT<sub>5</sub> human *MIF* promoter phenotype (Figure 2). Age-matched cohorts of mice were administered a single 1 mg intravenous injection of  $\beta$ -glucan and mortality assessed over time. As shown in Figure 4, *Mif*lung-Tg2.1 mice showed greater mortality than WT mice (15% vs. 75% survival, p=0.009), which in turn showed a trend toward improved survival over *Mif*-KO mice (75% vs. 100% survival, p=.070). The enhanced lethality attributable to *Mif* overexpression in the lung was abrogated by administration of a neutralizing anti-MIF monoclonal antibody (Figure 4).

To determine the effect of MIF on pulmonary granuloma formation in this model of experimental vasculitis, lungs were resected 48 hours following the injection of  $\beta$ -glucan and examined histologically. The *Mif*lung-Tg2.1 mice, which showed the highest mortality in this model, exhibited more pulmonary granulomas than WT or *Mif*-KO mice (*Mif*lung-Tg2 =  $10.5 \pm 1.2$  /mm<sup>2</sup> vs, WT =  $5.6 \pm 0.9$  /mm<sup>2</sup> [p=0.002] vs. *Mif*-KO =  $1.8 \pm 0.2$  /mm<sup>2</sup> [p<0.0001]) (Figure 4). To examine whether MIF is overexpressed in the pulmonary granulomas, lung sections from *Mif*lung-Tg2 mice were obtained 48 hours after  $\beta$ -glucan injection and stained with anti-MIF or control Ig. Representative lung sections demonstrating MIF immunostaining in alveolar epithelial cells and mononuclear cells within pulmonary granulomas (Figure 4).

Neutrophils are considered to play an important role in the pathogenesis of GPA (31). Because MIF attracts neutrophils via the non-cognate MIF receptor CXCR2 (32) and in some disease setting promotes the downstream expression of specific neutrophil chemoattractants (10, 33) we measured plasma and bronchoalveolar (BAL) neutrophil and

monocyte chemokines in *Mif*lung –Tg2.1 and WT mice. The neutrophil chemo-attractants KC and MIP-2 (CXCL2) were significantly elevated in the plasma and BAL of *Mif*lung –Tg2.1 mice as compared with WT (Figure 5). The monocyte chemokine MCP-1 (CCL2), which is MIF-dependent (26, 34), also was significantly elevated in the *Mif*lung –Tg2.1 mice, suggesting an additional role for MIF in recruiting the monocytes/macrophages that persist in granulomatous inflammation. An elevation in the levels of IL-6 – known to be MIF-dependent (35), also was observed in BAL samples from the *Mif*lung –Tg2.1 mice.

## DISCUSSION:

This study demonstrates that high *MIF* expression >–794 CATT<sub>5</sub> containing genotypes are over-represented in patients with GPA as compared with healthy controls. We also show that *Mif*lung transgenic mice engineered to express a similar two-fold increase mRNA level as cells transfected with >–794 CATT<sub>5</sub> human *MIF* promoter, have increased pulmonary granuloma formation, higher neutrophil/macrophage chemokine production, and increased mortality after  $\beta$ -glucan injection when compared to wild type and *Mif*-KO mice. Furthermore, the administration of anti-MIF monoclonal antibody to *Mif*lung –Tg2.1 mice improved survival. These data collectively support the role of MIF in the pathogenesis GPA.

MIF is an upstream regulatory cytokine that plays an essential role in both the expression of innate and adaptive immunity. MIF inhibits activation-induced apoptosis to sustain inflammatory signaling, prevents the egression of macrophages, and promotes the migration of neutrophils and lymphocytes into sites of inflammation (36). More recently MIF has been implicated in the formation of tissue granulomas; it is expressed in tuberculous granulomas to maintain cellular aggregation within the granuloma microenvironment (7, 25). Granuloma formation is a major component in GPA and in a relevant mouse model, *Mif*lung transgenic mice show higher pulmonary granuloma density than wild type or *Mif*-KO mice. One can hypothesize that higher MIF expression in the lungs of mice injected with  $\beta$ -glucan inhibits macrophage egress and promotes neutrophil and lymphocyte migration leading to higher granuloma density and higher mortality.

Neutrophils are considered a major cellular component in the pathogenesis of AAV (31). ANCA immunoglobulin G activates both neutrophils and monocytes, leading to the generation of reactive oxygen species, the degranulation of tissue-damaging proteases, and the formation of neutrophil extracellular traps (31, 37). Neutrophil and macrophage chemo-attractants such as IL-8 and MCP-1 correlate with disease activity in AAV and outcome (38). In our experimental model, *Mif*lung-Tg2.1 mice showed elevated production of KC and MIP-2, the murine homologues of IL-8, following  $\beta$ -glucan injection. In addition, the monocyte chemo-attractant MCP-1 - a key mediator for the infiltration of monocytes/macrophages, was elevated in the serum of *Mif*lung-Tg2.1 mice following  $\beta$ -glucan administration. These results suggest that MIF-dependent neutrophil and macrophage/monocytes chemokines contribute to the pathogenesis of granulomatous vasculitis in this murine model and support the potential pathogenic role of high expression *MIF* alleles in human AAV.



The mechanism(s) underlying the high mortality observed in the *Mif*<sup>lung-Tg2.1</sup> mice after  $\beta$ -glucan injection remain to be elucidated but may be due to higher pulmonary granuloma burden and an elevation in the systemic inflammatory response leading to shock.  $\beta$ -glucan administration to mice has been reported to result in an anaphylactoid reaction (39). That lethality was abrogated by anti-MIF mAb is noteworthy and warrants further study given the ongoing clinical testing of humanized anti-MIF and anti-MIF receptor antibodies (40).

Our genetic data indicate that patients with GPA have a greater prevalence of high expression (*i.e.*, > -794 CATT<sub>5</sub>) *MIF* genotypes when compared to healthy controls. These findings mirror the associations that have been reported previously between *MIF* polymorphisms and disease susceptibility or clinical severity in several autoimmune diseases, including systemic lupus erythematosus (13, 41), scleroderma (14), rheumatoid arthritis (15, 42), autoimmune liver disease (43), and celiac disease (44), as well as in granulomatous conditions such as sarcoidosis (6) and tuberculosis (25, 45). Furthermore, higher MIF plasma levels were noted in patients with GPA when compared to healthy controls, a finding previously reported in ANCA-associated vasculitis and other granulomatous diseases (8, 46, 47). Recent genome-wide association studies have identified several gene polymorphisms that are associated with susceptibility to GPA. These analyses have been limited to the study of selected SNPs however, and have not examined potentially important structural variants such as promoter microsatellites (48, 49). While patients with GPA had a greater prevalence of high expression *MIF* genotypes when compared to healthy controls, healthy controls also had a fair percentage of high expression MIF genotypes. We suspect that other genetic predispositions contribute to higher susceptibility for GPA by interacting with the *MIF* gene.

This study has several strengths and weaknesses. While there is no validation cohort for this candidate gene study, the sample size is large considering the rarity of GPA, and the results confirm an *a priori* hypothesis derived from a functional locus defined previously in studies of autoimmunity (13, 14, 50) and of unrelated granulomatous conditions (6, 25). Importantly, this study also provides the first concordance in findings between a GPA genetic susceptibility and a relevant mouse model of vasculitis. The exact cellular composition and molecular expression profile of the pulmonary granulomas observed in the mouse model remains to be examined to better define MIF-dependent pathways of granulomatogenesis.

In conclusion, MIF appears to play an important role in the susceptibility and pathogenesis of GPA. Therapies aimed at blocking MIF-directed pathogenesis pathways may offer a promising and precision medicine approach to treating vasculitis in high genotypic *MIF* expressers.

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## Abbreviations

<b>GPA</b>	granulomatosis with polyangiitis
<b>MIF</b>	macrophage migration inhibitory factor
<b>MIF</b>	human gene for macrophage migration inhibitory factor
<b>Mif</b>	mouse gene for macrophage migration inhibitory factor
<b>SNP</b>	single-nucleotide polymorphism

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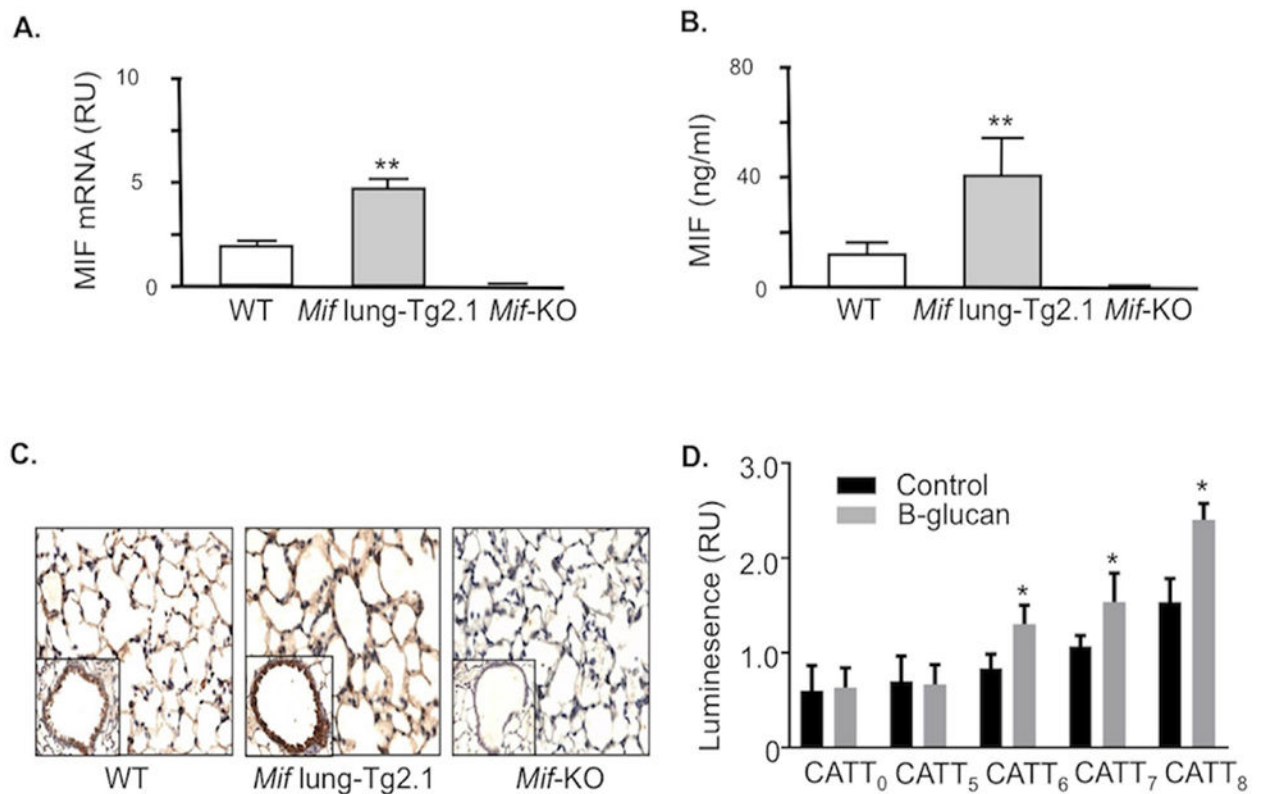
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**Figure 1. The human *MIF* -794 CATT<sub>5-8</sub> promoter polymorphism.**

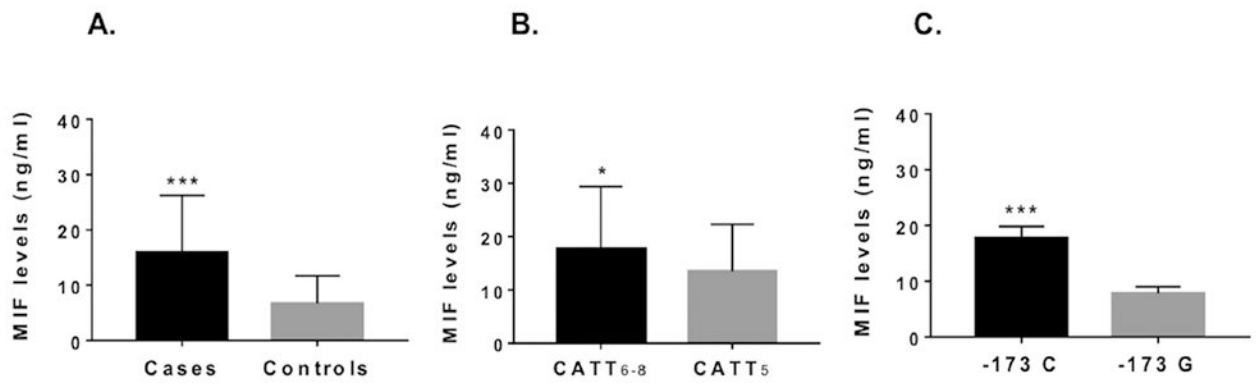
Schematic diagram of the human *MIF* gene with its three exons and the two studied promoter polymorphisms. The CATT<sub>5-8</sub> promoter microsatellite is the binding site for the ICBP90 transcription factor (15).



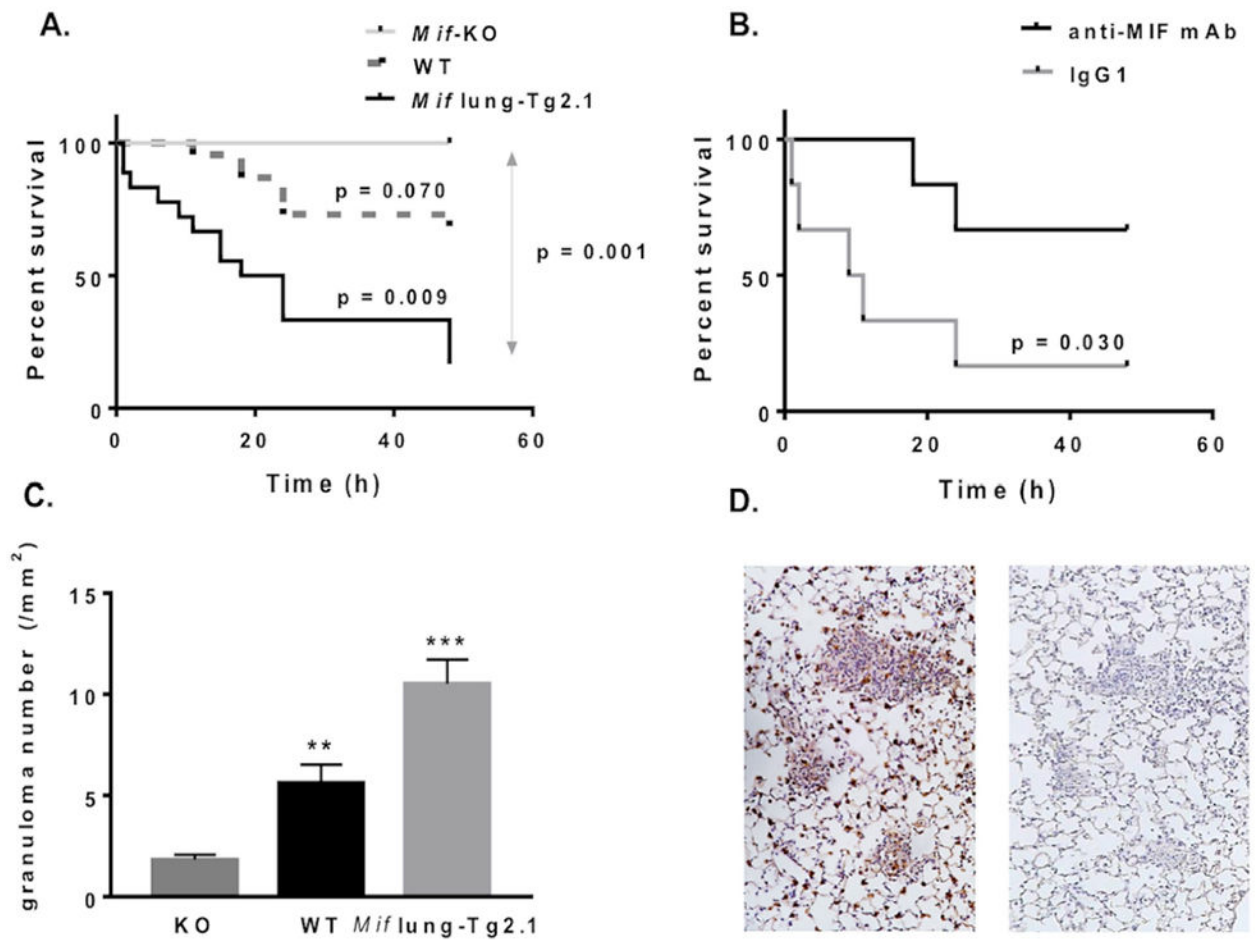


**Figure 2. MIF overexpression in *Mif*-lung Tg2.1 founder mice and regulation transcription by The *MIF* -794 CATT<sub>5-8</sub> promoter polymorphism in response to  $\beta$ -glucan in a CATT-length dependent manner.**

(A) Quantitative PCR analysis of *Mif* mRNA in lung tissue harvested from 3 representative wild type (WT), *Mif*lung-Tg2.1, and *Mif*-KO mice at 6 weeks of age. (B) ELISA analysis of MIF content in bronchoalveolar lavage fluid obtained from three mice of indicated strains. (C) Immunohistochemistry analysis of mouse lung tissue stained with anti-MIF pAb showing prominent staining in alveolar epithelium and increased MIF content in *Mif*lung-Tg2.1 mice. Tissue sections from a *Mif*-KO mouse lung was used as an antibody specificity control (200 $\times$ ). Insets show a representative terminal bronchus and sections are representative of three mice per genotype. \*\* $p < 0.01$  by Student's t test, two tailed. (D) Human pulmonary A549 cells transfected with MIF promoter/luciferase reporter fusion plasmids bearing 0, 5, 6, 7, and 8 CATT repeats were stimulated with 100 ng/ml  $\beta$ -glucan for 8 hrs and luciferase expression measured as luminescence relative units (RU). Mean  $\pm$  SD for quadruplicate determinations, replicated twice. \* $p < 0.05$  for comparisons to 5-CATT or to  $\beta$ -glucan stimulation versus control by Student's T test (two-tailed).

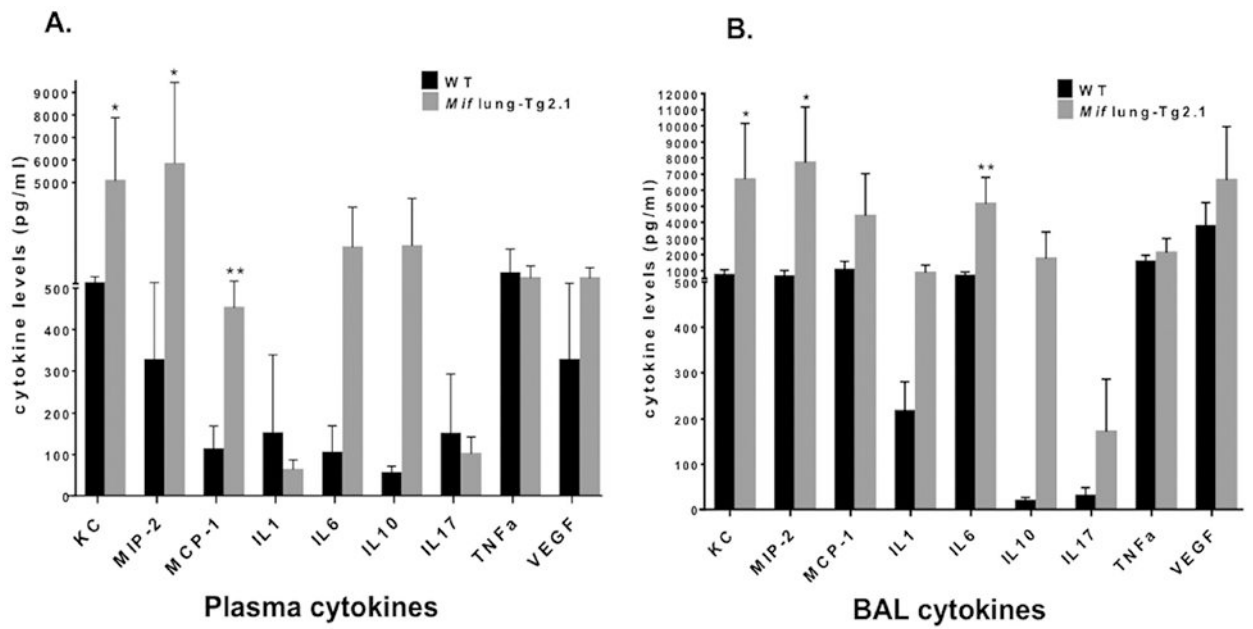


**Figure 3. MIF plasma levels in patients with granulomatosis with polyangiitis (GPA).** (A) Mean MIF plasma levels (ng/ml) in patients with GPA (n=73) and healthy controls (n=45). (B) Mean MIF plasma levels (ng/ml) in patients with GPA carrying high *MIF* expression - 794 CATT<sub>6-8</sub> containing genotypes (n=35) and patients carrying low *MIF* expression - 794 CATT<sub>5</sub> containing genotypes (n=38). (C) Mean MIF plasma levels (ng/ml) in patients with GPA carrying high *MIF* expression -173 C containing genotypes (n=23) and low *MIF* expression -173 G containing genotypes (n=50).



**Figure 4. MIF overexpression enhances  $\beta$ -glucan lethality and pulmonary granuloma.**

(A) Kaplan-Meier survival analysis of *Mif* lung-Tg2.1 transgenic mice, wild-type (WT), and *Mif*-knockout (*Mif*-KO) mice after intravenous injection of  $\beta$ -glucan (1 mg per mouse, iv). (B) Survival of *Mif* lung-Tg2.1 transgenic mice treated with anti-MIF mAb versus an isotypic control immunoglobulin (IgG<sub>1</sub>). Mice received a single i.p. injection of antibody (20 mg/kg, ip) at time 0. Data are compiled from 4 independent experiments, with A and B each replicated. A total of 44 mice were studied. (C) Granuloma density measured by histologic enumeration of mouse lungs obtained from *Mif*-KO, WT and *Mif* lung-Tg2.1 transgenic mice. Values represent 2 high power fields per section in >10 representative lung sections obtained from 36 mice. Values represent means  $\pm$  SEM. (D) Representative lung sections showing immunostaining for MIF in alveolar epithelial cells and infiltrating mononuclear cells in pulmonary granulomas in *Mif* lung-Tg2.1 mice. Sections were obtained 48 hrs after  $\beta$ -glucan injection and stained with anti-MIF (left panel) or control antibody (right panel). Magnification = 100 $\times$ . P values by Student's t test (two-tailed).



**Figure 5.** Cytokine levels (pg/ml) of KC (CXCL1), MIP-2 (CXCL2), MCP-1 (CCL2), IL-1, IL-6, IL-10, IL-17, TNF, and VEGF in wild type (WT) and *Miflung-Tg2.1* mice in plasma (A) and bronchoalveolar lavage (BAL) fluid (B). Samples were obtained 24–48 hrs after  $\beta$ -glucan injection. Data are mean  $\pm$  SD of cytokine levels in blood and BAL samples harvested from 8 WT mice and 4 *Miflung-Tg2.1* mice. P values by Student's t test (two-tailed).

**Table 1.**

Baseline demographics of study participants (patients with granulomatosis with polyangiitis and healthy controls) and percentages of *MIF* genotypes in each group.

	Cases (n=501)	Controls (n=576)	p-value
Female %	52	40	p<0.001
Age (Mean yrs $\pm$ SD)	52.8 $\pm$ 15.6	47.2 $\pm$ 15.7	p<0.001
- 173 GG (%)	65.7	59.8	
- 173 GC (%)	30.8	36.2	
- 173 CC (%)	3.5	4.0	
-173 C containing genotypes	34.2	40.2	0.210*
-794 CATT <sub>5/5</sub> (%)	5.6	8.2	
-794 CATT <sub>5/6</sub> (%)	26.9	29.0	
-794 CATT <sub>5/7</sub> (%)	6.8	8.9	
-794 CATT <sub>5/8</sub> (%)	0.4	0.0	
-794 CATT <sub>6/6</sub> (%)	41.0	36.6	
-794 CATT <sub>6/7</sub> (%)	17.5	15.7	
-794 CATT <sub>7/7</sub> (%)	0.2	0.5	
-794 CATT <sub>7/8</sub> (%)	1.6	1.1	
> -794 CATT <sub>5</sub> containing genotypes	60.2	53.9	0.049*

\* p adjusted for age and sex.