# MIF and D-DT are potential disease severity modifiers in male MS subjects

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**Little is known about mechanisms that drive the development of progressive multiple sclerosis (MS), although inflammatory factors, such as macrophage migration inhibitory factor (MIF), its homolog D-dopachrome tautomerase (D-DT), and their common receptor CD74 may contribute to disease worsening. Our findings demonstrate elevated MIF and D-DT levels in males with progressive disease compared with relapsing-remitting males (RRMS) and female MS subjects, with increased levels of CD74 in females vs. males with high MS disease severity. Furthermore, increased MIF and D-DT levels in males with progressive disease were significantly correlated with the presence of two high-expression promoter polymorphisms located in the *MIF* gene, a −794CATT$_{5-8}$ microsatellite repeat and a −173 G/C SNP. Conversely, mice lacking MIF or D-DT developed less-severe signs of experimental autoimmune encephalomyelitis, a murine model of MS, thus implicating both homologs as copathogenic contributors. These findings indicate that genetically controlled high MIF expression (and D-DT) promotes MS progression in males, suggesting that these two factors are sex-specific disease modifiers and raising the possibility that aggressive anti-MIF treatment of clinically isolated syndrome or RRMS males with a high-expresser genotype might slow or prevent the onset of progressive MS. Additionally, selective targeting of MIF:CD74 signaling might provide an effective, trackable therapeutic approach for MS subjects of both sexes.**

multiple sclerosis | disease modifier | sex differences

Multiple sclerosis (MS) is a chronic, immune-mediated demyelinating disease of the CNS (1–3). MS is categorized into subtypes according to its clinical course. Most patients diagnosed with MS begin with a relapsing-remitting course (RRMS). However, 10–15 y after disease onset, more than half of RRMS patients develop secondary progressive MS (SPMS). MS patients not initially diagnosed with RRMS have primary progressive MS (PPMS) in which there is gradual progression of impairment and disability from disease onset without an initial relapsing-remitting phase. Furthermore, while MS is two to three times more common in women than men, this ratio decreases with older age and there is an equal sex ratio among PPMS patients (4–6).

Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine that is secreted from various cell types in different tissues (7–13). It was suggested that during neuroinflammation, macrophages and microglia are a major source of MIF in the CNS (14). Niino et al. (15) demonstrated that the concentration of MIF in the cerebrospinal fluid of MS subjects was significantly elevated during disease relapse compared with healthy control subjects. In the experimental autoimmune encephalomyelitis (EAE) model, MIF was found to support EAE progression through its ability to activate and recruit CNS macrophages and microglia, which could impact CNS repair mechanisms during chronic EAE (16).

MIF expression is regulated by both genetic and epigenetic factors, such as posttranscriptional modification by histone deacetylases (17) and transcription factors such as ICBP90 (18). As for the genetic regulation of MIF expression, there are two functional polymorphisms located in the *MIF* gene: alleles of the −794 CATT$_{5-8}$ microsatellite repeat and the −173 G/C SNP have been reported to modulate *MIF* promoter activity and to correlate with MIF expression levels. *MIF* promoter activity is proportional to increased numbers of the CATT repeats at position −794, whereas the −173 C allele may be associated with increased *MIF* promoter activity by its linkage disequilibrium with the high-expression −794 CATT$_7$ variant (19).

CD74 (HLA-class II invariant chain) is a type II transmembrane glycoprotein containing a trimerization domain flanked by two highly unstructured regions (20). In addition to its role as a chaperone for MHC class II proteins, 2–5% of cellular CD74 is expressed on the cell surface independently of MHC class II (21).

## Significance

The biological processes that are involved in the progression of multiple sclerosis (MS) are far from complete. Macrophage migration inhibitory factor (MIF) and its homolog, D-dopachrome tautomerase (D-DT), are immunoregulatory cytokines known to be involved in the worsening of various autoimmune disorders. We demonstrate that genetically controlled high MIF expression (and D-DT) promotes MS progression in males, suggesting that these two factors are sex-specific disease modifiers. In addition, we show that MIF or D-DT deficiency ameliorates the disease severity of the murine model of MS. Our data suggest that targeting CD74, the common receptor for MIF and D-DT, with therapies such as partial MHC class II constructs could be therapeutically beneficial for inhibiting MS clinical progression in selected patients.

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CD74, in combination with CD44, CXCR2, or CXCR4, transduces MIF signaling (13, 22, 23), thus indicating an additional role in immune cell stimulation (23). We recently demonstrated enhanced CD74 cell surface expression on monocytes in mice with EAE, suggesting its involvement in disease course (24).

Recently, a second ligand for CD74 that is an ancestral homolog of MIF, called D-dopachrome tautomerase (D-DT, also known as MIF-2) was identified and reported (25–27). D-DT has similar properties and functions as MIF (26). Like MIF, D-DT has a vestigial enzymatic activity and catalyzes a similar reaction with model nonnaturally occurring substrates, such as D-dopachrome (28, 29). The human D-DT protein has only 35% identity with MIF [27% in mice (27)] and both proteins are expressed at equivalent levels in most tissues, with one report suggesting higher expression in the murine brain (30). However, the role of D-DT in MS and EAE has not yet been studied.

In the current study, we performed a comprehensive evaluation of the involvement of MIF, D-DT, and CD74 in MS by evaluating MIF and D-DT plasma levels, gene-expression levels, and frequencies of functional *MIF* promoter variants. In addition, we determined CD74 expression levels on $CD11b^+$ cells and CD74 gene-expression levels in peripheral blood mononuclear cells (PBMC) and brains of MS subjects and healthy controls (HC). Furthermore, we demonstrate the independent roles of MIF and D-DT as copathogenic factors in EAE.

## Results

**Patient Demographics.** Demographic data of the study participants and clinical characteristics are presented in Table 1. There was no statistical difference in the mean age between the HC, clinically isolated syndrome (CIS), and RRMS subjects, but the mean ages of these groups were expectedly significantly lower when compared with the SPMS and PPMS subjects ($P < 0.0001$), since the onset of progressive MS is usually at an older age. There was no statistical difference in the mean age between males and females in each of the different clinical groups. Also as expected, the disease severity in CIS and RRMS subjects as measured by the Expanded Disability Status Scale (EDSS) metric was significantly lower compared with SPMS and PPMS subjects ($P < 0.0001$). Disease severity did not differ between males and females with the same disease type. The majority of RRMS and SPMS subjects were treated with disease-modifying therapies (DMT), whereas only about one-third of the PPMS were treated with DMT. In addition, about 85% of the CIS subjects exhibited MRI abnormalities indicative of eventual development of MS.

**MIF and D-DT Levels in MS Subjects.** To date, only a few studies have evaluated the involvement of MIF in MS (15, 31, 32). Moreover, there are no available data about the role of the newly discovered MIF homolog, D-DT, in MS or other autoimmune diseases. We evaluated MIF and D-DT concentrations in the plasma of HC and MS subjects. MIF was significantly higher in the plasma of MS subjects compared with HC ($P = 0.041$) (Fig. 1*A*). Since MIF expression correlates with disease severity in unrelated autoimmune diseases (33, 34), SPMS subjects were grouped with PPMS subjects (e.g., progressive MS). Further analysis according to gender and disease type demonstrated that MIF plasma levels were significantly higher in male progressive MS subjects compared with CIS ($P = 0.004$) or RRMS subjects ($P = 0.047$), but only nominally higher compared with HC. MIF plasma levels did not differ strongly among disease subtypes in female MS subjects. In contrast, MIF plasma levels in male progressive MS subjects were significantly higher compared with female progressive MS subjects (mean of 56.1 ng/mL vs. 32.7 ng/mL, respectively; $P = 0.001$) (Fig. 1*B*). Similarly, analysis of MIF plasma levels of MS subjects according to their disease severity (EDSS) revealed that MIF concentration in the plasma of male MS subjects was significantly elevated in those with higher disease scores ($P = 0.011$), whereas for female MS subjects the observed regression was consistent with no trend ($P = 0.679$). Mean MIF concentrations across all disease severities in females were similar to those of male MS subjects with low severity (Fig. 1*C*).

D-DT levels were also somewhat elevated in MS subjects compared with HC, but this difference did not reach significance (Fig. 2*A*). Analysis of D-DT plasma levels according to sex did not reveal strong differences among the different groups, although the directions of the observed differences were similar to the MIF results (Fig. 2*B*). However, just as we observed with MIF concentrations, analysis of D-DT plasma levels in MS subjects according to their disease severity revealed that male MS subjects with higher disease scores tended to have significantly higher D-DT concentrations in plasma ($P = 0.017$), whereas female MS subjects showed no significant trend ($P = 0.642$), remaining on average at D-DT levels similar to those of males with low disease severity (Fig. 2*C*).

Previously, it was demonstrated that MIF is highly expressed in white matter brain tissues of MS subjects (16). We evaluated the relative mRNA expression levels of MIF and D-DT in brain tissues of two male and three female SPMS subjects. As demonstrated in Fig. 3*A*, RNA was isolated from 30 mg (~3 $mm^2$) of tissue from three representative white matter areas (for each brain slice). Real-time PCR analysis revealed that the expression of MIF and D-DT was significantly higher in brains of male SPMS relative to female SPMS subjects ($P = 0.007$ and $P = 0.006$, respectively).

Relative mRNA expression of MIF and D-DT levels in PBMC did not statistically differ between any of the groups (Fig. S1). We further demonstrated that MIF and D-DT mRNA expression

**Table 1. Characteristics of MS subjects and healthy controls**

| Characteristic | Sex | HC | CIS | RRMS | SPMS | PPMS |
|---|---|---|---|---|---|---|
| No. subjects | F | 25 | 22 | 52 | 50 | 30 |
| | M | 24 | 14 | 151 | 161 | 111 |
| Age ± SD | F | 47 ± 9 | 35 ± 12 | 42 ± 9 | 54 ± 8 | 53 ± 7 |
| | M | 41 ± 13 | 44 ± 7 | 39 ± 10 | 51 ± 6 | 52 ± 10 |
| Caucasian ethnicity | F | 96% | 95% | 100% | 98% | 100% |
| | M | 91% | 100% | 90% | 100% | 97% |
| Disease duration ± SD | F | N/A | 2.3 ± 2 | 10 ± 6 | 23 ± 11 | 9 ± 7 |
| | M | N/A | 3.85 ± 3 | 13 ± 12 | 18 ± 9 | 13 ± 6 |
| EDSS ± SD | F | N/A | 1.57 ± 0.9 | 2.76 ± 1.6 | 5.74 ± 1.2 | 5.1 ± 1.3 |
| | M | N/A | 1.81 ± 1.3 | 2.81 ± 1 | 5.68 ± 1.3 | 5.64 ± 1.5 |
| DMT | F | N/A | 36% | 95% | 76% | 33% |
| | M | N/A | 35% | 76% | 64% | 30% |

N/A, not applicable.

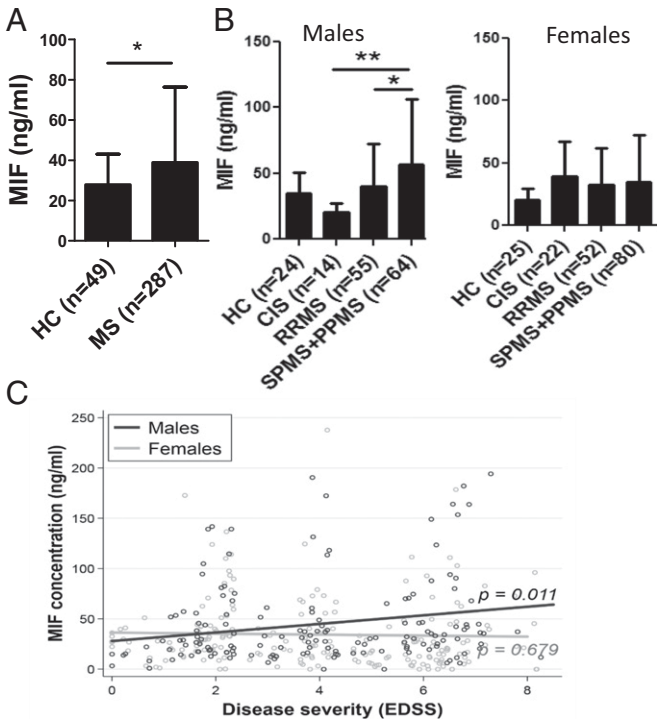


**Fig. 1.** Plasma levels of MIF in MS and HC subjects. MIF concentrations were evaluated in (*A*) all HC ($n$ = 49) and MS ($n$ = 287) subjects and stratified according to (*B*) gender and disease type or (*C*) gender and disease severity according to their EDSS scores. Data are presented as mean ± SD, $*P < 0.05$, $**P < 0.01$, one-way ANOVA with Tukey comparison (*A* and *B*) or $t$ test of coefficient in ordinary linear regression as a test of linear trend (*C*). Scatterplot points were jittered slightly along the EDSS axis for better visualization.

levels in all of the samples were highly correlated with each other ($r^2 = 0.732$, $P < 0.0001$), whereas MIF and D-DT mRNA levels were not as well correlated with CD74 expression levels ($r^2 = 0.03$, $P = 0.02$ and $r^2 = 0.01$, $P = 0.056$, respectively) (Fig. 3*B*). Taken together, our data suggest that expression of MIF vs. D-DT is strongly correlated and that each factor correlates with MS disease progression in male but not female subjects.

**MIF Promoter Polymorphisms.** The prevalence of the *MIF* −173 G/C SNP and −794 $CATT_{5–8}$ microsatellite polymorphisms was evaluated in male and female HC and MS subjects. Since *MIF* polymorphisms show population stratification (11), the genetic analyses were limited to samples from Caucasian patients. No deviation from Hardy–Weinberg equilibrium was detected for the polymorphisms in the male or female control groups ($CATT_{5–8}$ $P = 0.149$, G/C $P = 0.4386$ and $CATT_{5–8}$ $P = 0.537$, G/C $P = 0.587$, respectively). Although we observed a small but significant difference in MIF plasma levels between MS subjects compared with HCs, we did not observe any significant association between high-expression *MIF* genotypes in male and female MS subjects compared with sex-matched HC subjects (Table 2 and Table S1). However, since MIF was associated with EAE progression (16) and our data indicated that MIF plasma levels were elevated in male MS subjects with progressive disease, we evaluated *MIF* promoter polymorphisms between male RRMS subjects with disease duration over 10 or 15 y that are less likely to convert to progressive disease. As our original sample of RRMS subjects with prolonged disease duration was small, we genotyped and evaluated additional DNA samples of Caucasian males with MS obtained from the University of California, San Francisco MS repository. Disease scores of the RRMS subjects with prolonged disease duration were significantly lower compared with progressive subjects (EDSS = 3.95 vs. EDSS = 6.04, respectively; $P < 0.0001$). The frequencies of the −173 G/C SNP and the −794 $CATT_{5–8}$ genotypes are presented in Table 2. The frequency of the −173 C-containing allele, which is in linkage disequilibrium with the high-expression −794 $CATT_7$ allele, was significantly underrepresented in RRMS subjects with disease duration of more than 10 or 15 y compared with progressive MS subjects (21.6% or 20.5% vs. 31.9%, $P = 0.029$ and $P = 0.034$, respectively). The frequency of the −794 $CATT_7$ or the high-expression −794 $CATT_8$-containing genotype alone also was lower in RRMS subjects with long disease duration compared with progressive MS subjects (19.8% or 16.7% vs. 23.7%, $P = 0.121$ and $P = 0.247$, respectively), but this difference was within statistical error bounds, perhaps due to limitations in sample size. An analysis of inferred haplotypes showed that frequency of the combined high-expression genotypes (−173 GC/CC and −794 CATT6X/7X) was significantly lower in RRMS subjects with disease duration of more than 10 or 15 y, compared with progressive MS subjects, with odds ratios of 0.59 ($P = 0.029$) and 0.551 ($P = 0.034$), respectively (Table 3).

Interestingly, the frequency of the high-expression GC/CC and CATT6X/7X haplotypes was significantly lower in male RRMS subjects with disease duration of over 10 y, compared with the small sample size of female RRMS subjects with the same disease duration (21.6% vs. 44.0%, $P = 0.04$). Additionally, the frequency of the high-expression *MIF* haplotypes was higher in progressive male subjects compared with progressive female subjects, but not significantly (31.9% vs. 24.3%, respectively, $P = 0.30$). These results are consistent with the conclusion that the predominant influence of high-expression *MIF* genotypes is to affect disease progression in male MS subjects and not on overall disease susceptibility. It is important to note that to date, there are no available data on D-DT polymorphisms.

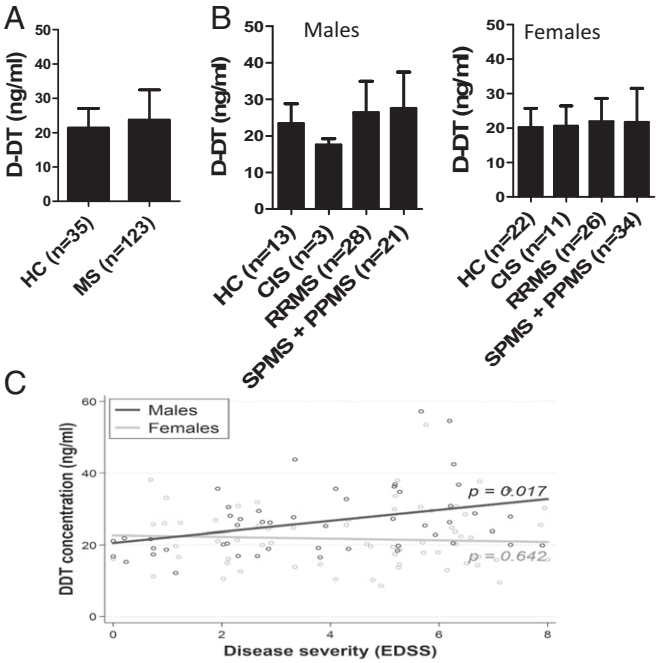


**Fig. 2.** Plasma levels of D-DT in MS and HC subjects. D-DT concentrations were evaluated in (*A*) all HC ($n$ = 35) and MS ($n$ = 123) subjects and stratified according to (*B*) gender and disease type or (*C*) gender and disease severity according to their EDSS scores. Data are presented as mean ± SD, analyzed for significant differences by one-way ANOVA with Tukey comparison (*A* and *B*) or $t$ test of coefficient in ordinary linear regression as a test of linear trend (*C*). Scatterplot points were jittered slightly along the EDSS axis for better visualization.

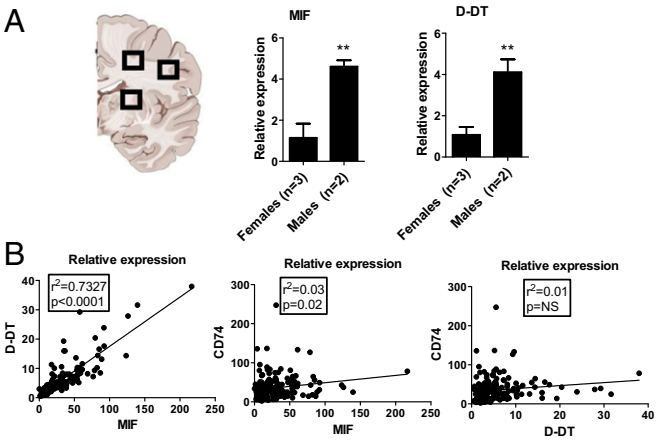


**Fig. 3.** MIF and D-DT relative expression in the brain and positive correlation between MIF and D-DT mRNA expression levels. (*A*) Relative mRNA expression levels of MIF, D-DT in three representative white matter areas of two males and three females SPMS subjects. Data are presented as mean ± SD, $^{**}P < 0.01$, Student's *t* test. (*B*) mRNA expression levels of MIF, D-DT and CD74 in PBMC of MS and HC subjects. Correlations were assessed by Spearman's correlation test.

**CD74 Cell Surface Levels in MS Subjects and the Effects of Partial MHC Class II Constructs.** Previously we demonstrated that CD74 cell surface expression on peripheral blood monocytes was up-regulated during EAE and in a small cohort of MS subjects compared with HC (24). To better evaluate CD74 cell surface expression in human monocytes, PBMC were evaluated from blood of a much larger cohort of HC and MS subjects. There was no significant difference in CD74 expression levels between HC and total MS subjects, or in MS males or MS females when stratified according to gender or disease type (Fig. 4 *A* and *B*). Evaluation of CD74 cell surface expression according to disease severity of MS subjects also did not reveal any significant association; rather, there was only a different trend line direction between males and females (Fig. 4*C*). However, females with high disease severity (EDSS > 5.5) had significantly higher CD74 levels than males with high disease severity (EDSS > 5.5; $P = 0.046$), suggesting a sex-dependent difference in subjects with advanced progressive MS. Relative CD74 mRNA expression levels in PBMC did not differ statistically between any of the groups (Fig. S1).

Partial MHC class II constructs bind to CD74 and competitively block MIF signal transduction, down-regulate CD74 cell surface expression, and ameliorate EAE (24, 35, 36). As shown in Fig. 4*D*, down-regulation of CD74 by the DRα1-human (h) MOG-35-55 construct also was affected by sex, with greater suppression in samples from males versus females ($P = 0.026$). Treating cells with the DR2β1 domain (as a control) did not significantly reduce CD74 surface expression on $CD11b^+$ cells in either males or females. Notably, the DRα1–(h)MOG-35-55 construct competed with both MIF and D-DT for binding to recombinant human CD74. This competition showed steep concentration-dependence and near-equivalence with respect to MIF versus its close homolog D-DT (Fig. 4*E*). These data suggest that partial MHC class II constructs are equally efficacious for inhibiting MIF vs. D-DT interaction with their common receptor, CD74.

**EAE in D-DT–KO and MIF-KO Mice.** MIF-KO mice exhibit acute EAE signs but no further progression of clinical disease, correlating with the role of CNS infiltrating monocytes in disease progression (16, 37, 38). However, the role of D-DT in EAE (or any other autoimmune disease model) has not been reported previously. To this end, we induced EAE in male and female C57BL/6 WT, MIF-KO and D-DT–KO mice and evaluated the disease course. As shown in Fig. 5, both male and female MIF-KO and D-DT–KO mice exhibited a significantly less-severe disease course compared with sex matched WT mice. Disease onset also was delayed in male D-DT–KO mice compared with WT, as seen in the mean daily scores for days 10–13 postimmunization ($P = 0.003$, $P = 0.005$, $P = 0.007$, and $P = 0.006$, respectively) and MIF-KO mice for days 10–13 postimmunization ($P = 0.008$, $P = 0.002$, $P = 0.001$, and $P = 0.041$). However, there was no difference in the cumulative disease index between MIF-KO and D-DT–KO mice in either sex. Both KO mice had significantly lower Cumulative Disease Index (CDI) compared with sex-matched WT mice.

Immunophenotypic analysis of male D-DT–KO, MIF-KO, and WT mice on day 18 postimmunization revealed that D-DT–KO and MIF-KO mice had a significantly lower frequency of activated $CD11b^+CD45^{hi}$ cells in spinal cords compared with WT

**Table 2. *MIF* promoter polymorphisms in male MS and HC subjects**

| *MIF* | Control *n* (%) | RRMS DS < 10 y *n* (%) | RRMS DS > 10 y *n* (%) | RRMS DS > 15 y *n* (%) | SPMS + PPMS *n* (%) |
|---|---|---|---|---|---|
| −173 SNP | | | | | |
| GG | 28 (63.6) | 18 (58.1) | 87(78.4) | 62 (79.5) | 184 (68.1) |
| GC | 15 (34.1) | 9 (29.0) | 20 (18.0) | 13 (16.7) | 79 (29.3) |
| CC | 1 (2.3) | 4 (12.9) | 4 (3.6) | 3 (3.8) | 7 (2.6) |
| −173*C containing genotypes | 16 (36.4) | 13 (41.9) | 24 (21.6)† | 16 (20.5)† | 86 (31.9) |
| −794 CATT | | | | | |
| 55 | 6 (15.4) | 2 (6.7) | 6 (5.4) | 4 (5.1) | 14 (5.2) |
| 56 | 8 (20.5) | 9 (30) | 40 (36.0) | 29 (37.2) | 85 (31.5) |
| 57 | 2 (5.1) | 0 | 7 (6.3) | 4 (5.1) | 11 (4.1) |
| 58 | 0 | 0 | 0 | 0 | 0 |
| 66 | 17 (43.6) | 13 (43.3) | 43 (38.7) | 32 (41.0) | 107 (39.6) |
| 67 | 6 (15.4) | 7 (23.3) | 11 (9.9) | 5 (6.4) | 50 (18.5) |
| 68 | 0 | 0 | 1 (0.9) | 1 (1.3) | 1 (0.4) |
| 77 | 0 | 1 (3.3) | 3 (2.7) | 3 (3.8) | 2 (0.7) |
| 78 | 0 | 0 | 0 | 0 | 0 |
| −794 $CATT_{7,8}$ containing genotypes | 8 (20.50) | 8 (26.6) | 22 (19.8) | 13 (16.7) | 64 (23.7) |

DS, disease duration.
†$P < 0.05$ compared with SPMS + PPMS group.

**Table 3. High expression *MIF* haplotypes in MS male subjects**

| *MIF* haplotype | RRMS DS > 10 or 15 y, % | SPMS + PPMS, % | Odds ratio (lower CI 95%) | *P* value Fisher exact one-tailed |
|---|---|---|---|---|
| CC/CG+ 6X/7X/8X | 21.6 (10 y) | 31.9 | 0.59 (<0.94) | 0.029 |
| CC/CG+ 6X/7X/8X | 20.5 (15 y) | 31.9 | 0.55 (<0.95) | 0.034 |

mice ($P = 0.0008$ and $P = 0.0002$, respectively). In addition, $CD11b^+$ cells in spinal cords of D-DT–KO and MIF-KO mice expressed lower levels of CD74 on their cell surface ($P = 0.0006$ and $P = 0.03$, respectively) (Fig. 6 *A* and *B*). Interestingly, D-DT–KO and MIF-KO mice with EAE had significantly larger spleens with increased numbers of splenocytes compared with WT mice ($P = 0.0084$ and $P = 0.0028$, respectively) (Fig. 6*C*). In addition, the frequencies of $CD4^+CD44^{hi}CD69^-$ cells ($P = 0.029$ and $P = 0.002$, respectively) and $CD11b^+CD74^+$ cells ($P = 0.035$ and $P = 0.006$, respectively) were significantly elevated in the spleens of D-DT–KO and MIF-KO mice compared with WT mice (Fig. 6*D*). Taken together, these data suggest that D-DT, like MIF, mediates EAE progression, and that D-DT absence is associated with reduced migration of memory and activated mononuclear cells from the periphery into the CNS.

## Discussion

Most patients with MS are diagnosed initially with the relapsing-remitting form of the disease before exhibiting continuous progressive disability over time (1–4, 39). Both inflammation and neurodegeneration may contribute to disease progression, although this process is still poorly understood. In the present study, we demonstrated that MIF and D-DT, and their common receptor CD74, affect disease severity and progression in a sex-dependent manner. In addition, we demonstrated the involvement of D-DT in autoimmunity.

MIF was one of the first cytokines to be described and is a key mediator of many autoimmune inflammatory diseases (8, 40). It was suggested that MIF contributes to the pathogenesis of EAE/MS by promoting leukocyte recruitment into the CNS, inhibiting apoptosis of activated monocytes, and enhancing the secretion of proinflammatory cytokines (16, 37, 41).

This comprehensive study evaluates functional *MIF* promoter polymorphisms and MIF expression levels in MS. Three prior studies evaluated MIF levels in the circulation or in active CNS lesions in MS subjects (16, 31, 32). Hagman et al. (31) demonstrated a positive correlation between MIF levels and EDSS score of MS subjects. Furthermore, MIF levels were shown to be increased in MS subjects with disability progression compared with subjects with stable disease.

Our human genetic data suggest that high genotypic MIF expression acts not as a disease susceptibility factor but rather as a disease modifier. Such a role for MIF was demonstrated previously in other autoimmune and inflammatory pathologies, including rheumatoid arthritis (19, 33), systemic sclerosis (42), systemic lupus erythematosus (34), and asthma (43). This observation is also in line with MIF's well-characterized role as an enhancer of inflammation in autoimmune diseases, although MIF genotype-dependent disease susceptibility also has been reported for several infectious diseases (44–47).

The analysis of *MIF* promoter polymorphisms in MS is consistent with this notion, and high genotypic *MIF* expressing male RRMS subjects may be more likely to convert to a progressive disease form compared with male RRMS subjects that carry low expression *MIF* genotypes. Although RRMS subjects with prolonged disease duration could still convert to SPMS, this usually

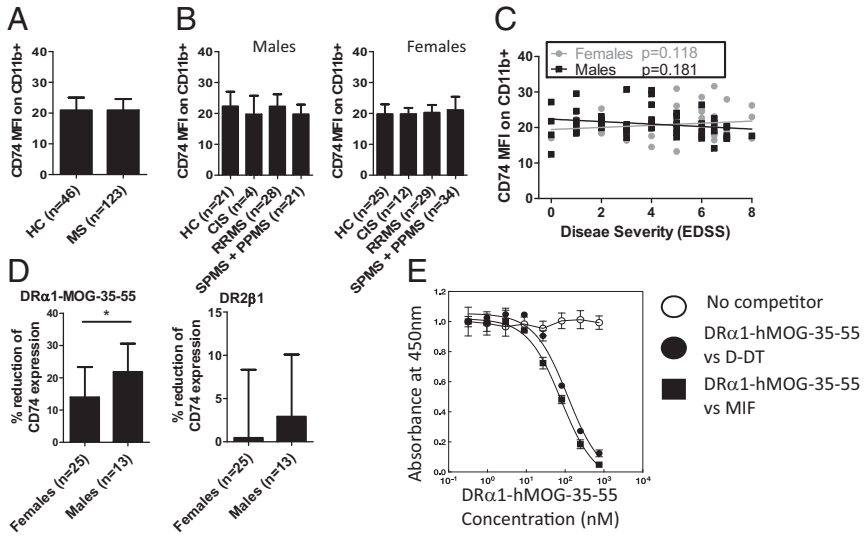


**Fig. 4.** CD74 cell surface expression on $CD11b^+$ cells of MS and HC subjects and inhibition of MIF and D-DT binding to recombinant human CD74 by the DRα1–hMOG-35-55 construct. CD74 cell surface mean fluorescent intensity on $CD11b^+$ cells was evaluated in (*A*) all HC ($n = 46$) and MS ($n = 123$) subjects and stratified according to (*B*) gender and disease type or (*C*) gender and disease severity according to their EDSS scores. Data are presented as mean ± SD, analyzed for significant differences by one-way ANOVA with Tukey comparison (*A* and *B*) or *t* test of coefficient in ordinary linear regression as a test of linear trend (*C*). (*D*) HC PBMCs were incubated with 5 μg of DRα1–hMOG-35-55 or DR2β1 constructs for 1 h and analyzed for CD74 expression on $CD11b^+$ monocytes. Data are presented as mean ± SD of reduction compared with untreated cells. *$P < 0.05$, Student's *t* test. (*E*) Serial 1:2 dilutions of DRα1–hMOG-35-55 were prepared in 10 nM rhCD74 and the mixtures were applied to wells previously coated overnight with 30 nM of either hMIF or hD-DT. IC50s were computed by fitting the data to a one-site competition using the Prism program (DeltaGraph). IC50 DRα1–hMOG-35-55 vs. hMIF = 78.5, $R^2 = 0.9720$; IC50 for DRα1–hMOG-35-55 for hD-DT = 116.5, $R^2 = 0.9821$.

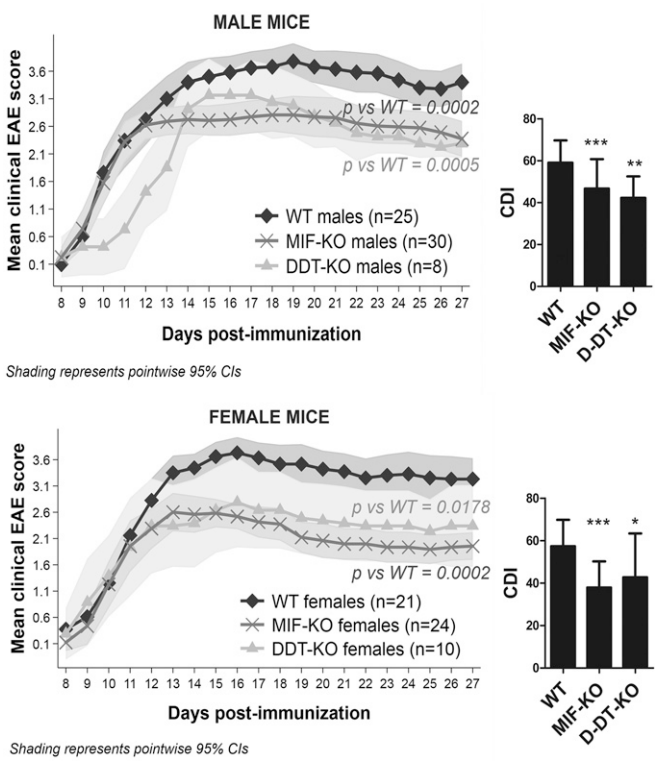


**Fig. 5.** EAE disease course in WT, D-DT–KO, and MIF-KO mice. EAE was induced with mouse (m)MOG-35-55 peptide in C57BL/6 WT, D-DT–KO, and MIF-KO mice. Mean clinical EAE daily disease scores (*Left*) and CDI scores (*Right*) are shown. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Daily mean score curves were compared using Fan and Lin's adaptive Neyman test (63) after discrete Fourier transformation of individual-level data, and CDIs were compared using Welch's one-sided *t* test after augmenting the within-group variances to include the CDI estimation error (64).

occurs within 10–15 y from disease onset. We did observe that the frequency of high-expressing *MIF* genotypes was slightly higher in RRMS subjects with disease duration of 10 y compared with 15 y. However, a larger study sample size would be needed to discern differences in RRMS subjects with different disease durations. It should be noted that two prior studies examined *MIF* polymorphisms in the Turkish population. One reported a protective role of low −794 CATT repeats (e.g., $CATT_{56}$) and a promotional role of the −173 C, which is in linkage disequilibrium with the high-expression −794 $CATT_7$, while a second smaller study that focused only on the −173 G/C SNP found no associations (48, 49). It is important to note that these studies focused mainly on RRMS subjects and did not evaluate males and females separately. Most of the RRMS subjects in our study were treated with DMT. While it is possible that DMT might differentially affect MIF plasma levels, our genetic findings, together with the gender differences in MIF plasma levels that were observed, support a role for MIF as a disease modifier. Concordance between MIF genotype and serum levels has been observed in some studies (33, 34) but not others (47). Generally, large and more clinically homogenous inflammatory syndromes have supported this relationship. However, we must emphasize that the serum compartment is an imperfect measure of MIF expression that may be genetically modulated at the site of inflammation.

Our human data also demonstrate a strong correlation between MIF and D-DT expression in plasma and CNS. Such correlation was demonstrated previously in sepsis patients and healthy subjects (50) in concert with MIF and D-DT cooperative regulation of lung carcinoma cells (51). The two proteins share 34% amino acid sequence identity and the common MIF superfamily protein fold (27). D-DT binds to CD74 with high affinity (D-DT: $K_D = 5.42 \times 10^{-9}$ M vs. MIF: $K_D = 1.40 \times 10^{-9}$ M) (27) and competes with MIF for binding to the CD74 receptor. Like MIF, D-DT triggers the CD74-dependent activation of ERK1/2 MAP kinase resulting in similar downstream proinflammatory signaling. Modeling studies predict three discrete binding regions between MIF or D-DT homotrimers with the CD74 homotrimer. Topologically, however, the location of the three CD74 binding regions for the D-DT homotrimer differs from that of the three MIF binding regions, with only a single functional D-DT binding site available per CD74 trimer (52).

Partial MHC class II constructs were previously shown to bind to CD74 through the DRα1 domain, block MIF binding, and down-regulate CD74 expression (24, 35). Herein, we also demonstrate that these constructs competitively inhibit D-DT binding to CD74. We also observed that the DRα1–MOG-35-55 construct induced a greater reduction of CD74 expression on $CD11b^+$ cells from male subjects compared with female subjects. Recently, we reported that effective treatment of chronic EAE in female mice with the DRα1–MOG-35-55 construct required higher doses compared with male mice. This effect was dependent upon estrogen signaling, through estrogen receptor (ER)α (53). Thus, it is possible that the sex differences that were observed in this study were affected by estrogen.

Whitacre and colleagues (37) were the first to describe reduced EAE progression in MIF-KO mice. Similar to MIF-KO mice, D-DT–KO mice developed acute EAE but showed less-severe disease progression compared with WT mice; the D-DT–KO mice also demonstrated delayed onset of disease compared with MIF-KO mice. This suggests that MIF and D-DT do not have entirely redundant effects in EAE, but that both of these factors contribute to disease progression. Our analyses also indicate that mice lacking D-DT had reduced migration of inflammatory cells into the CNS, but increased frequencies of activated monocytes and memory T cells in spleen, due perhaps to residual MIF-dependent inflammatory responses. Residual D-DT activity likely accounts for a similar pattern of responses as that observed in MIF-KO mice (16). To this point, Honigman et al. (30) demonstrated that D-DT, like MIF, is widely expressed in the brain parenchyma of adult mice (54). Taken together with our observation that both MIF and D-DT are expressed in white matter tissue of SPMS subjects, these findings indicate that like MIF, D-DT has a neuroinflammatory role and similarly affects cell migration. Nevertheless, additional studies are needed to more precisely clarify the roles of MIF and D-DT in MS, especially with respect to the mechanistic role of their common receptor CD74 and that of the noncognate chemokine receptors CXCR2 and CXCR4 (9). Furthermore, it would be interesting to evaluate whether increased MIF and D-DT levels in MS might promote the activation of $Ly6C^{hi}$ inflammatory monocytes similar to their effects described in parasite infections (55).

In conclusion, the combination of human genetic data and mouse modeling strongly implicate MIF and D-DT as disease modifiers in MS. High genotypic *MIF* expression likely underlies the elevated levels of MIF and additional downstream proinflammatory factors that enhance disease progression. These findings suggest that the MIF signaling pathway is a sex-specific, MS disease modifier, raising the possibility that pharmacologic MIF antagonism of CIS or RRMS males with a high *MIF* expresser genotype might slow or prevent development of progressive MS. Additionally, targeting CD74 signaling potentially could provide an effective, trackable therapeutic approach for MS subjects of both sexes. Thus, the coordinate targeting of MIF and D-DT with therapies such as partial MHC class II constructs could be therapeutically beneficial for inhibiting MS clinical progression in selected patients.

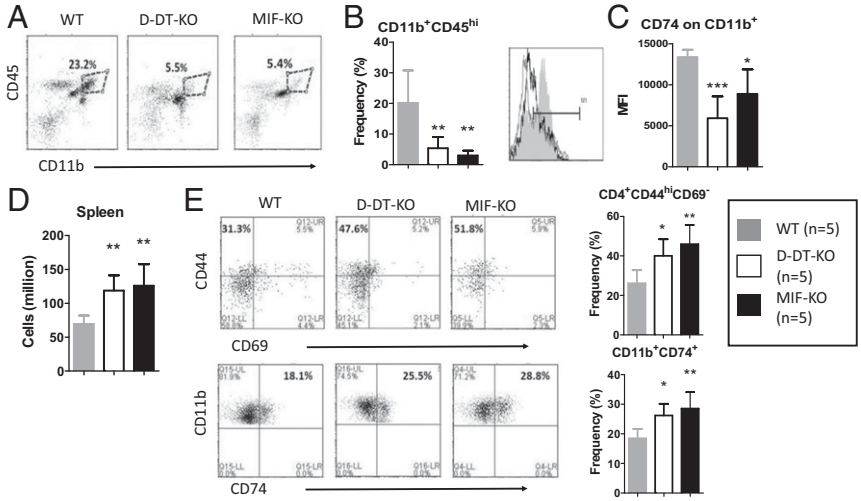


**Fig. 6.** Reduced CNS inflammation in D-DT–KO and MIF-KO mice. (*A* and *B*) Frequency of $CD11b^{+}CD45^{high}$ in spinal cords of WT, D-DT–KO, and MIF-KO male mice on day 18 postdisease induction. (*C*) CD74 cell surface expression (mean fluorescence intensity, MFI) on $CD11b^{+}$ cells in spinal cords. (*D*) Absolute cell numbers in spleen of WT, D-DT–KO, and MIF-KO mice. (*E*) Frequencies of $CD4^{+}CD44^{hi}CD69^{-}$ and $CD11b^{+}CD74^{+}$ cells in spleen. Data are presented as mean ± SD $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ one-way ANOVA with Tukey comparison.

## Patients and Methods

**Study Participants.** One-hundred seventeen participants (>18 y) were recruited at the Oregon Health & Science University (OHSU) MS Center. One-hundred seventy plasma and 474 DNA samples of MS subjects were also received from the University of California, San Francisco MS repository. Participants included subjects with clinically definite or laboratory-supported MS diagnosis according to Poser and MacDonald criteria, as well as subjects with CIS. Forty-nine HC were enrolled at OHSU. Exclusion criteria for controls included MS in family members or the presence of any autoimmune or chronic inflammatory condition. Participant ethnicity was determined by self-reporting. Over 90% of the study participants were of European ancestry.

**Analysis of Plasma MIF and D-DT Levels by ELISA.** Plasma MIF concentration was measured by the human MIF Quantikine ELISA kit (#DMF00B; R&D Systems) according to the manufacturer's instructions. Plasma D-DT concentration was measured by ELISA, as reported previously (27).

**Human Brain Tissue.** Frozen sections of five human CNS tissue specimens were obtained from the Rocky Mountain MS Center Tissue Bank. Postmortem intervals were between 7 and 42 h. Female subjects were 63, 64, and 68 y old and male subjects were 61 and 62 y old. All subjects were diagnosed with SPMS.

**Real-Time PCR.** Total RNA was isolated from CNS tissue or PBMC using an RNeasy cultured cell kit according to the manufacturer's instructions (Qiagen). Quantitative real-time PCR was performed using the StepOne Plus system with gene-on-demand assay products (Applied Biosystems) for TaqMan array mouse immune response or for CD74 (Assay ID: Hs00269961_m1), MIF (Assay ID: Hs00236988_g1), and D-DT (Assay ID: Hs00758115_m1). HPRT1 (Assay ID: Hs02800695_m1) and IPO8 (Assay ID: Hs00914057_M1) housekeeping genes were amplified as endogenous controls. Primers were used according to the manufacturer's instructions.

**MIF-173G/C Genotyping.** DNA was isolated from whole blood using a Blood & Cell Culture DNA Midi Kit (#13343; Qiagen). A 366-bp fragment of DNA containing the *MIF* −173 polymorphism (rs755622) was amplified using 200 ng of DNA, 0.5 mM of primers, and REDExtract-*N*-Amp PCR Ready Mix (#R4775; Sigma Aldrich). The PCR product was digested overnight using 0.4 units/μL of Alu I Restriction Enzyme (#R0137S; New England BioLabs) at 37 °C. The digested products were separated on a 3% agarose gel. The G allele contains one *Alu* restriction site and was cut into two fragments resulting in a 98- and a 268-bp band, while the C allele contains two *Alu* restriction sites and was cut into three fragments, resulting in 205-, 98-, and 63-bp bands.

**MIF −794 CATT5–8 Genotyping.** The *MIF −794 CATT*$_{5-8}$ microsatellite (*rs5844572*) was analyzed following the methodology described by Sreih et al. (34). [We note that neither the microsatellite repeat nor the nearby −173 G/C SNP is represented within the high-density genotyping chips used previously for genome-wide association studies in GPA (56, 57)]. Briefly, *MIF* −794 $CATT_{5-8}$ genotyping was carried out by PCR using a forward primer (5′-TGCAGGAACCAATACCCATAGG-3′) and a TET fluorescent reverse primer (TET lab5′-AATGGTAAACTCGGGGGAC-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 −794 $CATT_{5-8}$ repeat length was identified using Genotyper 3.7 software (Applied Biosystems). Although at least eight 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′UTR (47).

**Cell Surface CD74 Measurements.** PBMC from MS and HC subjects were isolated from whole blood using a Ficoll gradient and stored in liquid nitrogen. CD74 expression was measured using four-color flow cytometry. Frozen PBMC were thawed in 5% Stim Medium (RPMI 1640 media, 5% FBS, 2 mL 100× glycine, and 2 mL pyruvate) and suspended in Staining Solution (1× PBS and 5% BSA). Cells were stained with FITC-conjugated CD74 (Clone: MB731; BD), APC conjugated CD11b (clone: ICRF44; BioLegend), and 7-aminoactinomycin D (7-AAD; BD). Data were collected and analyzed on an Accuri C6 flow cytometer (BD).

**CD74 Down-Regulation Assay.** PBMCs were incubated in 5% Stim Media with 25 μg/mL DRα1-hMOG35-55, or DR2β1 proteins for 1 h at 37 °C. Cells were stained with APC-conjugated CD11b (clone:ICRF44; BioLegend) and PE-conjugated CD74 (Clone: LN-2; Santa Cruz) and 7-AAD (BD). Data were collected and analyzed on an Accuri C6 flow cytometer (BD).

**Competition Assay.** ELISA plates were coated with rhMIF or rhD-DT overnight at 4 °C and washed. After extensive washing, recombinant human (rh)CD74 (produced in our laboratory) at a constant concentration was combined with serially diluted DRα1–hMOG-35-55 concentrations in the competitive mixture and added to the wells for binding to coated rhMIF or hD-DT onto ELISA plate wells. Briefly, the production of the rhCD74 was built by using the N terminus and transmembrane domain of the mouse CD74 linked to the 135 residue extracellular domain of the human homolog encompassing residues 56 through 191. In addition, to produce a nondisulfide-linked polypeptide, the C residue at position 27 was substituted for an S residue. The recombinant protein was purified using standard purification protocols and tested with several monoclonal antibodies known to cross react with the wild-type human version. After binding, plates were washed again and bound rhCD74 was evaluated using a specific monoclonal anti-human CD74 antibody (PIN1). The reaction was allowed to initiate by adding the HRP substrate and stopped with 0.1 N HCl. The plate was read at 450 nm and the absorbance was plotted versus DRα1–hMOG-35-55 concentration. Curves were produced and analyzed using Prism and adjusted to a nonlinear regression for competitive

binding with the same program. Each point was performed by triplicate and the mean and SD were considered in the analysis.

**Mice.** C57BL/6 WT males and females were purchased from the Jackson Laboratory. MIF and D-DT–KO mice (C57BL/6) were produced at Yale (58, 59) and bred in-house at the Veterinary Medical Unit, VA Portland Health Care System. All mice were used at 8–12 wk of age.

**Induction of EAE.** Male and female mice between 8 and 12 wk of age were immunized subcutaneously at four sites on the flanks with 0.2 mL of an emulsion of 200 μg mouse (m) MOG-35-55 peptide and complete Freund's adjuvant containing 200 μg of heat-killed *Mycobacterium tuberculosis* H37RA (Difco) (60). In addition, mice were given Pertussis toxin (Ptx) from List Biological Laboratories on days 0 and 2 postimmunization (75 and 200 ng per mouse, respectively). Immunized mice were scored (not blindly) for clinical signs of EAE graded on a six-point scale of combined hindlimb and forelimb paralysis scores (60). For hindlimb scores: 0 = no signs; 0.5 = limp tail or mild hindlimb weakness (i.e., the mouse cannot resist inversion after a 90° turn of the base of the tail); 1 = limp tail and mild hindlimb weakness; 2 = limp tail and moderate hindlimb weakness (an inability of the mouse to rapidly right itself after inversion); 3 = limp tail and moderately severe hindlimb weakness (inability of the mouse to right itself after inversion and clear tilting of hind quarters to either side while walking); 4 = limp tail and severe hindlimb weakness (hind feet can move but drag more frequently than face forward); 5 = limp tail and paraplegia (no movement of hindlimbs). Front limb paralysis scores are either 0.5 for clear restriction of normal movement or 1 for complete forelimb paralysis. The combined score is the sum of the hindlimb score and the forelimb score. Rarely, there is mortality of mice with severe EAE, and in these cases mice are scored as a 6 for the remainder of the experiment. Mean EAE scores and SDs for mouse groups were calculated for each day from day 8 through day 27 postimmunization and summed for each mouse by numerically integrating the EAE score curve over the entire experiment (CDI, represents total disease load). A power analysis indicates that groups of at least six mice are sufficient to detect a difference of 2.0 EAE units with an SD of 1.0 and a power of 0.88.

**Flow Cytometry.** Four-color [FITC, phycoerythrin, (PE), 7-AAD, and allophycocyanin, (APC)] fluorescence flow cytometry analyses were performed to determine the phenotypes of cells following standard antibody staining procedures. Tissues were harvested from random mice in each group. For splenocytes, single-cell suspensions of spleens were prepared by homogenizing the tissue through a fine mesh screen. Cells were pelleted after lysis of red cells followed by washing twice with RPMI. Mononuclear cells from the spinal cord were isolated by Percoll gradient centrifugation as previously described (61). Cells from spleen or spinal cord were resuspended in staining medium (5% BSA, 1× PBS and 0.02% sodium azide) for FACS staining. Fc receptors were blocked with mouse Fc receptor-specific mAb (2.3G2; BD Pharmingen) before cell-surface staining. Data were collected and analyzed with Accuri C6 (BD Biosciences). All antibodies were purchased from BD PharMingen or eBioscience unless otherwise indicated. Cells were stained with a combination of fluorescent labeled antibodies to CD11b (clone: M1/70), CD74 (clone: In1), CD45 (clone: 30-F11), CD4 (clone: GK1.5), CD44 (clone: IM7), CD69 (clone: HI.2F3).

**Statistics.** Cohort data were reported using GraphPad Prism (v5.0) and expressed as the mean ± SD or percentage frequencies, and group comparisons were assessed by one-way ANOVA or Fisher's exact test, as appropriate. Sample size was supported by power analysis based on preliminary pilot data showing an effect size of approximately Cohen's $W = 0.22$ (62) on the association of genotype to MS disease type in males; with this sample size we anticipated power in excess of 95% to observe a similar effect in the current cohort. Evaluation of gender differences in linear trend of MIF and D-DT concentrations over increasing MS disease severity (as measured by the EDSS) was assessed by tests of the relevant interaction coefficients in ordinary linear regressions.

For MIF polymorphisms, Pearson's $\chi^2$ test was used to analyze Hardy–Weinberg equilibrium. Allele and genotype frequencies were compared using Fisher's exact test.

For nonparametric comparison between WT C57BL/6, MIF-KO, and D-DT–KO mice in the EAE experiments, daily EAE response values for each mouse were organized into a time curve for the mouse's follow-up and preprocessed using the discrete Fourier transform to decorrelate the values obtained on successive days; the sets of curves for each group of mice were then averaged in the frequency domain, and the group mean curves compared using Fan and Lin's adaptive Neyman test (63). Mean CDIs were compared between pairs of groups using Welch's one-sided $t$ test after augmenting the within-group variances to include the CDI estimation error (64). Tests with $P \leq 0.05$ were considered significant.

**Data Availability.** The datasets generated during or analyzed during the current study are available from the corresponding author upon request.

**Study Approvals.** The relevant Institutional Review Boards approved the study, including the Joint OHSU/VA Human Studies Institutional Review board and the VA Portland Institutional Animal Care and Use Committee. All subjects gave their signed informed consent to participate and Health Insurance Portability and Accountability Act notification was completed by each subject. All animal studies were approved and performed according to federal, state, and institutional guidelines.

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