Macrophage Migration Inhibitory Factor Potentiates Autoimmune-Mediated Neuroinflammation

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Macroencephalomyelitis (EAE) is a widely employed animal model for studying the mechanisms underlying MS pathogenesis. The progression of MS has been attributed to several mechanisms; however, increasing evidence suggests that microglia and macrophages play an essential role (6, 7). Experimental autoimmune encephalomyelitis (EAE) is a widely employed animal model for studying the mechanisms underlying MS pathogenesis. The generation of myelin-reactive T cells is an immunological hallmark of both EAE and MS (8). Pathogenic T cells traffic to the CNS; however, they represent only a small percentage of the CNS infiltrate in MS and EAE lesions (9). Rather, activated microglia and macrophages dominate inflammatory lesions and have been shown to contribute to disease progression through multiple mechanisms, including secretion of neurotoxic molecules and proinflammatory cytokines/chemokines, presentation of self Ag, and direct contribution to tissue damage and demyelination (7, 10). Although microglia and macrophages represent morphologically indistinguishable populations in the CNS, it is proposed that they have unique contributions to the CNS inflammatory response during EAE (11). Microglia are derived from myeloid progenitors and are resident in the CNS. Their activation has been shown to precede the development of EAE, suggesting that microglia participate in the early events leading to EAE, and several studies have demonstrated the clinical benefit of dampening microglial responses (6, 12). Recently, chronically activated microglia have been shown to negatively impact CNS repair mechanisms during chronic EAE (13). Importantly, chronically activated microglia have been identified in MS plaques, and are associated with local tissue damage (14). CNS infiltrating macrophages are also critical effector cells in EAE, because preventing their activity is highly effective at suppressing EAE (15–17). Thus, it is likely that both of these myeloid cell populations are involved in disease progression. Based on these studies, it is of therapeutic interest to study the processes that mediate myeloid effector cell function in the CNS and identify ways to attenuate chronic activation.

A body of evidence supports the fact that MIF is involved in proinflammatory mechanisms of myeloid cell function and, therefore, may serve as a therapeutic target for dampening cellular responses in the CNS. MIF was among the first cytokines to be discovered, and new insights into its role in inflammatory responses continue to be elucidated (18). Recently, a new member of the MIF superfamily, D-dopachrome tautomerase, a structural homolog dubbed MIF-2, has been described (19). MIF is a multipotent cytokine that is associated with clinical worsening and relapses in multiple sclerosis (MS) patients. The mechanism through which MIF promotes MS progression remains undefined. In this study, we identify a critical role for MIF in regulating CNS effector mechanisms necessary for the development of inflammatory pathology in a mouse model of MS, experimental autoimmune encephalomyelitis (EAE). Despite the ability to generate pathogenic myelin-specific immune responses peripherally, MIF-deficient mice have reduced EAE severity and exhibit less CNS inflammatory pathology, with a greater percentage of resting microglia and fewer infiltrating inflammatory macrophages. We demonstrate that MIF is essential for promoting microglial activation and production of the innate soluble mediators IL-1β, IL-6, TNF-α, and inducible NO synthase. We propose a novel role for MIF in inducing microglial C/EBP-β, a transcription factor shown to regulate myeloid cell function and play an important role in neuroinflammation. Intraspinal stereotaxic microinjection of MIF resulted in upregulation of inflammatory mediators in microglia, which was sufficient to restore EAE-mediated inflammatory pathology in MIF-deficient mice. To further implicate a role for MIF, we show that MIF is highly expressed in human active MS lesions. Thus, these results illustrate the ability of MIF to influence the CNS cellular and molecular inflammatory milieu during EAE and point to the therapeutic potential of targeting MIF in MS.
cytokine that exerts a variety of mitogenic and proinflammatory functions (20). MIF is constitutively expressed, stored intracellularly, and is rapidly released upon encountering activating stimuli. Once secreted, MIF has been shown to exert control over a variety of cellular responses, including cell survival, cytokine production in macrophages, and chemotactic responses (21–24). Because MIF-induced responses support sustained inflammation, evidence to date points to the detrimental role of MIF in autoimmune and chronic inflammatory disease. Neutralization of MIF by Ab depletion or genetic deletion has been shown to suppress inflammatory responses in a variety of murine models of human disease, including EAE (25–29). Currently, small-molecule inhibitors of MIF are being explored for the treatment of inflammatory and autoimmune diseases (29).

MIF is secreted from a variety of cellular sources, both in peripheral tissues and within the CNS (22, 30–32). Although much evidence supports a systemic role for MIF, there are few studies examining the activities of MIF in the context of neuroinflammation. Basal levels of MIF are detected in the uninfamed brain; however, expression is dramatically increased in the brain and CSF in response to inflammatory stimuli (31, 33). Specifically, in LPS-induced neuroinflammation, MIF expression localized with cells of the monocyte/macrophage lineage, indicating that infiltrating cells or microglia may be a significant source of MIF during inflammation (31). In MS, elevated levels of MIF were detected in the CSF of relapsing MS patients relative to samples collected during remission (34). Furthermore, a recent study concluded that increased serum levels of MIF were indicative of progressive disease (5).

In the current study, we sought to determine the contribution of MIF to the CNS inflammatory milieu and determine its potential to mediate disease progression in an animal model of MS. We show that MIF-deficient mice are fully capable of driving peripheral Th1 immune responses necessary for the generation of pathogenic T cells. However, MIF-deficient mice are protected from the transfer of pathogenic myelin oligodendrocyte glycoprotein (MOG)–specific cells, suggesting that protection from EAE occurs at the level of the CNS. We further show that MIF supports disease progression through its ability to skew the CNS inflammatory milieu and promote the activation and recruitment of CNS macrophages. Finally, we demonstrate that MIF creates a permissive environment for inflammation within the CNS, as intraspinal microinjection of MIF fully reconstitutes the ability of MIF-deficient mice to develop autoimmune-mediated inflammatory pathology.

Materials and Methods

Mice

Age-matched (8- to 10-wk-old) male C57BL/6 wild-type (WT) (Jackson ImmunoResearch Laboratories) and C57BL/6 MIF−/− mice were used (29, 33). MOG TCR transgenic mice on a C57BL/6 background (2D2) were a gift of V. Kuchroo (Center for Neurologic Disease, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA) (30). The 2D2 mice were crossed with MIF−/− mice to generate MIF−/− 2D2 mice for adoptive transfer studies. Offspring were genotyped using PCR to confirm deletion of mif. All 2D2 mice were screened using flow cytometric analysis on PBMCs using specific Abs to Vα3.2 and Vβ11 (BD Biosciences). Mice were maintained on a 12-h light/dark cycle and given food and water ad libitum. All mice were housed in specific pathogen-free conditions in individually ventilated cages and cared for according to Ohio State University Institutional Animal Care and Use Committee–approved protocols.

Antigens

MOG35–55 (MEVGYWRSPFSRVHLYRNGK) was purchased from C S Bio and was purified by HPLC (purity >95%).

Induction of EAE

C57BL/6 WT and MIF−/− mice were immunized s.c. over four sites on each of the flanks, with 0.1 ml of an emulsion containing 200 μg MOG35–55 in PBS mixed with an equal volume of CFA containing 200 μg heat-killed Mycobacterium tuberculosis, Jamaica strain. Pertussis toxin (250 ng in 0.2 ml PBS; List Biological Laboratories) was injected i.p. on the day of immunization and 2 d later. Mice were monitored daily for clinical signs of disease and were scored as follows: 0, no observable signs; 1, limp tail; 2, limp tail and ataxia; 3, paralysis of one hind limb; 4, complete hind limb paralysis; 5, death. EAE was also induced via adoptive transfer of 2D2 cells according to a protocol established by Williams et al. (37). Briefly, Th1-differentiated 2D2 cells were generated in culture with 20 μg/ml MOG35–55 and 0.5 mg/ml IL-12p70 (BD Pharmingen). After 48 h, cells were washed in PBS and transferred to naive, age-matched C57BL/6 WT and MIF−/− recipient mice.

Intraspinal stereotaxic microinjections

For analysis of MIF–vector (viral)–PBS-injected naive mice, serial sections of spinal cord tissue (2 mm rostral and caudal from the center of the microinjection site) were analyzed. For each animal, sections containing the largest area of macrophage/microglia activation (as determined by Iba-1 staining) were quantified using an Axiosplan 2 imaging microscope (Zeiss) with motorized X-Y-Z stage that was controlled by MCID Image Analysis quantification software. Within each section, the area of Iba-1–positive immunoreactivity was determined relative to the total area of the section (area demarcated with dashed line in Fig. 6A). Data are expressed as a proportional area (39). Similarly, Image J analysis software (National Institutes of Health) was used to determine C/EBP-β–positive immunoreactivity relative to the total sample area.

Isolation of CNS mononuclear cells

Spinal cords were homogenized, and CNS cells were separated from lipid/myelin debris with an OptiPrep gradient system (Fisher Scientific), as previously described (40). Briefly, spinal cords were mechanically dissociated with fine scissors in HBSS. Cells were recovered by centrifugation and enzymatically dissociated in 2.5 mg trypsin and 5 ml DMEM for 20 min at 37°C before trituration. DMEM supplemented with 10% FBS was added, and cells were filtered through a 70 μm cell strainer. Dissociated cells were pelleted, resuspended in HBSS, and layered over an OptiPrep gradient. The gradient was centrifuged at 450 × g for 15 min at 20°C, resulting in a cell pellet containing inflammatory and glial cells. Pelleted cells were washed and resuspended in media for immunolabeling.

Isolation and culture of primary microglia

Mixed glial cultures were generated from C57BL/6 WT and MIF−/− animals by day 1–3 mice. Microglia were removed from brains and cerebral cortices were minced and triturated, and then digested for 15 min at 37°C in SMEM containing 0.1% trypsin. Next, the cell mixture was triturated in DMEM supplemented with 10% FBS and DNase I (Sigma-Aldrich). Dissociated cells were centrifuged, resuspended in complete DMEM, and seeded in poly I-lysine (Sigma-Aldrich)–coated 75-cm² tissue culture flasks. Cells were maintained in DMEM containing 10% FBS with 1% glutamate and 1% Pen/Strep at 37°C in a 5% CO₂ humidified incubator for 7–10 d or until cells were confluent. Microglia were removed from the astrogial layer by shaking the flasks on an orbital shaker at 250 rpm for 15 h, followed by an additional 45 min at 300 rpm. Detached cells were collected and placed on tissue culture dishes for 30 min at 37°C, during which time microglia adhere. Nonadherent cells were removed after 30 min. Microglia (purity >97%, as determined by flow cytometry) were maintained in complete DMEM for at least 24 h before performing the indicated experiments. Primary WT microglia were incubated with medium alone or in the presence of MIF (10 or 100 ng/ml; Piramal Life Sciences) for 6 h prior to RNA isolation.

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Flow cytometry and intracellular cytokine staining

Flow cytometric analysis was performed to evaluate intracellular cytokine expression by Th1-differentiated 2D2 cells. Following 2D2 cell culture, BD GolgiPlug (brefeldin A; BD Pharmingen) was added to the culture for 3.5 h. Cells were labeled with PerCP-conjugated anti-CD4 mAb (BD Biosciences). After cells were washed and permeabilized (Cytofix/Cytoperm Kit; BD Biosciences), cells were labeled with the following mAbs: FITC T-bet (Santa Cruz Biotechnology), PE IL-17, and allophycocyanin IFN-γ (BD Pharmingen). OptiPrep gradient-isolated CNS cells were labeled with the following mAbs for surface phenotype analysis: CD45, CD11b, CD4, CD44, CD86, MHC class II, and CD40 (BD Pharmingen).

For ex vivo microglia/macrophage analysis, cells were suspended in DMEM containing 10% FBS and BD GolgiPlug for 5 h, fixed, permeabilized, and stained with allophycocyanin TNF-α. For ex vivo T cell analysis, cells were suspended in supplemented RPMI 1640 medium with Leukocyte Activation Cocktail with GolgiPlug (BD Pharmingen) for 5 h, fixed, permeabilized, and stained with allophycocyanin IFN-γ or FITC T-bet. For in vitro microglia studies, BD GolgiPlug was added for the last 5 h of culture, and cells were labeled with the following mAbs: V450 CD11b, allophycocyanin TNF-α, PE IL-6 (BD Pharmingen), and FITC MCP-1 (eBioscience). For intracellular analysis of C/EBP-α and C/EBP-β, primary microglial cells were fixed, permeabilized (IntraStain Kit; Dako), and labeled with primary Abs for C/EBP-α and C/EBP-β (Epitomics), followed by secondary staining with DyLight-488 and DyLight-405 (Epitomics). Data were acquired on a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Mouse tissue processing and immunohistochemistry

At various time points after spinal microinjection or EAE induction, mice were anesthetized and perfused intracardially with 100 ml PBS (pH 7.4), followed by 100 ml 4% paraformaldehyde. Perfused spinal cords were postfixed in 4% parafomaldehyde for 2 h, rinsed, and stored overnight at 4˚C in 0.2 mol/l phosphate buffer. Tissues were cryoprotected by immersing them in increasing concentrations of sucrose (10–30%) over several days. Spinal cords were blocked into 5-mm segments centered on the site of stereotactic microinjection, embedded in OCT compound, and frozen on dry ice. Cryostat sections (10 μm) were cut through each block using a Microm cryostat (HM 505 E), collected on Super Frost plus slides (Thermo Fisher Scientific), and stored at −20˚C until use. For immunohistochemical labeling, sections were washed in PBS, permeabilized with 0.1% Triton-X 100, and incubated overnight with primary Abs prepared in blocking solution. Primary Abs were used to label microglia/macrophages (ionized calcium-binding adapter molecule 1 [Iba-1]; 1:500; rabbit; Wako Chemicals, 1:500; rat; Serotec), T cells (CD3; rat; 1:100; BD Pharmingen), macrophage MIF (rabbit; 1:500; Abcam), or C/EBP-β (rabbit; 1:50; Epitomics). Alexa Fluor 488 (1:1000) or Alexa Fluor 546 (1:1000) conjugated secondary Abs were used to visualize primary Ab labeling. DAPI (Sigma-Aldrich) was added to secondary Abs to label cell nuclei. Other sections were stained for CD45 (1:4000; rat; Serotec) using peroxidase-based immunohistochemistry. Bone marrow was visualized using Elite-ABC reagent (Vector Laboratories) and diaminobenzidine as a substrate (Vector Laboratories). Confocal images were captured using an Olympus Fluoview FV1000 spectral confocal microscope.

Human tissue processing and immunohistochemistry

Human CNS tissue was obtained at autopsy according to an Institutional Review Board–approved protocol. Postmortem intervals were between 4 and 12 h. Brain tissue was stored frozen or formalin fixed for histological examination. CNS tissue was obtained from six subjects with MS and four subjects with nonneurologic diseases. Fresh-frozen blocks of CNS tissue were embedded in optimum cooling temperature medium using an acetone-dry ice bath and stored at −80˚C until used. Other tissue was fixed by immersing the brain in 10–20% formalin for 2–4 wk to obtain samples for paraffin embedding. Patient 1 was a 32- y-old woman, 4-y disease duration; patient 2 was a 42-y-old man, 6-y disease duration; patient 3 was a 38-y-old woman, 11-y disease duration; and patient 4 was a 31-y-old woman, 8-y disease duration. Three patients had secondary progressive MS and displayed chronic silent lesions only, as follows: patient 5 was a 59-y-old woman, 32-y disease duration, and patient 6 was a 69-y-old man, 10-y disease duration. Non-MS CNS tissue was from three patients dying from nonneurologic causes (myocardial infarct [three cases] and acute myeloblastic leukemia).

Lesion classification. MS lesions were classified histopathologically using H&E and Luxol fast blue staining and CD68 and glial fibrillary acidic protein (GFAP) immunolabeling. Cellular infiltration, demyelination, and presence of macrophages/microglia were used to classify lesions. Early active lesions were characterized by intense inflammation, demyelination, hypercellularity, edema, and astroglial hypertrophy, but no obvious fibrous astroglia. Chronic-active lesions were areas of long-term demyelination and gliosis with recent inflammatory activity, astroglial hypertrophy, and ongoing demyelination at the margins. Chronic silent lesions consisted of areas of demyelination and gliosis, but no inflammation.

Human tissue processing and immunohistochemistry. Frozen sections of OCT-embedded blocks were air dried and acetone fixed for 10 min. Paraflim-embedded sections were deparaffinized. After quenching with 0.03% hydrogen peroxide (10 min) and blocking with normal serum, sections were incubated with the following primary Abs overnight at 4˚C: mouse anti-CD68 mAb (macrophages/microglia; 1:100; Dako), anti-IFN-γ Ab (astrocyes; 1:100; Covance), anti–myelin basic protein (MBP) Ab (myelin; 1:16,000; Dako), and anti-MIF Ab (1:300; Invitrogen). Next, appropriate biotinylated secondary Abs were applied and visualized using Elite-ABC reagent and diaminobenzidine as a substrate.

RNA preparation and quantitative real-time PCR

RNA was extracted using a standard TRizol-based protocol, according to manufacturer’s instructions. For spinal cord microinjection studies, lumbar-thoracic spinal cord segments (5-mm segments centered on the microinjection site) were suspended in ice-cold TRizol (Invitrogen) and homogenized. Known concentrations of purified RNA were used for cDNA synthesis. cDNA was reverse transcribed using SuperScript II and random primers (Invitrogen) to obtain cDNA. Quantitative real-time PCR analysis was conducted by using specific primers for TNF-α, IL-1β, IL-6, inducible NO synthase (iNOS), MCP-1, MIF-1α, and MIP-1β, as previously described (41, 42). The following primers were used to amplify mRNA: C/EBP-α (forward, 5'-TGGCAAGAAGCAGCAACGATC-3'; reverse, 5'-GCAGTTGGCCCATGGCTTGAC-3'; forward, 5'-GTTTCCGGAGCTTGTAGCA-3'; reverse, 5'-CAACACCCCGGACGAAAC-3'). PCR cycles were carried out using 10 ng cDNA, 500 nM forward and reverse primers, and SYBR green master mix (Applied Biosystems). Each sample was analyzed in triplicate, and SYBR green fluorescence was detected using the Applied Biosystems 7900HT real-time PCR system. A standard curve was generated for each gene by using a cDNA dilution series, and melting point analysis was performed to confirm a single amplification product. Data were normalized to the 18S gene, and expressed as fold change from control-treated cells (medium) or tissue (PBS microinjection).

Statistical analysis

All data are graphed as mean ± SEM. A Mann-Whitney U test was used to determine significant differences in mean clinical disease score. A two-tailed Student t test was used to determine differences in mRNA expression, flow cytometric data, and immunohistochemistry data. A one-sample Student t test was used to detect statistical differences in proportional area or cEBP mRNA expression relative to a hypothetical value of 0 or 1, respectively. GraphPad Prism software was used to analyze data.

Results

MIF is required for the effector phase of EAE

To study the pathogenicity of MIF in autoimmune-mediated demyelinating disease, we compared the susceptibility of WT and MIF-deficient mice to EAE induction. Mice were immunized with MOG35–55 peptide in adjuvants, which produces progressive demyelinating disease, we compared the susceptibility of WT and MIF-deficient mice to EAE induction. Mice were immunized with MOG35–55 peptide in adjuvants, which produces progressive demyelinating disease. We have recently demonstrated that treatment with a small-molecule inhibitor of MIF attenuated EAE disease progression in C57BL/6 mice and prevented relapses in the PLP-induced SJL mouse model of relapsing-remitting MS, suggesting that MIF inhibitors may be efficacious in treating ongoing disease (29).

Adoptive transfer studies were used to dissect the role of MIF in the priming and differentiation of MOG-reactive T cell responses in the periphery as compared with its role in effector mechanisms in the CNS during EAE. For this study, splenocytes from MOG TCR transgenic (2D2) mice were used to adoptively transfer EAE following in vitro differentiation (36, 37). WT 2D2 mice were
CROSSED WITH MIF<sup>−/−</sup> MICE TO GENERATE MIF<sup>−/−</sup> 2D2 MICE. FOLLOWING CULTURE WITH MOG PEPTIDE UNDER TH1 POLARIZING CONDITIONS, 2D2 CELLS WERE ANALYZED FOR THEIR ABILITY TO GENERATE TH1 RESPONSES. IN SUPPORT OF OUR PREVIOUS STUDIES, BOTH WT AND MIF-DEFICIENT T CELLS SHOWED A SIMILAR CAPACITY TO PRODUCE IFN-γ AND UPREGULATE THE TH1 TRANSCRIPTION FACTOR T-BET (FIG. 1C, 1D). THUS, WE PREDICTED THAT BOTH WT AND MIF-DEFICIENT T CELLS WOULD EFFECTIVELY TRANSFER EAE. DIFFERENTIATED WT AND MIF-DEFICIENT 2D2 LYMPHOCYTES WERE TRANSFERRED INTO EITHER WT OR MIF-DEFICIENT RECIPIENT MICE, AND CLINICAL DISEASE COURSE WAS MONITORED. BOTH WT AND MIF-DEFICIENT T CELLS WERE CAPABLE OF TRANSFERRING EAE. HOWEVER, REGARDLESS OF THE PHENOTYPE OF THE TRANSFERRED DONOR CELLS, MIF-DEFICIENT RECIPIENT MICE SHOWED LESS SEVERE EAE (FIG. 1B), SUGGESTING THAT HOST-EXPRESSIONED MIF PLAYS A ROLE IN THE CNS INFLAMMATORY RESPONSE. COLLECTIVELY, THESE DATA INDICATE THAT MIF DEFICIENCY DOES NOT IMPAIR THE ABILITY TO DRIVE TH1 IMMUNE RESPONSES IN EAE, BUT RATHER CONTRIBUTES TO CNS EFFECTOR MECHANISMS IMPORTANT FOR THE PROGRESSION OF CLINICAL EAE.

MIF DRIVES THE ACTIVATION AND RECRUITMENT OF MYELOID CELLS TO THE CNS DURING EAE

The cellular composition of CNS infiltrates influences CNS inflammation and the clinical course of EAE (43). To explore the contribution of MIF to the CNS cellular milieu in EAE, spinal cords were isolated from WT and MIF-deficient immunized mice at peak EAE clinical disease (15 days postimmunization [dpi]). The percentage of CD45<sup>+</sup> cells, which includes both infiltrating lymphoid and myeloid populations as well as activated microglia, was dramatically reduced in the CNS of MIF-deficient mice (Fig. 2A). Additionally, the absolute number of CD45<sup>+</sup> cells isolated from the CNS at peak disease was significantly reduced in MIF-deficient mice relative to WT controls (Fig. 2B). We quantified distinct immune populations in the CNS during EAE in both groups. Resting microglia and macrophages both express CD11b; however, they differ in their relative expression of CD45, that is, resting microglia are CD45<sup>low/−</sup>/CD11b<sup>+</sup>, whereas inflammatory macrophages/activated microglia are CD45<sup>high</sup>/CD11b<sup>+</sup> (12, 44). Using these phenotypic criteria, the most abundant population in WT mice was inflammatory macrophages/activated microglia, whereas a greater percentage of resting microglia was isolated from the CNS of MIF-deficient mice with EAE (Fig. 2C). Similar proportions of lymphocytes (CD45<sup>high</sup>/CD11b<sup>−</sup>) were observed between groups (Fig. 2C). In support of previous studies, TNF-α expression in MIF-deficient CNS macrophages (CD45<sup>−</sup>/CD11b<sup>−</sup>) was reduced relative to the same population of WT cells (Fig. 2D).

The activation of CNS-resident microglia has previously been shown to provide an inflammatory milieu critical for maintaining T cell encephalitogenicity within the CNS. Subsequent to the effects of MIF on myeloid cells, we predicted the T cell component would also be affected. In the absence of MIF, reduced numbers of CD4<sup>+</sup> T cells are detected in the CNS at peak disease relative to WT controls (Fig. 3A, 3B). Furthermore, MIF-deficient mice exhibit dampened Th1 responses relative to WT mice. This is demonstrated by a reduction in intracellular IFN-γ and T-bet expression in CD4<sup>+</sup> T cells in the CNS of MIF-deficient mice (Fig. 3D, 3E). However, the expression of CD44, a marker of activated/memory T cells, was similar between groups, suggesting that MIF-deficient cells were effectively activated in the periphery (Fig. 3C). Notably, changes in immune cell trafficking associated with MIF deficiency were not accompanied by reduced markers of Ag presentation on CNS APCs (Supplemental Fig. 1). Collectively, these results implicate MIF in the activation of resident microglia and the recruitment of inflammatory macrophages, both effector mechanisms important for the progression of autoimmune-mediated neuroinflammation.
Cultured microglia are responsive to MIF

Several studies have implicated microglia as key cellular mediators of EAE pathogenesis (6, 45). To further investigate the inflammatory potential of MIF, primary microglia were stimulated with increasing concentrations of MIF, and their ability to alter gene expression was measured. RT-PCR analysis revealed dose-dependent changes in expression of several inflammatory mediators, as follows: TNF-α, IL-1β, IL-6, and iNOS (Fig. 4A–D). Confocal imaging of coverslips containing primary microglia revealed morphological differences between microglia treated with MIF versus media. Control cells had a ramified configuration with thinner processes, a morphology consistent with resting microglia, whereas MIF-treated microglia displayed fuller cell bodies with smaller processes, indicative of an activated and/or phagocytic phenotype (Fig. 4E). These changes were more pronounced with increasing doses of MIF (100 ng/ml MIF shown).

The diversity of MIF-mediated responses suggests that its activities are connected to a complex signaling cascade. Of interest, the C/EBP family of transcription factors has received increasing attention for their role in neuroinflammation and, more specifically, their ability to regulate myeloid cell effector function (42, 46–48). Interestingly, C/EBP-β gene expression was dose dependently increased in primary microglia following MIF treatment (Fig. 5A). In contrast, there was decreased expression of the C/EBP-α family member (Fig. 5B). To determine whether these changes in the transcriptional profile translated to changes at the protein level, we examined intracellular protein expression following MIF stimulation. Analysis revealed increased intracellular C/EBP-β protein expression in MIF-treated microglia, with no induction of C/EBP-α (Fig. 5C, 5D). These findings suggest a molecular mechanism by which MIF coordinates its inflammatory activities.

Intraspinal administration of MIF alone is capable of inducing neuroinflammation in the naive spinal cord

During MS and EAE, a dynamic CNS microenvironment controls the magnitude of CNS inflammation, making it difficult to detect...
The effects of individual cytokines. To study the direct contribution of MIF to neuroinflammation, we performed stereotaxic spinal cord microinjection of MIF into naïve spinal cords. When compared with vehicle injection (PBS), MIF microinjection resulted in a focal inflammatory response (Fig. 6A). Localized Iba-1 staining, indicating activation of resident microglia and/or recruitment of an inflammatory macrophage population, was quantified in microinjected tissue (Fig. 6B). Notably, no tissue damage was noted in the control (PBS)-microinjected group. To understand the molecular basis for these observations, we performed quantitative real-time PCR analysis of spinal cord mRNA from PBS- and MIF-microinjected tissue homogenates to determine the immune profile induced with MIF microinjection. A panel of inflammatory cytokines and chemokines known to influence MS and EAE pathogenesis was examined. Significant induction of MIP-1α, MIP-1β, IL-β, and TNF-α was observed in MIF-microinjected tissue relative to vehicle-injected control mice (Fig. 6C). Additionally, expression of MCP-1 was increased, but did not reach significance. Interestingly, this analysis did not reveal an increase in IL-6 or iNOS. Additionally, we have extended our analysis to examine C/EBP-β expression in vivo following MIF microinjection. C/EBP-β expression was significantly upregulated in MIF-microinjected tissue relative to vehicle-injected control mice and can be visualized in microglia/macrophages within inflammatory foci (Fig. 6D–F).

Together, these results demonstrate a proinflammatory role for MIF in the CNS and provide a molecular basis for the trafficking defect observed in MIF-deficient mice with EAE. We next explored whether MIF-induced changes in the CNS inflammatory profile conferred susceptibility to the development of inflammatory pathology during EAE. Could spinal microinjection of MIF restore autoimmune-mediated neuroinflammation in MIF-deficient mice?

**Spinal microinjection of MIF is sufficient to restore autoimmune-mediated neuroinflammation in MIF-deficient mice**

In light of our findings regarding the contribution of MIF to the CNS inflammatory milieu, along with the reported involvement of MIF in the regulation of immune cell trafficking in other organ-specific chronic inflammatory conditions, we next tested whether MIF microinjection could target EAE-mediated neuroinflammation to the CNS in MIF-deficient mice.

To test this hypothesis, we used a modified model of focal EAE adopted from Kerschensteiner et al. (49). This model is optimal for targeting EAE lesions to predetermined sites for their precise analysis and eliminating the disseminated inflammatory pathology that occurs with conventional EAE. By combining the suboptimal transfer of MIF-deficient 2D2 cells with local MIF spinal cord microinjection, we could observe whether MIF was capable of restoring inflammatory pathology in MIF-deficient mice with EAE.

During our initial experiments, we titrated the number of 2D2 cells that could be transferred to recipient mice without inducing clinical EAE. Based on these preliminary experiments, 5 × 10^6 MIF-deficient 2D2 cells were transferred to MIF-deficient recipients without inducing clinical signs of disease. Following the transfer of a suboptimal number of 2D2 cells, recipient mice were subjected to MIF spinal microinjection of the dorsal white matter, as described earlier. Spinal cords from recipient mice were isolated at designated time points for histopathological examination.

Our results indicated remarkable augmentation of neuroinflammatory pathology at the microinjection site 6 d post-2D2 cell transfer (4 d postmicroinjection), as visualized by CD45 staining (Fig. 7A). In the PBS-microinjected spinal cord, which served as control, no infiltrating cells were observed (Fig. 7A). Further characterization of MIF-induced inflammatory foci revealed that lesions were comprised of activated CNS macrophages, as well as T cells (Fig. 7B, 7C). Thus, MIF-targeted inflammatory lesions importantly share similar histopathological hallmarks with respect to EAE.

Additional histological assessment revealed that inflammatory lesions were not only targeted to the site of microinjection, but inflammatory foci could be visualized in areas distal to the site of microinjection (Fig. 7C), suggesting that the inflammatory signals initiated by MIF microinjection are propagated along the spinal cord. Inflammatory foci could be identified in tissue as far as 2 mm rostral and caudal to the point of microinjection, with the greatest pathology observed at the site of microinjection (Fig. 7E).

Furthermore, we examined spinal cord tissue at a later time point to determine whether we had induced a sustained inflammatory response. Notably, at 13 d post-2D2 transfer (11 d postmicroinjection), there was a marked reduction in the magnitude of inflammation at the site of microinjection (Fig. 7D). We attributed this resolution to the diminished bioavailability of MIF following a single microinjection. Using this model of focal EAE, we determined that MIF-dependent changes in the CNS microenvironment drive susceptibility to the development of inflammatory pathology, as a single MIF microinjection is capable of targeting inflammatory lesions to the CNS during EAE. Additionally, the loss of MIF bioavailability...
correlated with a decrease in inflammatory pathology, suggesting the importance of MIF for sustaining chronic inflammation in the CNS.

**MIF is highly expressed in active MS lesions**

Earlier studies led us to conclude that MIF functions at the level of the CNS to mediate progression of EAE. To determine the relevance of these findings to MS, we analyzed MIF expression in the brains of healthy controls and MS patients. We extended our analysis to include active and inactive white matter lesions, as well as cortical lesions as determined by MBP staining (Fig. 8A). Active white matter lesions were identified as areas of intense CD68-positive macrophages and GFAP-positive hypertrophic astrocytes (Fig. 8B, 8C). In contrast, inactive lesions demonstrated myelin loss, astrogliosis, and the virtual absence of CD68-positive cells (Fig. 8D, 8E). In tissues from MS patients, MIF expression was highly concentrated at the edge of active white matter lesions and predominantly associated with reactive hypertrophic GFAP+ astrocytes and macrophages, suggesting MIF may contribute to the actively demyelinating lesion (Fig. 8H). MIF expression was also present in astrocytes within the inactive center of white matter lesions (Fig. 8I), however absent from cortical lesions (Fig. 8I). Notably, no MIF expression was observed in normal-appearing white matter of MS patients and white matter from control brains (Fig. 8K, 8L). Collectively, these findings place MIF within areas of active inflammation in MS tissue with the potential to participate in the disease process.

**Discussion**

Increased MIF levels in serum and CSF have been shown to positively correlate with disease relapse and progression in MS patients (5, 34). Given this linkage to clinical worsening in MS and its macrophage-activating properties, it is suggested that MIF may enhance the inflammatory context of MS lesions. This study provides new insights into the contribution of MIF to the CNS inflammatory milieu during autoimmune-mediated neuroinflammation, and specifically highlights the ability of MIF to influence microglia and macrophage responses. Because aberrant microglia and macrophage activation is an immunological hallmark of several diseases of the CNS, our findings have applicability to other inflammatory neurodegenerative conditions.

MIF deficiency, whether achieved through genetic deletion or Ab neutralization, results in protection from EAE clinical disease (27–29). Several investigations have addressed the immunological basis for the effects of MIF on MS and EAE, and, although different potential mechanisms have been considered, most focus on the ability of MIF to influence adaptive immune responses. Denkinger
reactivity in each section was determined relative to the total sample area. Using Image J analysis software, the area of C/EBP-

Each group (MIF, EAE-/-) statistical evaluation of the experiment shown in (6). Data are expressed as a proportional area. Statistical evaluation of the experiment shown in (A) (mean ± SEM; *p < 0.05). (C and D) In a separate set of studies, spinal cords were isolated from MIF- or vehicle (PBS)-microinjected mice 48 h postmicroinjection for gene expression profile analysis. Relative gene expression of MCP-1, MIP-1α, MIP-1β, IL-1β, IL-6, iNOS, TNF-α, and C/EBP-β was determined by RT-PCR. Bar graphs show mean ± SEM of each group (MIF n = 9; PBS n = 6; *p < 0.05, **p < 0.01). (E) Tissue cross-sections from the experiment shown in (A) were used to visualize C/EBP-β staining in microinjected spinal cords (n = 3–5 per group, ×40). Using Image J analysis software, the area of C/EBP-β-positive immunoreactivity in each section was determined relative to the total sample area. (F) Statistical evaluation of the experiment shown in (E). Data are expressed as a proportional area (mean ± SEM; *p < 0.05).

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et al. (28) reported that, in the absence of MIF, fewer MOG-specific T cells access the CNS and exhibit an increased activation threshold. They attributed this finding to the reduced capacity of T cells to home to the CNS via their interaction with adhesion molecules on the blood–brain barrier (BBB). In a viral neurotropic infection model, MIF deficiency resulted in a reduced inflammatory response in the CNS, owing to its effects on the BBB (50). Recently, we demonstrated the clinical benefit of a small-molecule inhibitor of MIF, and found that it prevented leukocyte trafficking to the CNS and expanded a protective regulatory T lymphocyte population (29). Interestingly, we have shown that MIF deficiency mediates protective effects on EAE (6, 52, 53). The majority of cells isolated from MIF-deficient mice were CD45low resting microglia, suggesting that, in the absence of MIF, microglia fail to become sufficiently activated. This effect is most likely responsible for the reduced activation status of the T cell pool in the MIF-deficient CNS (54–56). Because activated microglia secrete a variety of inflammatory mediators, including TNF-α and macrophage-attracting chemokines, it is also likely that the dampened microglial responses contribute to the reduction of macrophage accumulation in the MIF-deficient CNS (7). We propose that MIF-mediated effects on trafficking are at least in part due to MIF’s ability to promote TNF-α expression in the CNS during EAE. Earlier studies showed that TNF-α supports an inflammatory milieu through its ability to promote adhesive properties of the BBB and production of monocyte chemoattractants. Furthermore, TNF-α can directly contribute to CNS pathology, as it has been shown to induce cytotoxicity in oligodendrocytes, the myelinating cell within the CNS (57).

To further assess the effects of MIF on microglial activation, the inflammatory profile of MIF-treated microglia in culture was examined. MIF treatment resulted in dramatic morphological changes in microglia consistent with cellular activation, further demonstrating the potent proinflammatory properties of MIF. The mitogenic effects of MIF have been extensively studied, and enhanced expression of several soluble mediators has been measured by macrophages in response to a variety of stimuli, including LPS and IFN-γ (22). In this study, we extend these findings to CNS-resident cells and report enhanced production of TNF-α, IL-1β, IL-6, and iNOS in response to MIF stimulation alone. Cytokine-directed tissue damage plays a pivotal role in mediating MS disease progression. We found that MIF promotes the differentiation of type I macrophages/microglia that are capable of mediating tissue damage and are characterized by the production of TNF-α and iNOS. Collectively, these data indicate that MIF is an upstream mediator of the production of tissue-destructive cytokines.
in microglia, and therefore, may represent a mechanism by which MIF promotes progressive disease. Interestingly, until recently, the beneficial effects of glatiramer acetate, an immunomodulatory MS drug, were not completely understood. This drug was thought to mediate a shift toward a protective Th2 response in MS patients. Macrophages have recently been proposed as the drug's target, because treatment dampens the production of inflammatory cytokines, including TNF-α, and promotes an anti-inflammatory type II macrophage phenotype (58). As repair processes are limited in the CNS, we propose that targeting MIF may represent a strategy for preventing the deleterious effects of activated myeloid cells within the CNS.

The diversity of cellular responses attributed to MIF suggests that its action is connected to a complex signaling network. MIF has been shown to engage several signaling pathways, including ERK1/2 MAPK and AP-1 pathways (59–61). Engagement of these pathways has been shown upon ligation with the MIF receptor, CD74 (61). There is an emerging role for the transcription factor C/EBP-β, as it has been shown to mediate mechanisms of neuroinflammation through its activity on myeloid cell function (42, 46–48). Of interest, C/EBP-β binding sites have been identified in the promoter regions of several inflammatory cytokines, including TNF-α, IL-1β, IL-6, and MIP-1α (42, 47). In the setting of adipogenesis, MIF has previously been shown to play a role in regulating adipocytes through modulation of C/EBP family members (62). Our observation that MIF induces C/EBP-β expression in microglia represents a novel pathway by which the myeloid-activating function of MIF may be potentiated. Notably, MIF treatment did not induce expression of the C/EBP-α family member; however, the reciprocal expression of these C/EBP family members has been previously described and may be attributed to functional overlap between family members (63, 64).

Elevated levels of MIF are detected in the CSF in MS as well as other inflammatory conditions, including Alzheimer's disease and CNS infection (34, 65, 66). Our findings in human MS tissue are consistent with previously published rodent literature, demonstrating that MIF is predominantly expressed in macrophages/microglia and can be taken up by astrocytes during pathological processes (22, 25, 31, 33). In light of these reports, along with our findings that MIF influences the development of EAE at the level of the CNS, we studied the potential of centrally secreted MIF to influence the CNS inflammatory milieu. Stereotaxic microinjections provide a useful means to study the site sp. act. of inflammatory mediators within the specialized CNS microenvironment. Our results showed that
intraspinal administration of MIF induces local activation of microglia/macrophages and polarizes a proinflammatory gene expression profile in naïve mice. Our studies demonstrate that MIF functions upstream of several inflammatory mediators that have been implicated in the pathogenesis of MS and EAE. Based on our earlier studies, we predict that microglia are the primary source of cytokines/chemokines following intraspinal microinjection of MIF; however, there is evidence that MIF can induce the expression of MCP-1 in microvascular endothelial cells (23). Additionally, a possible direct role for MIF in mediating chemotactic responses cannot be eliminated, as MIF has been shown to recruit both monocytes and T cells via its interaction with CXCR2 and CXCR4, respectively (24). Collectively, these findings provide a molecular basis for the trafficking defects exhibited by MIF-deficient mice and further implicate a central role for MIF in regulating CNS immune responses.

We further demonstrated the relevance of these findings in vivo by introducing MIF via intraspinal microinjection into an otherwise MIF-deficient EAE scenario. This system proved to be highly sensitive for detecting MIF-mediated effects on the development of CNS inflammatory pathology. MIF microinjection was sufficient to target EAE lesions to the spinal cord, suggesting that MIF-dependent changes in the CNS microenvironment drive susceptibility to the development of autoimmune-mediated inflammatory pathology. Similarly, it has been shown that stereotoxic microinjection of the proinflammatory cytokines IFN-γ and TNF-α targets EAE lesions to the CNS (49, 67, 68). However, because MIF has been shown to augment the production of IFN-γ and TNF-α, we propose that MIF functions upstream of many inflammatory mediators previously implicated in the development of autoimmune-mediated neuroinflammation. Upon examination of tissue at later time points, we noted a reduction in MIF-mediated inflammatory pathology, which we attributed to the diminished bioactivity following a single microinjection of MIF. These findings identify an additional role for MIF in sustaining immune-mediated inflammatory pathology in the CNS. It has previously been suggested that MIF may function to sustain inflammation through several mechanisms, including antagonizing the anti-inflammatory effects of glucocorticoids and preventing cellular apoptosis (21, 69). However, another likely possibility is that MIF, through its newly identified chemotactic properties, functions to retain cells at sites of inflammation.

An increasing body of evidence supports a role for MIF in coordinating the cellular response in target tissues during organ-specific autoimmunity. In this study, we extend these findings to the CNS and characterize the effects of MIF on the dynamic microenvironment during MS and EAE. Additionally, our studies have identified mechanisms of MIF that are broadly applicable. For example, it is conceivable that elevated levels of MIF resulting from CNS infection may provide a basis for infection-associated relapses that have been documented in MS patients (70, 71). Collectively, these findings attribute to MIF an essential role in mediating CNS effector function.

Conclusions

Our investigation provides new insights into the role of MIF in mediating microglia and macrophage responses during autoimmune-mediated neuroinflammation. Furthermore, these data suggest that the interplay between MIF and the inflammatory mediators released downstream of its secretion influences the population dynamics as well as activation status of inflammatory cells recruited to the CNS. In this way, MIF activates the inflammatory milieu and may dictate the composition and severity as well as the persistence of MS lesions. The outcomes of these studies point to the therapeutic potential of targeting MIF for the treatment of MS as well as other CNS pathologies with inflammatory components.

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Disclosures

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