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A Small-Molecule Macrophage Migration Inhibitory Factor Antagonist Protects against Glomerulonephritis in Lupus-Prone NZB/NZW F1 and MRL/lpr Mice

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Autoimmunity leads to the activation of innate effector pathways, proinflammatory cytokine production, and end-organ injury. Macrophage migration inhibitory factor (MIF) is an upstream activator of the innate response that mediates the recruitment and retention of monocytes via CD74 and associated chemokine receptors, and it has a role in the maintenance of B lymphocytes. High-expression MIF alleles also are associated with end-organ damage in different autoimmune diseases. We assessed the therapeutic efficacy of (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1), an orally bioavailable MIF antagonist, in two distinct models of systemic lupus erythematosus: the NZB/NZW F1 and the MRL/lpr mouse strains. ISO-1, like anti-MIF, inhibited the interaction between MIF and its receptor, CD74, and in each model of disease, it reduced functional and histological indices of glomerulonephritis, CD74+ and CXCR4+ leukocyte recruitment, and proinflammatory cytokine and chemokine expression. Neither autoantibody production nor T and B cell activation were significantly affected, pointing to the specificity of MIF antagonism in reducing excessive proinflammatory responses. These data highlight the feasibility of targeting the MIF–MIF receptor interaction by small-molecule antagonism and support the therapeutic value of downregulating MIF-dependent pathways of tissue damage in systemic lupus erythematosus. The Journal of Immunology, 2011, 186: 527–538.

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease that is characterized by the loss of immune tolerance and the production of autoantibodies to nucleic acids and nucleoproteins (1). Immunopathology results primarily from immune complex deposition in the small vessels of the skin, kidney, and other organs; this leads to the activation of complement and Ig Fc receptors and the recruitment of neutrophils and monocytes. Monocytes/macrophages are retained and persist within inflammatory sites, producing cytokines that propagate inflammatory tissue damage. In the kidney, for instance, infiltrating monocytes/macrophages are major constituents of the crescentic lesions that develop in rapidly progressive lupus nephritis, and their presence signifies severe glomerular injury (2).

Macrophage migration inhibitory factor (MIF) inhibits the movement or egress of macrophages, and it exerts an upstream role in regulating the innate immune response (3, 4). MIF is present preformed within monocytes/macrophages, and its rapid release results in the autocrine/paracrine activation of both immune and nonimmune cell types (5, 6). MIF counterregulates the immunosuppressive actions of glucocorticoids, and it promotes TNF-α and IL-1β production, leading to further MIF release and a reentrant activation response that supports the maximum expression of cytokines, matrix-degrading enzymes, and cyclooxygenases (3, 7, 8). Genetic knockout studies additionally have established an important role for MIF in inhibiting activation-induced apoptosis (9), which sustains monocyte/macrophage activation within inflammatory sites and contributes to the maintenance of mature immune cell populations (10–12). MIF signal transduction is initiated by high-affinity binding to CD74 (13). Recent studies indicate that MIF also may act as a noncognate ligand for the chemokine receptors CXCR2 and CXCR4; these proteins form complexes with CD74 and are necessary for MIF-driven atherogenic leukocyte recruitment (4, 14).

Evidence for a role of MIF in autoimmunity has been provided by studies showing that immunoneutralization or genetic deletion of MIF confers protection from pathologic progression in different experimental models of disease (15–17). MIF is known to be expressed in increased levels in the SLE-prone, MRL/MpJ-lpr mouse, and an intercross between this strain and mif−/− mice reduces glomerular injury and lethality (18). Both the circulating level and the tissue expression of MIF are elevated in patients with autoimmune inflammatory disorders, and high-expression MIF alleles have been associated with more severe end-organ damage in rheumatoid arthritis (19, 20), asthma (21), scleroderma (22), and with disease risk in SLE (23). Circulating levels of MIF are increased in patients with SLE and may correlate with indices of disease severity, renal dysfunction, and steroid resistance (24).
MIF is encoded by a unique gene, and crystallographic studies have revealed the protein to share structural homology with a class of prokaryotic tautomeres (25). Whereas in vitro studies have shown that MIF also tautomerizes model substrates (26), a physiologic role for this tautomerization activity has not been established. Indeed, genetic knock-in studies with a catalytically inactive MIF have led to the conclusion that enzymatic activity is a vestigial property of the protein that may have originated from the gene’s ancestral role in invertebrate immunity (27). The MIF tautomerase site nevertheless has been proposed to be an attractive entry point for the design of small molecules that might be targeted to the protein surface to inhibit receptor interaction, and proof-of-concept for this approach has been provided by the observation that covalent modification of MIF’s catalytic, N-terminal proline reduces both MIF bioactivity and its binding to target cell receptors (28, 29).

The investigation of new treatments for SLE remains challenging, and several recently developed biologic agents that are effective in other autoimmune disorders have not shown benefit in lupus (30). Given the unmet need for new therapeutic approaches in SLE, we tested the efficacy of a small-molecule MIF antagonist, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1), which binds to the MIF tautomerase site (31) in two different experimental models of SLE: the NZB/NZW F1 and the MRL/lpr mouse strains. In this study, we report that in each model of spontaneous lupus, treatment with ISO-1 reduced MIF-dependent proinflammatory cytokine production and leukocyte recruitment and ameliorated immune-mediated renal injury.

Materials and Methods

Reagents
ISO-1 was synthesized in three steps from 4-hydroxy-benzaldehyde by minor modifications of a previously reported procedure (32). The structure and purity of the synthetic product was verified by 1H-NMR and electrospray mass spectrometry (M’ = 236.1). N-acetyl-p-benzoquinone imine (NAPQI) was prepared as previously described (28). A neutralizing murine monoclonal anti-MIF IgG1, (NHIIID.9) (15, 33) was produced from ascites and an IgG1 isotypic control Ab (clone HB9) was obtained from American Type Culture Collection (Manassas, VA). Recombinant human and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified and mouse MIF were prepared as described by Bernhagen et al. (34), and the soluble MIF receptor ectodomain (CD74(23–232) = sCD74) was purified from an Escherichia coli expression system as previously reported (13).

MIF binding studies
For epitope mapping, individual human MIF 10-mer peptides were synthesized on polyethylene rods compatible with 96-well ELISA assays (35). The rod-coupled peptides were incubated in 96-well plates for 1 h with 1% BSA, 1% OVA, 0.1% Tween 20 in PBS, pH 7.4. Diluted anti-MIF or control Ab was incubated overnight with peptides in the 96-well plates at 4˚C and washed four times for 10 min in PBS with 0.05% Tween 20. Abs bound to peptide were detected with a peroxidase-coupled goat anti-rabbit IgG, the addition of substrate solution, and measurement of absorption at 405 nm (OD405).

The binding of MIF to the MIF receptor (CD74) was quantified by an in vitro competition assay employing immobilized MIF receptor ectodomain (CD74(23–232)) and 2 μg/ml biotinylated human MIF (13). The OD405 was measured after addition of test inhibitors and the values plotted as percentage OD405 relative to wells containing biotinylated human MIF alone.

Mice and study design
Female NZB/NZW F1 and MRL-Fas+/− (MRL/lpr) mice were obtained from Charles River (Wilmington, MA) and acclimated for 2 wk prior to study. All mice were maintained under specific pathogen-free conditions, and studies were performed in accordance with an approved institutional animal care and use committee protocol. Blood was obtained for baseline studies, following which the mice were divided into groups of 10–11 individuals. The NZB/NZW F1 mice were treated for 12 wk beginning at 22 wk of age, and the MRL/lpr mice were treated for 10 wk beginning at 9 wk of age. ISO-1 was administered in sterile 10% DMSO/H2O at a dose of 40 mg/kg by daily i.p. injection. Control mice received vehicle alone. Anti-MIF mAb or control IgG1 was administered i.p. in sterile saline at a dose of 20 mg/kg twice weekly. All mice were observed daily and weighed weekly for evidence of drug toxicity. Midway through the treatment protocol, blood was sampled from the retroorbital plexus for measurement of blood urea nitrogen (BUN), cytokines, and autoantibodies. At the completion of the studies, mice were euthanized by CO2 asphyxiation, blood sampled by cardiac puncture, and tissues removed and processed for flow cytometric, histologic, and mRNA and protein analysis.

Analyses for autoantibodies, cytokines, and urea nitrogen
Serum anti-dsDNA IgG Abs were measured by ELISA using S1 nucleosetreated DNA as described previously (36). A positive serum sample from a 20-wk-old MRL/lpr mouse was used as an internal control. MIF was measured using a murine-specific ELISA and native-sequenced recombinant mouse MIF as a standard (21). The IFN-α ELISA kit was from PBL Laboratories (Piscataway, NJ). The remaining cytokines were measured using a multicytokine bead master kit (Luminex, Billerica, MA). BUN levels were quantified by the Clinical Chemistry Laboratory of Yale–New Haven Hospital (New Haven, CT).

Renal histopathology and immunohistochemistry
To assess pathologic changes, kidney tissues were stained with H&E and with periodic acid–Schiff reagent, and numbered slices were evaluated by a pathologist (M.K.) blinded to the treatment protocol. Scoring was on a scale of 0 to 4+ and included the assessment of endocapillary proliferation, capillary loop thickening, leukocyte exudation, and glomerular necrosis (karyorrhexis, fibrinoid changes, cellular crescents, and hyaline deposits) (36, 37). Sections were examined in 8–10 individual kidneys from each treatment group. Ig deposition was assessed by immunofluorescence staining with anti-mouse IgG (A11001; Invitrogen, Carlsbad, CA). Slides were analyzed at the lowest positive dilution (1:25,000), and the fluorescence intensity within glomeruli was evaluated with ImageJ software (National Institutes of Health, Bethesda, MD) and expressed on a scale of 1–4 (38). Kidney tissue additionally was processed (n = 4 per group) and each section stained individually for MIF+ cells (anti-MIF R102) (39), F4/80+macrophages (clone BM8), CD3+T cells (anti-CD3; Abcam, Cambridge, MA) (40), CD74+ cells (clone sc-5438), and CXCR4+ cells (clone 247506; R&D Systems, Minneapolis, MN) (41). An avidin–biotin–HRP system or secondary Abs conjugated with fluorescent dyes were used, and nonimmune IgG was used as a specificity control. Immunoreactive cells were enumerated in ~50 glomeruli within at least four sections per experimental condition (33). The presence of interstitial nephritis was assessed by enumerating F4/80+macrophages in at least 20 grid-defined (100× magnification) fields of renal interstitium.

Flow cytometry analysis
Spleen and cervical lymph nodes were harvested, weighed, cleared of erythrocytes, and the cells pooled from individual mice for phenotypic analysis using four-color flow cytometry (FACS-Calibur; BD Biosciences, San Jose, CA), commercially available Abs, and FlowJo software (Tree Star, Ashland, OR) as previously described (36).

Quantitative PCR analysis
RNA was extracted from frozen tissue samples using the RNeasy extraction kit (Qiagen, Valencia, CA), and cDNA was synthesized from 1 μg RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR was carried out with the iQ SYBR Green system (Bio-Rad) using previously published primers (21, 42). The emitted fluorescence for each reaction was measured during the annealing/extension phase, and relative quantity values were calculated by the standard curve method. The quantity value of GAPDH in each sample was used as a normalizing control. Differences were evaluated by non-parametric testing using the Mann–Whitney U test.

Transcriptome analysis
Total RNA from kidneys (three samples per experimental group) was isolated using RNeasy miniprep columns (Qiagen), and labeling and hybridization were performed with the Genisphere (Hatfield, PA) Array900 Expression Array Detection kit (http://www.genisphere.com/array_detection_900.html) according to the manufacturer’s protocol. The OMM25K oligonucleotide gene array set (18,000 genes) from Yale University Keck Facility (New Haven, CT) was used (http://keck.med.yale.edu/microarray/s), and the cDNA probe and the fluorescent 3DNA reagent were hybridized to the microarray in succession. Hybridization was performed with an Advalytix Slide Booster hybridization.
MIF is expressed in elevated levels in lupus-prone mice

In preparation for studying the potential therapeutic effect of MIF inhibition, we examined MIF expression in two experimental models of lupus, the NZB/NZW F1 and the MRL/lpr mouse strains. The NZB/NZW F1 mouse strain is a useful model for autoimmune B cell and T cell interactions and for the time-dependent diversification of the autoimmune response. A progressive serum autoantibody response results in the development of severe nephritis at 24–48 wk of age. The MRL/lpr mouse develops a lymphoproliferative autoimmune syndrome that mimics several features of SLE; these include a similar spectrum of autoantibodies and an immune complex glomerulonephritis that develops over 12–24 wk of age (44).

Both the NZB/NZW F1 and the MRL/lpr mice manifest a time-dependent elevation in circulating MIF at ages that correspond with disease progression and the development of glomerulonephritis (Fig. 2) (45). MIF mRNA and protein expression in kidneys also increased significantly with inflammatory progression in the

FIGURE 1. Pharmacologic targeting of the MIF N-terminal region. A, Competition studies of MIF interaction with its receptor in the presence of NAPQI, ISO-1, or anti-MIF. The MIF receptor ectodomain (CD74^{73–232}) was immobilized in 96 wells and recombinant MIF added together with increasing concentrations of antagonists as described in Materials and Methods. Data are shown for a biologically neutralizing anti-MIF IgG1 (15). Symbols depict means of quadruplicate measurements, and lines show log regression analyses. No influence of vehicle or control IgG1 was observed (data not shown). B, Amino acid epitope scan of human MIF performed by reacting a neutralizing anti-MIF polyclonal Ab (15) with sequential peptide 10-mers (each offset by two residues). Peptide 4 shows the highest reactivity and corresponds with MIF^{--15} (NTNVPRASVP). The inset shows the MIF primary sequence. C, Simulated view of the immunoreactive epitopes superimposed on the MIF homotrimer. Axial and lateral views of MIF are shown with the model dopachrome tautomerase substrate, D-dopachrome methyl ester (blue space-filling model). The epitopes defined by peptides 2–6 and 23–26 are in red and green in one subunit, revealing their position adjacent to the tautomerase site.
Previously described (45). MIF protein and cell count data are expressed as mean ± SD, with p values calculated by two-tailed Student t test. *p < 0.005; **p < 0.001.

![FIGURE 2](image)

**FIGURE 2.** Plasma and intrarenal MIF expression increases during disease progression in lupus-prone mice. Plasma and renal MIF was assessed in NZB/NZW F1 (upper panels) and MRL/lpr (lower panels) mice. MIF levels in plasma and renal tissue lysates were measured by specific ELISA and renal MIF mRNA by qPCR (n = 4 per experimental group; see Materials and Methods). Plasma samples were obtained at the ages shown and compared with plasma from healthy, C57BL/6 mice. Renal histologic sections were stained with anti-MIF (R102) and the MIF-positive cells enumerated within 50 individual glomeruli of representative kidney sections (n = 4 kidneys per group). Inset images show MIF immunostaining within a representative glomerulus of an NZB/NZW F1 mouse at 22 and 34 wk (original magnification ×200). Diffuse staining for MIF within proximal tubular epithelial cells also is evident, as previously described (45). MIF protein and cell count data are expressed as mean ± SD, with p values calculated by two-tailed Student t test. For the qPCR data, the upper and lower edges of the boxes represent the 75th and 25th percentiles, respectively, the error bars denote the range of observations, and the horizontal line shows the median, with p values calculated by the Mann–Whitney U test. *p < 0.005; **p < 0.001.

Pharmacologic inhibition of MIF attenuates renal dysfunction and glomerulonephritis in lupus-prone mice

We initiated a therapeutic trial of ISO-1 and anti-MIF mAb in the NZB/NZW F1 and the MRL/lpr mouse strains. Mice were divided into groups (n = 10 to 11 per group) and treated daily with ISO-1 or its vehicle, or twice weekly with anti-MIF or an isotypic control (IgG1) Ab. In NZB/NZW F1 mice, anti-dsDNA autoantibodies become detectable in the circulation at ∼24 wk of age, and renal disease may be detected at 28 wk. Treatment was begun at 22 wk, which is prior to the onset of nephritis, and continued until 34 wk of age to encompass the period of autoimmunity. ISO-1 or anti-MIF mAb were well-tolerated, and treatment for up to 12 wk was not associated with any evident toxicity or change in body weight in the animals compared with vehicle-treated controls (data not shown). NZB/NZW F1 mice treated with ISO-1 or anti-MIF showed a significant reduction in the progressive rise in serum BUN, which is a sensitive indicator of renal function (Fig. 3A). Renal tissue that was examined at the end of the treatment protocol and scored histologically for glomerular damage confirmed that ISO-1 or anti-MIF ameliorated the development of glomerulonephritis (Fig. 3B, 3C). The kidneys from treated mice showed a decrease in glomerular crescents and karyorrhexis. Neither of the MIF antagonists reduced the glomerular deposition of circulating Ig (Fig. 3D). MIF inhibition did reduce interstitial inflammation, as assessed by the enumeration of F4/80+ macrophages (Fig. 3E, 3F), and it reduced the intraglomerular content of infiltrating F4/80+ macrophages, CD3+ T cells, and cells expressing MIF (Fig. 3G). A corresponding decrease in the glomerular content of infiltrating MIF receptor-positive (CD74+, CXCR4+) cells also was observed.

For the study of the MRL/lpr mice, treatment was initiated at 9 wk of age, continued for 10 wk, and terminated when mice reached 19 wk of age. Circulating anti-DNA Abs become evident in MRL/lpr mice at 9 wk, and their appearance precedes the development of glomerulonephritis (44). ISO-1 reduced the time-dependent increase in renal insufficiency and histologic indices of renal damage (Fig. 4A–C). Although anti-MIF also ameliorated the development of glomerular disease, its effect was not significantly different from that observed by treatment with control IgG1. A protective action of nonspecific IgG on renal immunopathology was observed, which may reflect an immunosuppressive action by IgG on FcR ITIM signaling or by depletion of serum complement (46, 47). The not-quite significant effect of anti-MIF mAb in MRL/lpr mice also may have resulted from an insufficient dose of anti-MIF in this model of renal immunopathology. Representative periodic acid–Schiff stained sections of kidneys nevertheless illustrate the reduction in glomerular cellularity, focal and segmental lesions, and glomerulosclerosis that was evident in the ISO-1–treated MRL/lpr mice compared with vehicle controls. As in the case of NZB/NZW F1 mice, ISO-1 treatment of MRL/lpr mice was unaccompanied by a reduction in immune complex...
FIGURE 3. ISO-1 or anti-MIF ameliorates glomerulonephritis in NZB/NZW F1 mice. A, Serum BUN levels in mice treated with ISO-1, anti-MIF mAb, or controls (vehicle or IgG1). Blood was sampled in four mice per group before treatment (22 wk), midway through treatment (28 wk), and at the end of the treatment protocol (34 wk). B, Glomerulonephritis scores (0 to 4+) reflecting indices of cellularity, focal and segmental lesions, and sclerosis in histologic sections after 12 wk of treatment ($n = 8$ kidneys per group). C, Representative, periodic acid–Schiff stained kidney sections obtained at 34 wk of age showing the typical inflammatory lesions that develop in NZB/NZW F1 and the less intense glomerulonephritis observed in mice treated with ISO-1 or anti-MIF (original magnification $\times 200$). D, Glomerular Ig deposition detected using fluorescein-conjugated anti-mouse IgG. Fluorescence intensity was examined microscopically and scored on a scale of intensity of 0–4. $p = NS$ among groups. E, Assessment of interstitial inflammation by enumeration of F4/80$^+$ macrophages in $\approx 20$ interstitial fields selected from four representative kidneys per group. F, Reduced inflammatory infiltration is evident in immunoperoxidase-stained images from mice treated with ISO-1 or anti-MIF (original magnification $\times 200$). G, Enumeration of intraglomerular F4/80$^+$ macrophages, CD3$^+$ T cells, MIF$^+$ cells, and MIF receptor-positive (CD74$^+$, CXCR4$^+$) cells. Data are summed from $\approx 50$ glomeruli visualized in four kidneys per experimental group. Values shown are mean $\pm SD$, and $p$ values are by two-tailed Student $t$ test. $^*p < 0.05; ^{**}p < 0.01$. 
FIGURE 4. ISO-1 ameliorates glomerulonephritis in MRL/lpr mice. A, Serum BUN levels in mice treated with ISO-1, anti-MIF mAb, or controls (vehicle or IgG1). Blood was sampled in four mice per group before treatment (9 wk), midway through treatment (14 wk), and at the end of the treatment (19 wk). B, Glomerulonephritis scores (0 to 4+) reflecting indices of cellularity, focal and segmental lesions, and sclerosis were obtained in kidney sections
\( n = 8 \) kidneys per group) after 10 wk of treatment. The \( p \) values are by two-tailed Student \( t \) test. C, Representative, periodic acid–Schiff stained kidney sections obtained at 19 wk of age showing inflammatory renal damage in MRL/lpr mice and the less intense glomerulonephritis observed in mice treated with ISO-1 (original magnification \( \times 200 \)). D, Glomerular IgG deposition detected using fluorescein-conjugated anti-mouse IgG. Fluorescence intensity was examined microscopically and scored as described (38). \( p = NS \) among groups. E, Assessment of interstitial inflammation by enumeration of F4/80+ macrophages in \( \approx 20 \) interstitial fields selected from four representative kidneys per group. F, Reduced inflammatory infiltration is evident in a representative immunoperoxidase-stained renal section from an ISO-1–treated mouse, which shows a few periglomerular F4/80+ cells (original magnification \( \times 200 \)). G, Enumeration of intraglomerular F4/80+ macrophages, CD3+ T cells, MIF+ cells, and MIF receptor-positive (CD74+, CXCR4+) cells. Data are summed from \( \approx 50 \) glomeruli visualized in four kidneys per experimental group. Values shown are mean \( \pm SD \), and \( p \) values are by two-tailed Student \( t \) test. \(* p < 0.05; ** p < 0.01.\)
deposition (Fig. 4D), but it significantly reduced the content of infiltrating inflammatory cells (F4/80+ macrophages) in renal interstitium (Fig. 4E, 4F) as well as the number of intraglomerular F4/80+ macrophages, CD3+ T cells, MIF+ cells, and MIF receptor-positive (CD74+, CXCR4+) cells (Fig. 4G).

**Influence of MIF inhibition on serum autoantibody and splenic lymphocyte populations in lupus-prone mice**

Serum anti-dsDNA autoantibody is a serologic hallmark of SLE. Circulating levels of anti-dsDNA in NZB/NZW F1 mice were unaffected by ISO-1 treatment, although a modest decrease in anti-dsDNA Ab titer was discernible at the 28-d time point in the anti-MIF–treated group (Fig. 5A). An analysis of pooled splenic and lymph node cells in NZB/NZW F1 mice did not show any effect of MIF inhibition on B cell (CD3− B220+) or T cell populations (CD3+CD4+, CD3+CD8−), and this included a specific analysis of double-negative (CD3−B220−), naive (CD3+CD4+CD44loCD62hi, CD3+CD8−CD44+CD62hi), and mature (CD3+CD4+CD44hi CD62hi, CD3−CD8−CD44+CD62hi) T cells (Fig. 5B). In the case of the MRL/prl lupus-prone mice, circulating concentrations of anti-dsDNA autoantibody were unchanged (Fig. 5C), however a modest increase in the percentage of total CD3+ T cells and a decrease in the percentage of B cell (CD3− B220+) and naive T cell (CD3+CD4+CD3+CD8−CD44+CD62hi) populations was noted after ISO-1 treatment (Fig. 5D). MIF has been described to provide survival signals to murine B cells via a Syk–Akt dependent pathway (11, 12), and it is plausible that pharmacologic MIF antagonism may influence the composition of the secondary lymphoid organs in the lymphoproliferative MRL/prl mice. Nevertheless, this modest difference in lymphoid subpopulations after ISO-1 treatment was not associated with a significant change in the circulating level of anti-dsDNA autoantibody (Fig. 5C).

**Influence of MIF inhibition on plasma cytokine expression**

We next measured the circulating levels of selected cytokines during disease development. Serum levels of the inflammatory effector, TNF-α, and the chemokine, MCP-1 (CCL2), increased significantly in both the NZB/NZW F1 and MRL/prl lupus strains, with the highest levels of these mediators observed in the lymphoproliferative MRL/prl mice (Fig. 6). MIF is known to upregulate innate cytokine production by suppressing activation-induced apoptosis (9, 10), and ISO-1 or anti-MIF treatment resulted in a significant reduction in plasma TNF-α and CCL2 in the MRL/prl and NZB/NZW F1 strains, respectively. The production of type I IFNs (IFN-α/β) is associated with the development of lupus immunopathology (1). Our measurements showed the highest circulating levels of IFN-α in the lymphoproliferative MRL/prl strain at 9 wk, however plasma IFN-α concentrations decreased during disease course irrespective of treatment. These data indicate that one impact of MIF inhibition is to reduce the systemic production of effector cytokines such as TNF-α, which initiates endothelial and end-organ damage by several mechanisms, and CCL2, which mediates monocyte/macrophage and T cell trafficking into inflammatory sites (1).

**Influence of MIF inhibition on renal cytokine and inflammatory gene expression**

An examination of kidney tissue from the two lupus-prone mouse strains by quantitative PCR (qPCR) showed a disease-associated increase in the expression of mRNA for several effector cytokines, including TNF-α, IL-1β, and CCL2, as well as an elevation in IL-12 and IFN-α (in the NZB/NZW F1 and MRL/prl strains, respectively) (Fig. 7). After administration of MIF inhibitors, a statistically significant reduction was observed in the NZB/NZW F1 strain for TNF-α, IL-1β, and CCL2, and in the MRL/prl strain for TNF-α, CCL2, and IL-1β (after anti-MIF). Of note, while control IgG1 treatment was noted to confer some protection on
glomerular disease in the MRL/lpr mouse (Fig. 4B), both ISO-1 and anti-MIF were associated with a significant reduction in the expression of renal TNF-α and CCL2 in this mouse strain. Overall, these data suggest a specific action for MIF antagonism in reducing proinflammatory cytokine expression in the NZB/NZW F1 and MRL/lpr models of spontaneous SLE.

FIGURE 6. Serum cytokine levels in NZB/NZW F1 and MRL/lpr mice before and after treatment with MIF inhibitors. Sera were isolated at the indicated times in the two mouse models and analyzed for cytokine content by Luminex beadlyte methodology (TNF-α, IL-1β, IL-12, CCL2) or specific ELISA (IFN-α). Values are the mean ± SD of four to five mice measured per group. Only significant differences are marked, and the 22 and 9 wk “before treatment” groups were compared with vehicle and IgG controls (mean ± SD; p values by two-tailed t test). *p < 0.05; **p < 0.005; ***p < 0.001.

FIGURE 7. qPCR analyses of mRNA expression in renal tissue in NZB/NZW F1 (A) and MRL/lpr (B) mice before and after treatment with MIF inhibitors. The mRNA values for each treatment group (n = 4 kidneys per group) are expressed in units relative to mRNA for GAPDH. The values for wild-type mice are from aged-matched, disease-free (C57BL/6) controls. The upper and lower edges of the boxes represent the 75th and 25th percentiles, respectively, the error bars denote the range of observations, and the horizontal line shows the median, with p values calculated by the Mann–Whitney U test. Only significant differences are marked (p values by Mann–Whitney U test). *p < 0.05.
To gain a more comprehensive assessment of the influence of MIF neutralization on the nephritic phenotype of the NZB/NZW F1 and MRL/lpr lupus-prone mice, we performed a comparative analysis of mRNA isolated from the kidney tissue of treated and untreated mice using genome-scale DNA microarrays. Using statistical and fold-change filtering procedures, a significant change in the expression of 50 genes was observed after ISO-1 or anti-MIF treatment in both the NZB/NZW F1 and MRL/lpr strains (Fig. 8). These genes could be grouped into three functional networks: cytokine/chemokine/receptor, T cell, and MIF receptor. Forty of these genes encoded proinflammatory cytokines, chemokines, and their receptors. In addition to the decrease in the expression of TNF-α, IL-1β, and CCL2 noted by qPCR and described earlier, reduced levels of IL-12, IL-17, MIF, and a number of additional proinflammatory cytokines as well as chemokine receptors (CCR 1, 4, 5, 6, 8; CXCR 1, 4, 6) was apparent. The treatment-induced reduction of this gene cluster appeared to be more marked in the MRL/lpr than in the NZB/NZW F1 strain, and there appeared to be a greater effect with ISO-1 than for anti-MIF in both strains, although exceptions are evident for particular genes. The presence of the CD2, CD4, and TCR-α gene transcripts also was reduced by MIF inhibition, which most likely reflects the reduced infiltration of kidneys by CD3+ T cells (Figs. 3G, 4G). Among genes known to be regulated by activation of the MIF receptor, MIF antagonism reduced the expression of the ERK effector, RhoGTPase (48), and the two tyrosine kinases, SRC and LCK (49, 50). MIF-dependent, Src family kinase activation is known to inhibit p53-mediated apoptosis (9, 51), and a corresponding decrease in the downstream expression of cyclin-dependent kinases also was observed (48, 52).

In summary, the amelioration in renal injury observed in the NZB/NZW F1 and the MRL/lpr strains, whether mediated by anti-MIF or by the small-molecule pharmacologic antagonist ISO-1, was associated with a broad downregulation in the expression of inflammatory cytokines and a reduction in the recruitment and retention of infiltrating immune cells.

**FIGURE 8.** Two-way coupled cluster analysis of gene expression in lupus-prone mice treated with ISO-1 or anti-MIF. Each column represents an experimental condition (ISO-1 or anti-MIF) relative to control (vehicle or IgG1). The genes selected for display showed significant differences ($p < 0.05$) in two or more of the experimental conditions shown ($n = 3$ samples analyzed per experimental group).
Discussion
MIF is an innate mediator that exists preformed in monocytes/macrophages and is released rapidly upon proinflammatory activation (3). Historically, the main role of MIF has been considered to be in the retention of infiltrating mononuclear cells within inflammatory lesions. More recent investigations have emphasized the ability of MIF to sustain cellular responses by inhibiting activation-induced apoptosis (10, 12) and to regulate leukocyte trafficking via noncognate interactions with CXCR2 and CXCR4, which are expressed in association with the MIF receptor, CD74 (4, 14). MIF also influences the differentiation of the adaptive T and B cell response (6, 21). Although MIF-knockout mice are developmentally normal, inflammatory or infectious provocation results not only in deficiencies in innate cytokine production (10, 53) but also in lymphocyte survival, T cell polarization, and Ab production (6, 12, 21).

Emerging clinical (24), genetic (23), and murine experimental data (18, 33) support an important role for MIF in the immunopathology of SLE. Serum MIF concentrations are elevated in lupus patients and are positively associated with end-organ damage (Systemic Lupus International Collaborating Clinics/American College of Rheumatology index) (24). MIF is encoded within a polymorphic genetic locus and high-expression (24) but also in lymphocyte survival, T cell polarization, and Ab production (6, 12, 21).

Intrarenal mRNA levels of TNF-α, IL-1β, and CCL2 also were reduced in response to anti-MIF or ISO-1 treatment. A reduction in the expression both of tissue-damaging cytokines such as TNF-α or IL-1β and of the chemokine CCL2 is consistent with MIF’s upstream role in the expression of these mediators (3, 33) and may explain in large part the protective action of MIF inhibition in these models of lupus nephritis (59, 60). CCL2 induces the transendothelial migration of monocytes, thereby facilitating tissue injury (61), and MIF itself directly activates the chemokine receptor CXCR4, which is expressed in association with CD74 (4, 14). Leukocyte recruitment is an important early event in autoimmune kidney injury (62), and the persistence of macrophages is a consistent feature of rapidly progressive lupus nephritis (2). CXCR4 is known to be upregulated in different mouse models of lupus, and treatment with a CXCR4 peptide antagonist has been shown recently to reduce intrarenal leukocyte trafficking and prolong survival in the B6.Sle1Yaa mouse model of SLE (63). The lower indices of inflammatory cytokine activation and intrarenal leukocyte content that were observed after anti-MIF and ISO-1 treatment was supported by the microarray-based survey of gene expression, which showed that in both lupus-prone mouse strains, there was a generalized downregulation in the expression of numerous proinflammatory cytokines, chemokines, and MIF-dependent signaling intermediates. These conclusions also appear in agreement with the protective effect of MIF deficiency on renal injury that was reported in MRL/MpJ-lpr/lpr mice back-crossed onto an mif<sup>−/−</sup> background (18). In that study, mif deletion reduced renal macropage recruitment and intrarenal TNF-α and IL-1β expression and urinary CCL2 excretion.

Experimental studies of murine lymphoid development and human lymphoproliferative disorders also support a functional role for MIF in B cell survival signaling (11, 12). Although neutralization of MIF in the MR1/lpr lupus-prone mice appeared to influence immune cell subpopulations in the spleen, it is unlikely that this effect was therapeutically beneficial because circulating anti-dsDNA levels and renal Ig deposition were not significantly affected. B cells are currently being targeted in the clinical application of anti-CD20 and soluble human B lymphocyte stimulator therapies, and it is conceivable that more potent MIF antagonists may exert a similar action in downregulating B cell responses (11).

In summary, the current data support the therapeutic value of reducing MIF-dependent effector responses in SLE, and they highlight the feasibility of targeting the MIF–MIF receptor interaction by a small-molecule approach. Recent disappointments in the application of biologically based therapies such as anti-TNF

parameters of glomerular injury and interstitial inflammation. Neither glomerular IgG deposition, circulating anti-dsDNA antibody levels, or major indices of splenic T or B cell activation were markedly affected by MIF inhibition, although in the case of the MRL/lpr mice, a modest reduction in the secondary lymphoid tissue content of B cells and naive CD4<sup>+</sup>, CD8<sup>+</sup> T cells was observed. Whether there is a greater reliance on MIF-dependent pathways for lymphocyte survival and the development of autoimmunity in the lymphoproliferative MRL/lpr mice remains to be more closely evaluated (11, 12, 44). An alteration in B and T cell populations was not observed in the NZB/NZW F1 lupus-prone mice, which harbor distinct abnormalities in these lymphocytes (58).
Development of chronic colitis is dependent on the cytokine MIF. J. Immunol. 2: 1061–1066.


