Identification of Iguratimod as an Inhibitor of Macrophage Migration Inhibitory Factor (MIF) with Steroid-sparing Potential*

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Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that has been implicated in a broad range of inflammatory and oncologic diseases. MIF is unique among cytokines in terms of its release profile and inflammatory role, notably as an endogenous counter-regulator of the anti-inflammatory effects of glucocorticoids. In addition, it exhibits a catalytic tautomerase activity amenable to the design of high affinity small molecule inhibitors. Although several classes of these compounds have been identified, biologic characterization of these molecules remains a topic of active investigation. In this study, we used in vitro LPS-driven assays to characterize representative molecules from several classes of MIF inhibitors. We determined that MIF inhibitors exhibit distinct profiles of anti-inflammatory activity, especially with regard to TNFα. We further investigated a molecule with relatively low anti-inflammatory activity, compound T-614 (also known as the anti-rheumatic drug iguratimod), and found that, in addition to exhibiting selective MIF inhibition in vitro and in vivo, iguratimod also has additive effects with glucocorticoids. Furthermore, we found that iguratimod synergizes with glucocorticoids in attenuating experimental autoimmune encephalitis, a model of multiple sclerosis. Our work identifies iguratimod as a valuable new candidate for drug repurposing to MIF-relevant diseases, including multiple sclerosis.

Macrophage migration inhibitory factor (MIF)3 is one of the first cytokine-like proteins to be discovered (1, 2). Although MIF was originally characterized as a T-cell factor that inhibits random migration of macrophages, subsequent investigations have shown that it is ubiquitously expressed and has pleiotropic effects on inflammation, chemotaxis, and cell survival and proliferation (3–5). As a cytokine, MIF binds a CD74-CD44 receptor complex to initiate downstream signaling via the p44/p42 mitogen-activated protein kinase pathway, which leads to up-regulation of prostaglandins and phospholipase A2, with subsequent pro-inflammatory effects (5–7). These effects are relevant to multiple inflammatory states, and MIF has been shown to have an important pathologic role in inflammatory conditions such as sepsis, rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (8–11).

Glucocorticoids are the cornerstone of treatment for several diseases due to their potent anti-inflammatory and immunosuppressive effects. Unfortunately, chronic systemic glucocorticoid use is also associated with a potentially severe adverse effect profile that is a significant source of iatrogenic illness among patients with chronic disease (12, 13). Glucocorticoids initiate their effects via a range of genomic and nongenomic mechanisms, many of which rely on interactions with membrane-bound or cytosolic glucocorticoid receptors (14). A common theory holds that most therapeutic effects are mediated by transrepression downstream of the glucocorticoid receptor that results in decreased production of pro-inflammatory proteins, whereas adverse (along with some therapeutic) effects appear to be caused by transactivation. Recent work has indicated that the actual mechanistic properties of glucocorticoids are likely to be more complex (14, 15). Nonetheless, dissociation of these events remains a key goal in steroid therapeutics, and a true “steroid-sparing therapy” would allow for lower doses of glucocorticoids to be used to achieve the same therapeutic result with fewer adverse effects (16). The plant-derived compound A is an important candidate in this growing field of therapeutics (17, 18).

Unlike other cytokines, MIF is capable of overriding the anti-inflammatory effects of glucocorticoids, likely based on interaction with the glucocorticoid receptor complex. Therefore, selective MIF inhibition is a relevant consideration in adjuvant steroid therapy.

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‡‡ The abbreviations used are: MIF, macrophage migration inhibitory factor; ANOVA, analysis of variance; AUC, area under the curve; EAE, experimental autoimmune encephalomyelitis; LAL, Limulus amebocyte lysate; M-CSF, macrophage colony-stimulating factor; MOG, myelin oligodendrocyte glycoprotein; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate-buffered saline; rMIF, recombinant MIF; RPMI, Roswell Park Memorial Institute; DEX, dexamethasone; VEH, vehicle; T₄, thyroxine.
In our study, we selected compounds from multiple classes of MIF small molecule inhibitors (detailed in Table 1) and screened them for anti-inflammatory activity in an LPS-treated monocyte system. We discovered that, even within our small cohort, compounds segregated into at least three groups with distinct anti-inflammatory profiles. We selected the clinically utilized chromene derivative T-614 (iguratimod), which had low anti-inflammatory activity in our screen, for further study. Using MIF-dependent in vitro and in vivo studies, we determined that T-614 inhibits MIF and attenuates inflammatory disease in an MIF-dependent fashion. When tested alongside glucocorticoids in an in vitro context, we found that T-614 has significant additive effects with glucocorticoids in suppressing inflammation. We verified this in vivo in experimental autoimmune encephalomyelitis (EAE), showing that combination therapy with dexamethasone and T-614 is more efficacious in treating the disease phenotype than either drug alone. Our data suggest that iguratimod may exert its clinically observed anti-inflammatory activities via MIF inhibition and that this drug should be explored further as a potential steroid-sparing therapeutic in diseases such as multiple sclerosis.

Results

Cytokine Release by Monocytes, Different Profiles of MIF Inhibitors—Small molecules of various classes with micromolar or lower IC$_{50}$ in the MIF tautomerase assay were selected for biologic characterization in the context of LPS-treated human monocytes (Table 1). None of these compounds exhibited significant toxicity up to 50 μM in this context (data not shown). We found that although these molecules all have a similar profile of inhibition of MIF enzymatic activity, they exhibit diverse profiles of anti-inflammatory activity in this bioassay. The clearest distinctions were observable in TNFα release. The coumarin derivatives K-679 and K-680 almost completely suppressed TNFα release in monocytes; two isoxazole compounds (ISO-1 and ISO-66) as well as the pyrimidazole compound K-664.1 exhibited moderate suppression of TNFα release; whereas the chromene-derived T-614, isoxazole ISO-92, carbonyl oxime OXIM-11, and hormone isomer D-T4 almost completely spared TNFα at concentrations up to 50 μM (Fig. 1A). The release of the chemokines MCP-1 (macrophage chemotactic protein 1) and IL-8 segregated similarly, with some exceptions: ISO-92 and K664.1 were both stronger suppressors of MCP-1 than TNFα; D-T4 almost completely spared TNFα and IL-8 in disease conditions where strong cytokine suppression may be undesirable, such as multiple sclerosis (48). Both T-614 and OXIM-11 exhibited minimal suppression for all the cytokines analyzed in this study. T-614 is clinically utilized in Asia, and its inventors have shown it to be strongly anti-inflammatory and efficacious in treating rheumatoid arthritis (49–51), which...
TABLE 1
Representative MIF inhibitory compounds selected for characterization
IC_{50} values are based on MIF dopachrome tautomerase activity as detailed under “Experimental Procedures”. Compounds previously characterized as MIF inhibitors are noted with a reference; other compounds are designated as “Novel”.

| Compound | Category       | Structure | IC_{50}, µM (±SD) | Reference |
|----------|----------------|-----------|-------------------|-----------|
| ISO-1    | Isoxazole      | ![Structure of ISO-1](image1) | 18.20 ± 2.90     | (31)      |
| ISO-66   | Isoxazole      | ![Structure of ISO-66](image2) | 1.47 ± 0.44      | (108)     |
| ISO-92   | Isoxazole      | ![Structure of ISO-92](image3) | 1.07 ± 0.01      | (39)      |
| K-679    | Coumarin       | ![Structure of K-679](image4) | 0.22 ± 0.04      | Novel     |
| K-680    | Coumarin       | ![Structure of K-680](image5) | 1.12 ± 0.04      | Novel     |
| T-614    | Chromene       | ![Structure of T-614](image6) | 6.81 ± 0.56      | Novel     |
| K-664.1  | Pyrimidazole   | ![Structure of K-664.1](image7) | 0.16 ± 0.06      | (41)      |
| OXIM-11  | Carbonyl oxime | ![Structure of OXIM-11](image8) | 1.57 ± 0.15      | (27)      |
| D-T4     | Hormone isomer | ![Structure of D-T4](image9) | 11.30 ± 0.29     | (45)      |
contrasts with the results of our phenotypic screen. Although putative mechanisms have been ascribed to the molecule, including COX-2 inhibition (52) or modulation of NFκB translocation (51), the data from these studies suggest that the molecular target of T-614 lies upstream of these factors. Identification of MIF as a molecular target for this drug would help clarify its experimental and clinical activities, as well as offering an anti-MIF clinical candidate with immediate applications as a repurposed drug. For these reasons, we determined that T-614 merited further investigation, and we sought to characterize it in MIF-specific assays.

**T-614 Inhibits MIF in Vitro—** Multiple approaches have been used to examine MIF activity in vitro, including measurement of glucocorticoid override and ERK phosphorylation. Our experience has been that these assays can produce inconsistent results and may involve non-MIF effects. To examine T-614 as a selective MIF inhibitor, we chose two bioassays where the readout is directly elicited by exogenously added rMIF protein. In the first assay, rMIF was found to significantly increase BrdU incorporation in synchronized Raji B cells at a concentration of 1 ng/ml, which mirrors the findings of Leng et al. (5) using thymidine incorporation in the same cells. T-614 did not affect BrdU incorporation on its own at concentrations as high as 200 μM, which is similar to previous observations (53); however, it did attenuate the rMIF effect at a concentration of 100 μM (Fig. 2A).

**T-614 Inhibits MIF in Vivo—** To test in vivo efficacy and selectivity of T-614 as an MIF inhibitor, we employed a murine endotoxemia model that has been well characterized in the context of MIF using both knock-out and inhibitory approaches (8, 33, 36). Using BALB/c mice that are vulnerable 1 ng/ml, which mirrors the findings of Leng et al. (5) using thymidine incorporation in the same cells. T-614 did not affect BrdU incorporation on its own at concentrations as high as 200 μM, which is similar to previous observations (53); however, it did attenuate the rMIF effect at a concentration of 100 μM (Fig. 2A). For the second bioassay, IL-8 production was analyzed in adherence-purified peripheral blood monocytes, where we observed 100 ng/ml rMIF triggered a significant increase in IL-8 production over 24 h. Although T-614 did not affect IL-8 on its own and did not attenuate IL-8 release from LPS stimulation in enriched monocytes (Fig. 1B), it did dose-dependently suppress exogenous rMIF-induced IL-8 release from adherence-purified monocytes (Fig. 2B).
to endotoxemia, T-614-treated animals showed significantly increased survival after a lethal dose of LPS compared with vehicle-treated controls (Fig. 3A). T-614 treatment also attenuated TNFα release measured in serum isolated 90 min post-LPS administration in wild-type C57BL/6 mice. In MIF−/− mice on the same background, however, there was no significant effect of T-614 on serum TNFα 90 min post-LPS administration (Fig. 3B). These data suggest that T-614 can attenuate a systemic inflammatory response in vivo and suggest that its effects are MIF-specific in this context.

**T-614 Is Additive with Glucocorticoids in Vitro**—Glucocorticoid synergy has been demonstrated in the context of MIF in previous studies using RNA silencing and anti-MIF antibodies (20, 30). Additionally, the small molecule ISO-1 has shown some synergy with glucocorticoids in suppressing cytokines in LPS-treated peripheral blood monocytes (31) as well as dexamethasone-treated cultured nasal polyps (32). Because these studies used murine RAW 264.7 macrophages, human THP-1 monocytes, and primary human peripheral blood monocytes, we adapted similar systems for our study. For RAW 264.7 cells, individual pretreatment with inhibitor and dexamethasone attenuated TNFα release induced by 4-h stimulation with LPS, and the combination of the two drugs had a significant additive effect (Fig. 4A). A similar additive effect was observed in the setting of THP-1 cells stimulated with LPS for 16 h (Fig. 4B). To confirm the effect in primary cells, we tested adherence-purified human peripheral blood monocytes as well as M-CSF-polarized macrophages and again found that both T-614 and dexamethasone can individually attenuate LPS-induced TNFα release and that the combination suppressed it further (Fig. 4, C and D). We noted that although T-614 was generally cytokine-sparing up to 50 μM in primary monocytes enriched by magnetic selection (Fig. 1A), in the context of primary adherence-purified monocytes the drug did suppress TNFα at concentrations as low as 10 μM (data not shown). These variations might be attributable to differences between these monocyte preparations, and, indeed, it has been reported that the standard adherence protocol yields a relatively lower purity monocyte population that may have distinct inflammatory responses (54, 55).

**T-614 Augments Low-dose Glucocorticoids in Vivo**—Although MIF emerged early as a potential target in diseases where steroid therapy is a mainstay of treatment, to our knowledge no study has been undertaken actually pursuing anti-MIF treatment as a steroid-sparing therapy in disease or a disease model (56). Here, we chose a model of EAE for a pilot study exploring this question. EAE is a model of multiple sclerosis, a disease characterized by immune-mediated demyelination of the central nervous system, frequently resulting in disability (57). Intravenous or oral glucocorticoid treatment is commonly used in combination with other drugs for treatment of acute episodes and progressive disease (58, 59). The EAE model is characterized by T-cell infiltration into the central nervous system causing inflammation and myelin damage (60). Notably, previous work has established that MIF plays a significant role in this model, likely related to its ability to override the immunosuppressive effects of glucocorticoids; deletion of the MIF gene significantly increases the efficacy of glucocorticoid treat-
ment after disease induction (61). T-614 has also been tested in this model and was found to significantly attenuate disease progression in vivo, likely in relation to inhibition of T-cell proliferation and cytokine production (62).

To examine potential synergy between glucocorticoids and T-614, we induced EAE in C57BL/6 mice by immunization with the MOG(35–55) peptide, and EAE disease peaked around day 20 post-immunization. Animals treated with either 0.1 mg/kg dexamethasone (3 days post-onset) or 12.5 mg/kg T-614 (daily post-onset) showed minor attenuation in disease scores (Fig. 5A). Animals co-treated with the two drugs showed a further decrease in disease scores compared with either drug alone, which was significantly different from control animals during peak disease (days 18–23). Additionally, animals receiving the combination therapy had a significantly lower area under the curve measure (AUC) for clinical scores, an indicator of less severe disease overall (Fig. 5B) (63). Together with our studies in vitro, these in vivo data suggest that T-614 may have promise as a steroid-sparing therapy.

Discussion

Biologic systems are complex, and this complicates target-based drug design. Although a target such as MIF may be implicated in multiple disease processes via well elucidated mechanisms, probing the full scope of interactions between that target and the vast array of proteins and small molecules in the cellular milieu can be a daunting task. MIF is classically viewed as a pro-inflammatory cytokine, with candidate anti-MIF therapies promoted as potentially valuable anti-inflammatory drugs (3, 40). Suppression of an acute-phase cytokine such as TNF would seem to be desirable in a general anti-inflammatory drug. However, several studies have shown that even this classical pro-inflammatory cytokine can have protective roles in tissue repair and regeneration. In the central nervous system, TNF has been shown to induce proliferation of neural stem cells, likely via interactions with TNFR2 (48, 64, 65). Treatment with anti-TNF has been linked to the development of demyelinating disease, which may relate to TNF’s roles in repair and neurogenesis (66–68). It stands to reason that when developing a drug for use in multiple sclerosis, such as an MIF inhibitor (61), it would be useful to design...
MIF Inhibition and Iguratimod

In our study, we have for the first time identified a molecular target that may explain some of the observed activities for T-614. T-614 interacts with the MIF trimer, inhibiting MIF's tautomerase enzymatic activity with an IC\textsubscript{50} value comparable with ISO-1, the best-characterized MIF inhibitor (Table 1) (40). This interaction is relevant to MIF biology because T-614 was able to inhibit MIF-induced proinflammatory effects, including proliferation of B cells and cytokine release from monocyes (Fig. 2, A and B). Moreover, these effects are selective for MIF, because T-614 did not suppress systemic inflammation in the absence of MIF (Fig. 3B). MIF is known to exist in human plasma in nanogram/ml concentrations, which based on our IC\textsubscript{50} data would be inhibited by T-614 at steady state concentrations (23, 82). All these data suggest that anti-inflammatory effects of T-614 may be mediated at least partially through MIF inhibition.

MIF is a pleiotropic molecule, and MIF inhibition could potentially underlie other observed activities of T-614. For
example, MIF may be involved in T-614-mediated inhibition of immunoglobulin production from B cells; MIF has been previously linked to immunoglobulin production (83) and has a well-known role in promoting survival of B cells (84). T-614 is known to suppress the IL-17 signaling axis, and MIF is known to stimulate this axis (85). T-614 has been shown to inhibit osteoclastic differentiation, which is induced by MIF (86, 87). It is not difficult to imagine that MIF may be targeted by T-614 in all of these activities, and it could be responsible for some or all of the efficacy of T-614 as a disease-modifying anti-rheumatic drug. Of further note, several studies have found that MIF has a significant role in the pathogenesis of rheumatoid arthritis and may be a relevant target in the disease (9, 88–91). Future studies of this drug may consider investigating the potential MIF dependence of these activities.

In addition to establishing T-614 as a MIF-targeting molecule, we also provide evidence for novel clinical applications of this drug. Also known as iguratimod, T-614 is currently clinically available in Japan and China as a daily oral formulation administered at 25–50 mg daily, which has shown safety and efficacy in improving symptoms and disease progression in rheumatoid arthritis both as a monotherapy (92–94) and in combination with methotrexate (95). Several preclinical studies have suggested that the drug may also have utility in other settings, such as multiple sclerosis (62) and cachexia in the context of adenocarcinoma (96). MIF is an influential player in a large variety of disease processes, including autoimmune (10, 11, 97, 98), neurologic (99, 100), metabolic (101), and oncologic conditions (102–105). Several of these disease processes benefit from treatment with glucocorticoids, of which MIF is an endogenous counter-regulator (19). Here, we show that T-614, acting as an MIF inhibitor, can synergize with glucocorticoids in an in vitro inflammatory model (Fig. 4, A–D) as well as in vivo in the EAE model (Fig. 5, A and B), suggesting potential clinical applications as a steroid adjunct. We have also determined that T-614 exhibits fewer effects on TNFα compared with other MIF inhibitors in the context of enriched primary human monocytes (Fig. 1, A and B). This latter finding stands in contrast to previous findings that found that T-614 has significant cytokine-suppressing effects (51, 74, 75) as well as our own findings in adherence purified monocytes (Fig. 4C), which we would attribute to differences in the preparation of these cells (54, 55). Because T-614 is steroid-sparing and lacks profound TNFα suppression in certain contexts, we believe that it would be an excellent candidate for preclinical study as a steroid adjunct in multiple sclerosis, where TNFα inhibition may exacerbate demyelination (66). However, further study is needed to determine whether T-614 impacts the transactivation effects of glucocorticoids, which would be undesirable in a steroid-sparing therapeutic (17, 18).

In sum, our findings highlight the complexities of target-based drug design, because we observed that even high affinity MIF inhibitors have distinct anti-inflammatory effects in vitro. Our screen highlighted the clinically available chromone derivative compound T-614 (iguratimod) as a MIF inhibitor with a low anti-inflammatory profile, which further investigation revealed has potential as a steroid-sparing therapeutic. We hope that our work encourages the repurposing of iguratimod to other MIF-relevant diseases, including multiple sclerosis.

**Experimental Procedures**

**Reagents**—All reagents were purchased from Sigma or Fisher unless otherwise indicated. Compound T-614 (iguratimod) was purchased from Ontario Chemical (Guelph, Ontario, Canada); it was solubilized in alkaline solution, pH 7.8 (106), for in vitro and endotoxemia studies and in 5% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS) for EAE studies. Recombinant human MIF protein for catalytic characterization and in vitro use was expressed in Escherichia coli BLD1(DE3) cells and purified as described previously (31). In vitro readouts of MIF bioactivity were confirmed (where applicable) with bioactive recombinant human MIF purchased from Shenaodao Biotechnologies (Warwick, PA). Prior to in vitro use, endotoxin content was confirmed to be less than 0.05 EU/μg protein by a colorimetric end point Limulus amebocyte lysate (LAL) assay (Lonza, Allendale NJ). Unless otherwise indicated, cytokine ELISAs were purchased as DuoSet kits from R&D Systems (Minneapolis, MN) and used according to the manufacturer’s instructions.

**MIF Dopachrome Tautomerase Activity**—The enzymatic activity of MIF on freshly prepared L-dopachrome methyl ester was assessed as described previously (44). Sterile recombinant human MIF was maintained in TBS, pH 7.4, at concentrations ranging from 0.5 to 1 mg/ml for up to 6 months without significant loss of activity. Inhibitory compounds were solubilized in DMSO, added to a cuvette containing 1 μg/ml rMIF in PBS, and mixed thoroughly; dopachrome substrate was then added, and absorbance at 475 nm was monitored for 20 s to measure activity.

**Cell Culture**—Human Raji B and THP-1 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM L-glutamine, and 55 μM 2-mercaptoethanol (THP-1 cells only) (Life Technologies, Inc.). Cells were passaged by dilution three times weekly with total media replacement every 3 weeks. Raji B cells were used for 2 months post-thaw, and THP-1 cells were used only during weeks 2 and 3 post-thaw. RAW 264.7 macrophages were maintained in adherent culture in DMEM, 4.5 g/dl glucose supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, and 55 μM 2-mercaptoethanol (THP-1 cells only) (Life Technologies, Inc.). Cells were passaged by scraping and used only until passage 20. All cells were cultured in a humidified incubator at 37 °C, 5% CO₂. Unless otherwise indicated, all cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA) and stored as passage 5 aliquots in liquid nitrogen.

**Preparation of Peripheral Blood Cells**—Human peripheral blood was collected in sodium heparin (IRB 12-200A) or obtained as Leuko PAKs from New York Blood Center (New York). Mononuclear cells were isolated by density gradient centrifugation in Ficoll-Paque Plus (GE Healthcare). Monocytes were either purified by 2-h adherence to Primaria culture plates (Corning Life Sciences) or enriched by negative magnetic selection using monocyte isolation kit II (Miltenyi Biotech, Auburn, CA). All monocyte preparations were cultured in RPMI 1640 medium supplemented with 10% human AB serum, 100
units/ml penicillin/streptomycin, and 2 mM L-glutamine and used within 24 h. Macrophages were differentiated from adherence-purified monocytes by incubation with 10 ng/ml human M-CSF (Sigma) for 7 days, with media replenishment performed on days 3 and 5.

Cytokine Production Assays—For LPS-induced cytokine release, negative selection-enriched human monocytes were plated at a density of $2 \times 10^5$ cells/ml in 96-well plates. Cells were pretreated with MIF inhibitors in 0.1% DMSO (final) for 1 h before stimulation with LPS from 

E. coli

R515 (Axxora, Farmingdale, NY) for 24 h. Cell-free supernatants were collected and stored at $-80^\circ$C for cytokine determinations by ELISA; 1:2 dilutions were used for TNF-$\alpha$ and 1:3 dilutions for IL-8 and MCP-1. Cytotoxicity was assessed using a Cytotox96 non-radioactive cytotoxicity kit (Promega, Madison, WI); no significant cytotoxicity was observed for the compounds used unless otherwise indicated.

Although the production of cytokines in response to MIF stimulation alone is controversial, the production of IL-8 by MIF stimulation of peripheral blood monocytes has been confirmed in multiple contexts (46, 69). Human peripheral blood monocytes purified by adherence were plated at a density of $2 \times 10^5$ cells/well in a 96-well plate. Cells were pretreated with T-614 or vehicle for 30 min prior to stimulation with MIF for 24 h. Cell-free supernatants were recovered and subjected to IL-8 MAX standard ELISA using a 1:100 dilution (Biologend, San Diego). Cytotoxicity was assessed by a neutral red assay as described previously, using a 1-h incorporation period (107); no significant cytotoxicity effects were observed unless otherwise indicated.

MIF-induced Proliferation—For this bioassay, we adapted the method of Leng et al. (5) with some modifications. Briefly, human Raji B cells were plated at a density of $0.5 \times 10^5$ cells/well in a 96-well plate and synchronized by incubation for 24 h in RPMI 1640 medium supplemented with 0.1–0.5% FBS. Synchronized cells were pretreated with T-614 or vehicle for 30 min prior to stimulation with MIF for 24 h. At 20 h BrdU was added to cells and quantified using a BrdU Cell proliferation assay kit (Cell Signaling Technology, Danvers, MA).

Glucocorticoid Synergy in Vitro—The methods of Roger et al. (20) and Kerschbaumer et al. were adapted for these experiments (30). RAW 264.7 cells were plated at a density of $1 \times 10^6$ cells/well in a 96-well plate and incubated overnight (16 h) before pretreatment with T-614 or vehicle for 20 min, treatment with dexamethasone (Alfa Aesar, Ward Hill, MA) solubilized in 0.01% DMSO for 20 min, and finally stimulation with LPS from 

E. coli

O111:B4 (Sigma) for 4 h. Cell-free supernatants were collected and analyzed immediately with a murine TNF-$\alpha$ ELISA at a 1:10 dilution. THP-1 cells were plated at a density of $2 \times 10^5$ cells/well in a 96-well plate and used the same day with the same treatment conditions as RAW 264.7 cells, except that THP-1 cells were incubated with LPS for 16 h prior to isolation of cell-free supernatants. Supernatants were analyzed using a human TNF-$\alpha$ ELISA at a 1:10 dilution. Human peripheral blood mononuclear cells isolated by adherence (described above) were plated at a density of $2 \times 10^5$ cells/well in a 96-well plate and used the same day with the same treatment conditions as THP-1 cells. Supernatants were analyzed using a human TNF-$\alpha$ ELISA at a 1:20 dilution. Human macrophages were differentiated from adherence-purified monocytes by a 1-week culture with 10 ng/ml M-CSF on cells plated at a density of $2 \times 10^5$ cells/well and used when an astrocytic morphology was clearly observed in $>50\%$ of the cells. Cells were treated under the same conditions as THP-1 cells, and supernatants were analyzed using a human TNF-$\alpha$ ELISA at a 1:10 dilution.

For all above experiments, cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (1–2-h incorporation); no significant cytotoxicity effects were observed under any treatment conditions unless otherwise noted.

Animal Experiments—The Institutional Animal Care and Use Committees of the Feinstein Institute for Medical Research and/or the University of Texas at San Antonio reviewed and approved all animal protocols prior to initiation of experiments. Male BALB/c animals were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for endotoxemia experiments at ages 8–10 weeks. MIF KO animals were maintained on a C57BL/6 NCr background from Charles River Laboratories (Stone Ridge, NY); these animals were used alongside matched wild-type animals for in vivo experiments at ages 8–12 weeks.

Endotoxemia—Endotoxemia was induced by intraperitoneal injection of LPS from 

E. coli

O111:B4 (Sigma). In BALB/c animals, 5 mg/kg LPS was used as a lethal dose for survival experiments; animals were treated with T-614 (20 mg/kg i.p.) 0.5 h prior to LPS, 6 h after LPS, and then once daily for 3 days and monitored for survival over 2 weeks. In C57BL/6 animals, 20 mg/kg LPS was used as non-lethal dose for plasma cytokine experiments; animals were pretreated with T-614 (20 mg/kg i.p.) twice, one dose each at 2 and 0.5 h prior to LPS administration, and euthanized at 90 min post-LPS by CO$_2$ asphyxiation with cervical dislocation. Blood was collected by cardiac puncture and allowed to clot 20 min at room temperature and 20 min at 4°C; sera were isolated by centrifugation at 300 $\times$ g for 10 min and stored at $-20^\circ$C for further analysis by TNF-$\alpha$ ELISA (1:3 dilution).

EAE—EAE was induced by subcutaneous injection of C57BL/6 mice with 200 $\mu$g of MOG(35–55) peptide (United Biochemical Research, Seattle) in 50 $\mu$l of complete Freund’s adjuvant, in addition to 400 ng of pertussis toxin administered intraperitoneally on days 0 and 2. Animals were monitored and scored daily for clinical signs of EAE as follows (61): 1, limp tail; 2, moderate hind limb weakness; 3, complete hind limb paralysis; 4, quadriplegia or perimorbid state; 5, death. Upon reaching a disease score of 1.0, mice were treated intraperitoneally with 0.1 mg/kg dexamethasone (DEX) or phosphate-buffered saline (PBS) daily for 3 days, as well as 250 $\mu$g of T-614 or vehicle (VEH) daily until the end of the study.

Author Contributions—C. M., T. C., and B. S. collaborated to produce the data in Fig. 1. K. F. C. and M. H. synthesized/provided MIF inhibitory compounds in Table 1, characterized their IC$_{50}$ values, and helped prepare T-614 for in vitro and in vivo experiments. J. B. conducted experiments encompassing Figs. 2–4 and wrote most of the paper. S. N., J. C., and T. F. collaborated to produce the data in Fig. 5. Y. A. conceived the idea of the project, oversaw the preparation of the paper, and gave final approval of the version to be published.
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