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Regulation of IL-1 and TNF Receptor Expression and Function by Endogenous Macrophage Migration Inhibitory Factor

Myew-Ling Toh,* Daniel Aeberli,* Derek Lacey,* Yuan Yang,* Leilani L. Santos,* Michael Clarkson,† Laveena Sharma,* Colin Clyne,† and Eric F. Morand3*

Macrophage migration inhibitory factor (MIF) has a key role in regulation of innate and adaptive immunity and is implicated in sepsis, tumorigenesis, and autoimmune disease. MIF deficiency or immunoneutralization leads to protection against fatal endotoxic, exotoxic, and infective shock, and anti-inflammatory effects in other experimental models of inflammatory disease. We report a novel regulatory role of MIF in type 1 IL-1R and p55 TNFR expression and function. Compared with wild-type cells, MIF-deficient cells were hyporesponsive to IL-1- and TNF-induced MAPK activity, AP-1 activity, and cellular proliferation, while NF-κB function was preserved. Hyporesponsiveness of MIF-deficient cells was associated with down-regulation of cytokine receptor expression, which was restored by reconstitution of either an upstream kinase of MAPK, MAPK/ERK kinase, or MIF. These data suggest that endogenous MIF is required for cytokine activation of MAPK/AP-1 and cytokine receptor expression. This autocrine regulatory pathway defines an important amplifying role of endogenous MIF in cytokine-mediated immune and inflammatory diseases and provides further molecular evidence for the critical role of MIF in cellular activation. The Journal of Immunology, 2006, 177: 4818–4825.

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acrophage migration inhibitory factor (MIF) is a pleiotropic cytokine with a crucial role in the regulation of innate and adaptive immunity (1). MIF is implicated in multiple autoimmune and inflammatory states, including sepsis, rheumatoid arthritis (RA), tumorigenesis, acute respiratory distress syndrome, glomerulonephritis, and enterocolitis (2). MIF immunoneutralization or MIF deficiency is associated with protection against endotoxic shock and lethality, and marked anti-inflammatory effects in several experimental models of autoimmune disease.

The underlying molecular mechanisms of these observed biological effects are less well-understood. MIF-induced cellular activation is mediated via MAPK and the AP-1 transcription factor. MIF is thought to signal through classical receptor-dependent activation of MAPK through binding to CD74, the cell surface form of the MHC class-II-associated invariant chain (3). In NIH3T3 cells, MIF induces sustained ERK MAPK activation associated with increased cellular proliferation and phospholipase A2 (PLA2) activity (4). In these same cells, MIF was shown to modulate adhesion-dependent sustained ERK MAPK activation and regulate cyclin D1 expression and cell cycle progression (5, 6). MIF activates MAPK and AP-1 pathways in neuroblastoma cell lines (7), osteoblasts, dermal fibroblasts (8), and RA fibroblast like-synoviocytes (FLS) (9–11). In contrast, at supraphysiological concentrations, MIF also signals through nonreceptor-based pathways, via intracellular binding and activation of JAB1 leading to selective down-regulation of AP-1 activation (12).

MIF, unlike other cytokines such as TNF, has a widespread tissue and cellular distribution including large preformed stores in immune cells and fibroblasts. This places MIF in an ideal position for regulation of immune and inflammatory events entrained by other factors such as endotoxin and cytokines. For example, endogenous MIF regulates TLR-4 expression to permit macrophage responses to endotoxin (13). Whereas the role of endogenous MIF in responses to endotoxin is well-documented, the role of endogenous MIF in regulating responses to proinflammatory cytokines implicated in autoimmune diseases is poorly understood. The hypothesis that endogenous MIF does play a role in regulating cellular responses to other cytokines is supported by several observations. For example, immunoneutralization of MIF with anti-MIF mAb inhibits IL-1- and TNF-induced proliferation and IL-1-induced PLA2 and cyclooxygenase-2 (COX-2) expression in RA FLS (9, 14). MIF is known to exert autocrine and paracrine effects on immune cell activation, inducing IL-1 and TNF, leading in turn to further MIF production (15, 16). We report here that endogenous MIF is required for IL-1- and TNF-induced MAPK activation and regulates the expression of receptors for these cytokines. These data provide a novel mechanism of action of MIF in the amplification of the immune response.

Materials and Methods

Cells

MIF−/− mice were provided by Dr. J. R. David (Harvard School of Public Health, Boston, MA) (17, 18), crossed for two generations with MIF wild-type (WT) C57BL/6 mice and experiments were performed with littermate MIF−/− and WT controls. Murine dermal fibroblasts (MDF) were isolated...
as described (18). Mouse embryonal fibroblasts (MEF) were generated from WT and MIF−/− embryos at day 14.5. Cells were propagated in RPMI 1640/10% FCS (ICN Biomedicals) and passages 4–6 used.

**Plasmid constructs**

The MIF coding region was amplified by PCR by using the following primers: 5'-CACCATGCGATGTCATCGAAAAC-3' and 5'-GCCGGA GGTGGAGGTGGTCC-3' (19). The MIF open reading frame was verified by sequencing and cloned into a eukaryotic expression vector pcDNA 3.1 (Invitrogen Life Technologies). The following plasmid vectors were used: NfB-Luc, AP-1-Luc, pF-C-MEKK, pPUC, pFA2-Elk1, pFA2-c-jun, or pFA2-ChOP (Promega).

**Flow cytometry**

The type 1 IL-1R (IL-1R) was detected following incubation with mAb against IL-1R. The p55 TNFR (p55 TNFR1) was detected following incubation with mAb against TNFR1, followed by biotin-conjugated Ab and allophycocyanin streptavidin compared with isotype control Abs (BD Pharmingen). For MIF reconstitution experiments, MIF−/− cells were transfected with 1 μg of pcDNA 3.1 or pcDNA3.1 MIF and 0.5 μg of pEGFP using Lipofectamine 2000 (Invitrogen Life Technologies) or Amaxa nucleofactor technology for 48 h. Transfection efficiency was 70–15%. To investigate the effects of MAPK/ERK kinase (MEKK) transfection on p55 TNFR expression, MIF−/− cells were nucleofected (Amaxa) with 50 ng of an irrelevant vector pPUC as a control or pF-C-MEKK and 0.5 μg of pEGFP. Following nucleofection, p55 TNFR expression was detected as above and measured in enhanced GFP (EGFP)-positive cells relative to control.

**Western blot**

Cells treated with 0.1 or 5 ng/ml IL-1β (Sigma-Aldrich), 1 ng/ml TNF-α (BioSource International) or 0.3 M sorbitol (Sigma-Aldrich) were examined for nuclear factor (NF)-κB translocation on p55 TNFR expression, MIF−/− cells were treated with 0.1 or 5 ng/ml IL-1 for 1 h. Complementary oligonucleotides representing the consensus NF-κB (5'-AGTGTAG GGG GAC TTT CCC AGG C-3') and AP-1 (5'-CGC TTGATG ACT CAAGG CAA-3') binding sites were annealed and labeled with [32P]. The NF-κB or AP-1 nucleotide motifs are underlined. Sequence-specific binding was determined by incubation with nonradiolebelled WT or mutated probe, and supershift was detected with Abs against p50, p65, or c-fos and c-jun (Santa Cruz Biotechnology). Bands were analyzed using a PhosphorImager (Molecular Dynamics) and Image Gauge software (version 3.46).

**Luciferase reporter assays**

For AP-1 Luc assays, cells were transfected for 24 h with 1 μg of AP-1 Luc reporter plasmid (Promega), and 50 ng of pCMV-β-GAL (Promega) using Fugene 6 (Roche), stimulated with 0.1 or 5 ng/ml IL-1β for 8 h. To investigate the effects of MEKK transfection on the transcriptional activity of AP-1, MIF−/− cells were cotransfected in DMEM/0% FCS with 50 ng of a negative control plasmid pF-C-dbl and pFA2-Elk1, pFA2-c-jun, or pFA2-ChOP. Cells were simultaneously transfected with 50 ng of pFR-luc (Promega) for 16 h. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized to β-galactosidase.

**Cellular proliferation assays**

DNA synthesis was measured by [3H]thymidine incorporation, as described (21). Cells were treated with IL-1β or TNF-α for 48 h, pulsed for 18 h with 1 μCi/ml [3H]thymidine (Amersham Biosciences) and radioactivity was determined by liquid scintillation counting.

**ELISA**

Cells were treated with 0.1 or 5 ng/ml IL-1β for 24 h, in the presence or absence of the IκB phosphorylation inhibitor BAY-11-70-82 (10 μM; Calbiochem) (22) and IL-6 was measured by ELISA.

**Real-time PCR**

WT and MIF−/− cells were treated with 0.1 or 5 ng/ml IL-1 for 3 h. RNA was extracted using TRizol reagent (Invitrogen Life Technologies). One microgram of RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen Life Technologies) and PCR amplification was performed on a Rotor-Gene 3000 (Corbett Research) using LightCycler DNA Master SYBR Green I kit (Roche). Marine IL-6 (23), IL-1R1 (forward, 5'-TCCGCGACTAAGGGAGAAA-3', reverse, 5'-CTCTTCCCAATTCGATCTCACA-3'), p55 TNFR (24), and β-actin (23) primers were used. mRNA was normalized to β-actin expression, and results were presented as the fold induction of mRNA expression relative to control samples.

**Statistical analysis**

Results are expressed as the mean ± SEM. Analysis was performed using the Student t test, and p values <0.05 were considered statistically significant. All data are the result of at least three separate experiments.

**Results**

**Endogenous MIF is required for IL-1-induced MAPK, AP-1 activation, and cell proliferation**

We examined IL-1-induced MAPK activation in WT and MIF−/− cells. A total of 0.1 ng/ml IL-1 induced robust MAPK activation in WT cells (Fig. 1A, left panel). In contrast, MIF−/− cells were markedly hyporesponsive to IL-1 activation of all three MAPK (p38, ERK, and JNK) phosphorylation at all time points. In dose response experiments, IL-1 induced dose-dependent phosphorylation of p38 in WT cells but this was impaired in MIF−/− cells (Fig. 1A, right panel).

We next examined downstream effects on AP-1 activation, using EMSA and reporter gene assays. For EMSA, cells were treated with 0.1 or 5 ng/ml IL-1 for 1 h and nuclear protein was extracted. Specific binding was determined using a radioiodinated AP-1 probe, and nonspecific binding with a mutated (mut) probe (Fig. 1B, left panel). Supershift was demonstrated with anti-fos or anti-jun Abs. Compared with WT cells, constitutive and IL-1-induced AP-1 DNA-binding activity was significantly reduced in MIF−/− cells (Fig. 1B, right panel; p < 0.05). Similarly, AP-1 luciferase activity was reduced in MIF−/− compared with WT cells (Fig. 1C; p < 0.05).

MIF has a key role in regulation of cell growth and proliferation via activation of MAPK in various cell types (3, 4, 9). To determine the functional significance of the observed MIF-dependent alterations in cytokine-induced signal transduction, we examined cell proliferation using [3H]thymidine incorporation in WT and MIF−/− cells. Both baseline and IL-1-induced proliferation were significantly reduced in MIF−/− fibroblasts compared with WT cells (Fig. 1D; p < 0.05). Thus, endogenous MIF was required for both basal and cytokine-induced cell proliferation. To ensure that MIF was indeed expressed by these cells, MIF expression in cell lysates was measured by Western blotting (Fig. 1E). MIF protein was readily detected in WT, but not MIF−/−, cells. Expression of MIF was also confirmed in WT MEF. Confirmation of the normal regulation of MIF expression in both cell types was confirmed by the observation of increased MIF expression in response to the glucocorticoid dexamethasone in WT but not MIF−/− cells (Fig. 1E).

**Endogenous MIF is not essential for IL-1-induced NF-κB activation and IL-6 expression**

We next investigated IL-1-induced NF-κB activation by analysis of IκBα degradation by Western blotting and NF-κB EMSA. IL-1-induced IκBα degradation was similar in WT and MIF−/− cells (Fig. 2A). For EMSA, specific binding was shown using a radioiodinated NF-κB probe, and nonspecific binding with a mutated (mut) probe (Fig. 2B, left panel). Supershift was demonstrated with anti-p50, anti-p65 Abs. There was no difference detected in IL-1-induced NF-κB DNA binding between MIF−/− and WT cells (Fig.
Endogenous MIF is required in IL-1-induced MAPK, AP-1 activation, and cell proliferation. A, WT and MIF−/− MDF were treated with 0.1 ng/ml IL-1 over 60 min (left panel). MAPK phosphorylation was measured by Western blot, and total ERK used as a loading control. The result is representative of three separate experiments. Hyporesponsiveness of MIF−/− cells to IL-1 is dose responsive (right panel). WT and MIF−/− cells were treated with IL-1 for 30 min, phosphorylated p38 was measured by Western blot, and total p38 was used as loading control (representative of n = 3). B, MIF WT MDF nuclear extracts bind AP-1 (left panel). Extracts were incubated with radiolabeled probe encompassing the AP-1 nucleotide motifs (ATG ACT CAG) (40,000 cpm) in the presence or absence of nonradiolabeled competitor WT (×100) or mutated (mut) probe (×100). For Ab supershift analysis, extracts were incubated with anti-c-fos or c-jun before addition of probe. DNA-protein complexes were separated from free probe by gel electrophoresis. MIF−/− cells are hyporesponsive to IL-1-induced AP-1 DNA binding by EMSA (right panel). Nuclear extracts from cells treated with IL-1 were incubated with the AP-1-radiolabeled probe, and DNA/protein complexes were separated from free probe by gel electrophoresis. Densitometry of AP-1 values were expressed as AU (mean ± SEM; n = 3; p < 0.05). *p < 0.05 compared with MIF WT cells. C, AP-1 transactivation is reduced in MIF−/− cells. MIF WT and MIF−/− cells were transfected with 1 μg of AP-1-Luc reporter plasmid, and 50 ng of pCMV5-β-GAL for 24 h. Luciferase activity was normalized to β-galactosidase activity (mean ± SEM; n = 3 in triplicate). *p < 0.05 compared with MIF WT cells. D, MIF WT and MIF−/− cells were treated with 0.1 ng/ml IL-1 for 48 h, labeled with 1 μCi/ml [3H]thymidine for 18 h, and cell proliferation was determined in duplicate cultures and expressed as [3H]thymidine incorporation (cpm) (mean ± SEM; n = 6). *p < 0.05 compared with WT MIF WT cells. E, MIF WT and MIF−/− cells (MDF, left panel; MEF, right panel) were treated in the absence or presence of 10−7 M dexamethasone for 24 h. MAPK expression was measured by Western blot and β-actin was used as a loading control (representative of n = 3).

The MAPK activation cascade, we next examined IL-1-induced activation of MKK3/6, the kinase upstream of p38 (26, 27) (Fig. 3A). Compared with WT cells, MIF−/− fibroblasts were hyporesponsive to IL-1-induced MKK3/6 phosphorylation. These data demonstrated that in the absence of endogenous MIF, impairment of IL-1-induced signaling events occurred upstream of MAPK activation. To examine whether this defect was specific to cytokine receptor-induced signaling, cells were treated with the osmotic stress agent sorbitol (0.5 M) for 30 min (Fig. 3B). In contrast to the impairment of p38 phosphorylation in IL-1-treated MIF−/− cells, phosphorylation of p38 induced by sorbitol was similar in MIF−/− and WT fibroblasts. This suggested that hyporesponsiveness of MIF−/− cell MAPK activation was stimulus specific, and that a reduction in endogenous MIF may impede MAPK signaling at some point between binding of IL-1 to the IL-1R complex and upstream of MEK/3/6 activation.

Decreased type 1 IL-1R expression in MIF−/− cells

To determine whether hyporesponsiveness in MIF−/− cells related to alterations in cytokine receptor expression, expression of the IL-1R1 was analyzed by flow cytometry and real-time PCR in WT and MIF−/− cells. As measured by flow cytometric analysis of IL-1R-positive cells, cell surface expression of IL-1R1 was 4-fold lower in MIF−/− compared with WT cells (Fig. 3C; p < 0.05). Similarly, expression of IL-1R as measured by
mean fluorescence intensity (MFI), was 6.7-fold lower in MIF−/− compared with WT cells (p = 0.01) (Table I). Correspondingly, expression of the IL-1R1 mRNA was 10-fold lower in MIF−/− cells compared with WT cells (Fig. 3D; p < 0.05).

Decreased p55 TNFR expression in MIF−/− cells
To investigate whether these effects were restricted to the IL-1R, we examined receptor expression of TNF. Cell surface p55 TNFR expression, as measured by flow cytometric analysis of TNFR-positive cells, was 3-fold lower in MIF−/− cells compared with WT cells (Fig. 3E; p < 0.05). The expression of p55 TNFR, as measured by FACS, was 2.7-fold lower in MIF−/− compared with WT cells (Table I; p = 0.0006). Expression of p55 TNFR mRNA was similarly 2.5-fold lower in MIF−/− cells compared with WT cells (Fig. 3F; p < 0.05).

Endogenous MIF is required for TNF-induced MAPK phosphorylation and cell proliferation
To confirm the functional significance of the observed reduction in TNF expression in the absence of MIF, we examined TNF-induced MAPK activation and proliferation. A total of 1 ng/ml TNF induced robust MAPK activation in WT fibroblasts (Fig. 4A). In contrast, TNF-induced ERK and JNK phosphorylation was impaired in MIF−/− cells, but in contrast to the results observed for IL-1 stimulation, p38 phosphorylation was intact. This indicates differential requirements for endogenous MIF in IL-1- compared with TNF-induced p38 MAPK activation. The role of endogenous MIF in MAPK-dependent TNF-induced cell activation was examined by cell proliferation. Similar to the results observed with IL-1 treatment, TNF-induced proliferation was significantly impaired in MIF−/−, compared with WT cells (Fig. 4B). Our data indicated that up-regulation of cytokine receptor expression and receptor-dependent signaling events by endogenous MIF were not specific to the IL-1R1, but included regulation of TNF-R1 expression and function.

Reconstitution of MIF in MIF−/− cells restores p55 TNFR expression
To confirm the regulation of cytokine receptors by MIF, we next investigated whether reconstitution of MIF in MIF−/− cells could restore cytokine receptor expression. We examined the effects of MIF transfection on p55 TNFR expression in studies using MIF−/− MEF. Reduced expression of p55 TNFR was confirmed in MIF−/− MEF compared with WT MEF by real-time PCR (Fig. 4C). MIF−/− MEF were cotransfected with an EGFP reporter plasmid (pEGFP) together with an empty vector (pcDNA3.1+) or an MIF-expressing vector (pcDNA3.1+MIF) for 48 h, then p55 TNFR expression was determined using flow cytometry as described in Materials and Methods. MIF transfection significantly increased p55 TNFR expression in MIF−/− MEF (Fig. 4D). These findings were also confirmed in MDFs (Fig. 4E). Expression of p55 TNFR was increased in both transfected (EGFP+) and untransfected (EGFP−) cells, indicating autocrine-paracrine effects of MIF.

p55 TNFR expression is regulated via MAPK/AP-1 activation
We next investigated the molecular mechanism by which MIF modulates p55 TNFR expression. We hypothesized that down-regulation of cytokine receptor expression in the absence of MIF is dependent on reduced MAPK/AP-1 transcriptional activity. The
upstream kinase MEKK is known to be a strong activator of the MAPK/AP-1 pathway (28–30). We examined whether activation of the MAPK/AP-1 pathway via MEKK could up-regulate TNFR expression in MIF−/− dermal fibroblasts. pFC-MEKK, or a control plasmid, were cotransfected with pEGFP in MIF−/− cells and p55 TNFR expression analyzed in transfected cells by flow cytometry. MEKK transfection of MIF−/− cells was associated with increased p55 TNFR expression (Fig. 4F), suggesting the involvement of the MAPK/AP-1 pathway in the reduced TNFR expression observed in MIF−/− cells. MEKK transfection was confirmed to induce activation of downstream components of the MAPK cascade Elk 1, CHOP, and c-jun, compared with control transfected cells.

**Discussion**

Unlike other cytokines, abundant preformed and renewable stores of MIF are present in multiple immune and nonimmune cell types (4, 16, 31–33). Constitutively expressed MIF has immunoregulatory functions in sepsis, inflammatory diseases, and tumorigenesis (4, 12, 13, 34, 35). Thus, MIF is well-placed to potentially mediate facilitatory effects on the actions of other cytokines. MIF, TNF, and IL-1 have established roles in the pathogenesis of multiple immunopathologies, however, the mechanism(s) underlying their interactions is unknown. MIF is released as early as or earlier than IL-1 or TNF from cellular stores. In vivo, MIF deficiency or neutralization leads to protection against lethal bacterial sepsis and septic shock induced by Gram-negative endotoxin (17, 36) or Gram-positive exotoxin (37), and amelioration of other chronic inflammatory diseases in the context of reduced MIF is associated with reduced circulating or local TNF and IL-1 production (17, 38, 39). In vitro, MIF-deficient cells exhibit impaired TNF production in response to LPS, an effect mediated via MIF regulation of TLR-4-dependent cellular responses. Herein, we demonstrate that MIF regulates cellular receptor-dependent MAPK/AP-1 activation responses to proinflammatory cytokines independent of NF-κB.

We observed an essential role for endogenous MIF in cytokine-induced MAPK activation and basal and cytokine-induced MAPK-dependent proliferation. This is consistent with the action of exogenous MIF on MAPK/AP-1 activation (3, 4, 8, 9, 40, 41), and MAPK-dependent control of cell growth and tumorigenesis (7, 42, 43). In NIH3T3 cells, MIF was required for ERK-induced cell proliferation (3, 4, 6). In these same cells, both exogenous and endogenous MIF modulated adhesion-dependent sustained ERK activation, cyclin D1 expression, and cell cycle progression (5). Similarly, MIF induces ERK-dependent RA FLS proliferation (9, 14), whereas MIF immunoneutralization prevents cytokine-induced RA FLS proliferation (9). MIF immunoneutralization also prevents IL-1-induced ERK-dependent PLA2, and p38-dependent COX-2 expression (9), further supporting a permissive role of MIF in cytokine-induced MAPK-dependent cellular activation.

**Table 1. IL-1R1 and p55 TNFR expression**

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<th>MIF WT</th>
<th>MIF−−−</th>
<th>p Value</th>
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<tbody>
<tr>
<td>IL-1R1 MFI</td>
<td>19.45 ± 7.50</td>
<td>2.90 ± 0.53</td>
<td>0.01</td>
</tr>
<tr>
<td>p55 TNFR MFI</td>
<td>7.73 ± 0.77</td>
<td>3.98 ± 0.56</td>
<td>0.0006</td>
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*IL-1R1 and p55 TNFR expression was measured by flow cytometry and MFI was calculated after subtraction of MFI in control-Ab-labeled cells. Values are expressed as the mean ± SEM for IL-1R1 (n = 3) or p55 TNFR (n = 6) positive cells. Values of p are for MIF WT compared with MIF−−− cells.
In contrast, our study, MIF was not required for cytokine-induced NF-κB activation or NF-κB-dependent IL-6 expression. This finding is in keeping with several previous reports. In RA FLS, for example, MIF does not induce NF-κB at concentrations that activate ERK (21). Moreover, anti-MIF mAb inhibits IL-1-induced cell activation, without effects on NF-κB, and NF-κB inhibition does not inhibit the biological effects of MIF (21). Up-regulation of NF-κB DNA binding by MIF has been reported in RA FLS, but only at supraphysiologic concentrations (1 μg/ml) (44), whereas induction of AP-1 DNA binding occurred at a more physiological concentration. MIF has been reported not to directly affect IκB kinase activity (45) TNF-induced NF-κB was identical in control and MIF antisense-transfected cells (46). In contrast, in the context of reduced TLR-4 expression, MIF-deficient macrophages were hypersensitive to LPS-induced NF-κB activity (13). Both p38 MAPK and NF-κB have been reported to be implicated in the regulation of IL-6 expression (10, 25). However, NF-κB is essential as NF-κB p50/p65-deficient fibroblasts do not express IL-6 in response to IL-1 (47). In our study, IL-1-induced IL-6 remained intact despite a profound reduction in IL-1-induced p38 phosphorylation in MIF−/− cells, and NF-κB antagonism lead to near complete abrogation of IL-1-induced IL-6. This suggests that NF-κB is essential for IL-1-dependent IL-6 expression, and that this is independent of MIF. This is supported by data demonstrating no difference in LPS-induced IL-6 expression in MIF−/− or WT peritoneal macrophages (17), and increased IL-6 expression in MIF−/− macrophages following cutaneous Leishmania major infection (48).

FIGURE 4. A, Endogenous MIF is required for p55 TNFR-induced MAPK activation. WT and MIF−/− MDF were treated with 1 ng/ml TNF over 60 min. MAPK phosphorylation was measured by Western blot. Membranes were serially stripped and reprobed for phospho-MAPK and total ERK was used as a loading control. The result is representative of three separate experiments. B, Endogenous MIF is required in TNF-induced cell proliferation. MIF WT and MIF−/− cells were treated with 1 ng/ml TNF for 48 h and labeled with 1 μCi/ml [3H]thymidine for 18 h. Cell proliferation was determined from duplicate cultures and expressed as [3H]thymidine incorporation (cpm) (mean ± SEM; n = 6). *, p < 0.05 compared with MIF WT cells. C, Down-regulation of p55 TNFR expression in MIF−/− MEF. p55 TNFR expression was measured in WT and MIF−/− MEF by real-time PCR. The results, based on a ratio of p55 TNFR mRNA/β-actin amplification, are presented as the fold induction in p55 TNFR mRNA expression relative to control samples (mean ± SEM; n = 3). *, p < 0.05 compared with MIF WT MEF. D and E, Reconstitution of MIF restores p55 TNFR expression. For MIF reconstitution experiments, MIF−/− cells were transfected with 0.5 μg of empty vector (pcDNA 3.1+) or 1 μg of pcDNA 3.1+ MIF together with 0.5 μg of pEGFP using Lipofectamine 2000 (MEF) (D) or Amaxa nucleofactor technology (MDF) (E) for 48 h. †, p < 0.05 compared with empty vector, pcDNA 3.1+. F. Reconstitution of MEKK restores p55 TNFR expression and MAPK/AP-1 activation. MIF−/− MDF were transfected with 50 ng of an irrelevant vector pPUC as a control or pFC-MEKK and cotransfected with 0.5 μg of pEGFP using Amaxa nucleofactor technology for 48 h. Cells were then incubated with mAb against p55 TNFR and EGFP-positive cells analyzed by flow cytometry. Representative histograms are shown with background isotype control staining in gray. The percentage of p55 TNFR-positive cells is shown adjusted for isotope control staining (mean ± SEM). G, Effects of MEKK transfection on the transcriptional activity of downstream components of the MAPK/AP-1 cascade. MIF−/− cells were cotransfected with 50 ng of pFC-dbd, FA2-Elk1, pFA2-c-jun, or pFA2-ChOP, and pFR-luc (Promega). Results are expressed as fold increase, mean ± SEM, n = 3 of luciferase activity relative to β-galactosidase activity of three separate experiments performed in triplicate.
MIF deficiency was associated with a marked reduction in TNF and IL-1R expression. An upstream defect in cytokine receptor-induced MAPK pathway activation was supported by impairment of cytokine-induced MKK3/6 phosphorylation in MIF−/− cells, whereas osmotic stress-induced p38 phosphorylation was intact. Reduced MAPK/AP-1 activation in MIF-deficient cells could be explained by, or alternately be the explanation for, reduced cytokine receptor expression. MIF up-regulates macrophage TLR-4 expression via the transcription factor PU.1 (13). The IL-1R shares homology with TLR-4, and the promoter region contains binding sites for the PU.1 and AP-1 transcription factors (49, 50). PU.1 is, however, only expressed in cells of myeloid differentiation lineage, and hence was not a candidate for regulation of IL-1R expression in fibroblasts (51). Although little is known about the transcriptional regulation of p55 TNFR, the p75 TNFR is known to contain binding sites for AP-1 (52). Transfection of MIF−/− cells with the upstream activator of MAPK, MEKK, was associated with activation of Elk 1, CHOP, and c-Jun, and up-regulation of p55 TNFR expression. This suggests that MIF deficiency may impair proinflammatory cytokine receptor expression via the effects observed here on the MAPK/AP-1 pathway.

In addition to the role of endogenous MIF in regulating IL-1R1 and TNFR1 expression, we observed uncoupling of cytokine-induced MIF-dependent cell proliferation from NF-κB-dependent IL-6 expression. Binding of TNF to the p55 TNFR leads to activation of a multiprotein signaling complex with release of slenker of death domains, recruitment of TNFR-associated death domain protein, and the adaptor proteins TNFR-associated factor 2 (TRAF2) and receptor-interacting protein, which ultimately activate MAPK and NF-κB pathways involved in inflammation, proliferation, and apoptosis (53). Uncoupled receptor-dependent MAPK and NF-κB signaling has been previously described for other TNFR superfamily members and TLR-4, either at the receptor level or through recruitment of other downstream components (54, 55). Recent studies suggest that regulation of p55 TNFR and downstream components such as TRAF2 may also lead to divergent effects on MAPK and NF-κB signaling. For example, down-regulation of p55 TNFR by phosphorylation has been shown to selectively decrease MAPK activation in the presence of preserved NF-κB function (56). In contrast, preferential activation of MAPK but not NF-κB has been reported to be determined by the location of the p55 TNFR in lipid or nonlipid raft components of the plasma membrane (57). Upon activation by TNF, p55 TNFR is segregated to predominantly lipid rafts in the plasma membrane. In parallel, ERK MAPK localizes to the same compartment whereas IkB remains in the nonlipid compartment. In addition, downstream components of the p55 TNFR complex such as TRAF2 recruited by slenker of death domains display similar diversity in regulation of MAPK independent of NF-κB (58, 59).

In conclusion, endogenous MIF has been shown here to exert a permissive regulatory role in cellular responsiveness to key proinflammatory cytokines TNF and IL-1 via up-regulation of cytokine receptor-dependent MAPK signaling independent of NF-κB. Autocrine up-regulation of cytokine-mediated MAPK-dependent events by endogenous MIF may provide a previously unrecognized mechanism for the amplifying role of MIF in immunity, and tumorigenesis, and the observed benefits of MIF deficiency in experimental models of inflammatory disease. Due to the widespread expression of endogenous MIF, IL-1R1, and the p55 TNFR in both immune and nonimmune cells, anti-MIF therapies would be anticipated to have significant anti-inflammatory effects. These data emphasize the role of MIF in regulating the set-point of the cytokine activation response and the potential impact of anti-MIF strategies in inflammatory disease.

Disclosures

The authors have no financial conflict of interest.

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