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Macrophage Migration Inhibitory Factor and CD74 Regulate Macrophage Chemotactic Responses via MAPK and Rho GTPase

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Macrophage migration inhibitory factor (MIF) promotes leukocyte recruitment to sites of inflammation. However, whether this stems from a direct effect on leukocyte migration is unknown. Furthermore, the role of the MIF-binding protein CD74 in this response has not been investigated. Therefore, the aim of this study was to examine the contributions of MIF and CD74 to chemokine-induced macrophage recruitment. Intravital microscopy studies demonstrated that CCL2-induced leukocyte adhesion and transmigration were reduced in $MIF^{-/-}$ and $CD74^{-/-}$ mice. $MIF^{-/-}$ and $CD74^{-/-}$ macrophages also exhibited reduced chemotaxis in vitro, although $CD74^{-/-}$ macrophages showed increased chemokinesis. Reduced CCL2-induced migration was associated with attenuated MAPK phosphorylation, RhoA GTPase activity, and actin polymerization in $MIF^{-/-}$ and $CD74^{-/-}$ macrophages. Furthermore, in $MIF^{-/-}$ macrophages, MAPK phosphatase-1 was expressed at elevated levels, providing a potential mechanism for the reduction in MAPK phosphorylation in MIF-deficient cells. No increase in MAPK phosphatase-1 expression was observed in $CD74^{-/-}$ macrophages. In in vivo experiments assessing the link between MIF and CD74, combined administration of MIF and CCL2 increased leukocyte adhesion in both $MIF^{-/-}$ and $CD74^{-/-}$ mice, showing that CD74 was not required for this MIF-induced response. Additionally, although leukocyte recruitment induced by administration of MIF alone was reduced in $CD74^{-/-}$ mice, consistent with a role for CD74 in leukocyte recruitment induced by MIF, MIF-treated $CD74^{-/-}$ mice displayed residual leukocyte recruitment. These data demonstrate that MIF and CD74 play previously unappreciated roles in CCL2-induced macrophage adhesion and migration, and they indicate that MIF and CD74 mediate this effect via both common and independent mechanisms. *The Journal of Immunology*, **2011, 186: 4915–4924.**

Recruitment of leukocytes to sites of inflammation involves a multistep cascade of interactions between leukocytes in the vascular lumen and endothelial cells in vessel walls (1, 2). After first rolling and adhering on the endothelial surface, arrested leukocytes migrate over the endothelial surface to find an optimal site for transmigration, and then exit the vasculature (3, 4). Upon entering the interstitium, leukocytes are directed toward the source of the inflammatory stimulus by chemoattractants. During this process, leukocytes are exposed to numerous inflammatory mediators, including multiple chemoattractant species. Studies suggest that leukocytes navigate this complex milieu using specific intracellular signaling molecules to prioritize chemotactic cues derived from multiple chemoattractants (5–7). Signals induced in response to ligation of G protein-coupled chemoattractant receptors and/or selectin ligands such as PSGL-1 mediate integrin activation required for leukocyte arrest (8–10). Subsequent leukocyte chemotaxis involves the coordinated actions of multiple intracellular signaling molecules, including the PI3K-Akt, MAPK, and Rho GTPase pathways (11–17). Additionally, leukocyte signaling during chemotaxis may be affected by mediators present in the inflammatory milieu other than classical chemoattractants. An example of such a mediator is macrophage migration inhibitory factor (MIF).

MIF is a pleiotropic proinflammatory protein that has been found to contribute to many inflammatory diseases (reviewed in Ref. 18). The effects of MIF include the promotion of cytokine expression, inhibition of apoptosis, and counter-regulation of the effects of endogenous glucocorticoids (19). A growing body of evidence now indicates that MIF also promotes leukocyte accumulation during pathological inflammatory responses (20–24). Local administration of exogenous MIF can induce leukocyte recruitment, via promotion of CCL2 release from endothelial cells (25), and induction of leukocyte arrest and chemotaxis (26). However, as MIF is widely expressed, including intracellularly in cells such as macrophages and endothelial cells, it is unclear whether the effects of locally administered MIF mimic the actions of endogenous MIF (27). Our intravital microscopy studies of leukocyte recruitment in MIF-deficient mice have demonstrated that endogenous MIF promotes leukocyte–endothelial cell interactions

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Abbreviations used in this article: MIF, macrophage migration inhibitory factor; MKP-1, MAPK phosphatase-1; WT, wild-type.

and leukocyte migration into tissues (28, 29). In recent in vitro work, we have observed that endothelial cell-expressed MIF increases leukocyte–endothelial cell interactions via promotion of endothelial adhesion molecule expression (30). However, whether endogenous MIF expressed by leukocytes has additional effects that modulate their migratory function has not been investigated.

The ability of MIF to modulate numerous intracellular signaling pathways, including several known to be involved in leukocyte migration, raises the possibility that endogenous MIF promotes leukocyte recruitment via effects on leukocyte migration. For example, MIF deficiency impairs MAPK signaling entrained by the proinflammatory cytokines IL-1 and TNF or by Ag-specific T cell activation (31–33), and MIF also activates the PI3K–Akt pathway (34, 35). Whether MIF affects leukocyte migration via effects on these pathways has not been examined. An additional poorly understood aspect of the MIF-dependent effect on leukocyte recruitment is the identity of the cellular receptor via which MIF mediates this effect. CD74 (the MHC class II-associated invariant chain, Ii) has been reported to bind MIF and to be required for MIF-dependent cellular responses such as PGE_2 production and proliferation (36). Blockade of CD74 reduces MIF-dependent monocyte arrest on ex vivo carotid arteries from atherosclerotic mice, and it reduces MIF-induced pulmonary chemokine expression and neutrophil recruitment (26, 37). CD74 has also been shown to contribute to MIF-induced Akt and ERK MAPK activation (36, 38). However, the role of CD74 in chemokine-mediated signaling has not been investigated. Therefore, the aim of these studies was to examine the role of MIF and CD74 in promoting chemokine-induced leukocyte migration, using CCL2 as a model monocyte chemoattractant.

Materials and Methods

Animals

The generation of $MIF^{-/-}$ and $CD74^{-/-}$ mice has been described elsewhere (39, 40). Mice were maintained on the C57BL/6 background and wild-type (WT) C57BL/6 mice were used as controls. All animal experiments were performed in accordance with the regulations of Monash University Animal Ethics Committee.

Cytokine/chemokines

Recombinant human MIF was produced in an *Escherichia coli* expression system (32). Recombinant murine CCL2 was purchased from PeproTech (Rocky Hill, NJ). Recombinant murine CSF-1 was purchased from R&D Systems (Minneapolis, MN). fMLF was purchased from Sigma-Aldrich (St. Louis, MO).

Intravital microscopy

Intravital microscopy of the cremaster muscle was performed as previously described (25). Briefly, the cremaster muscle of anesthetized (ketamine/xylazine) mice was exteriorized onto an optically clear viewing pedestal and the cremasteric microcirculation was visualized using an intravital microscope (Axioplan 2 imaging; Carl Zeiss, North Ryde, NSW, Australia). Three postcapillary venules (25–40 μm in diameter) were examined for each experiment. Images were visualized using a video camera and recorded on videotape for subsequent playback analysis. Leukocyte–endothelial cell interactions (rolling, adhesion, and emigration) were assessed as described previously (25). CCL2 (345 ng) or MIF (1 μg) were injected intrascrotally in 200 μl saline 4 h prior to examination via intravital microscopy, as previously described (25, 41).

Cell isolation

Bone marrow-derived macrophages were generated via differentiation from bone marrow precursors by culturing bone marrow cells for 5 d in RPMI 1640 (Sigma-Aldrich, Castle Hill, NSW, Australia) supplemented with 10% FCS, 50 U/ml penicillin, and 50 ug/ml streptomycin (Invitrogen, Carlsbad, CA) in the presence of CSF-1 (1 ng/ml). Peritoneal macrophages were isolated from mice using a modification of a published technique (42). Briefly, mice were injected i.p. with 4% thioglycollate (2 ml), and cells were harvested 4 d later via peritoneal lavage. Cells were incubated for 1 h and nonadherent cells discarded. Cells harvested were routinely >90% macrophages as defined by expression of F4/80 and CD115.

In vitro macrophage migration assays

In vitro macrophage migration assays were performed using a chemotaxis chamber (Neuro Probe, Gaithersburg, MD) as described previously (43). Briefly, CCL2 (10–200 ng/ml) or fMLF (10^{-11} M) in RPMI 1640/0.1% BSA was added to the bottom chamber, on top of which was placed a 5-μm pore size membrane filter followed by a silicone gasket and the top chamber. Thioglycollate-elicited peritoneal macrophages (1×10^5 in 50 μl RPMI 1640/1% FCS) were applied to the top chamber and then incubated for 2.5 h at 37°C, 5% CO_2. Macrophage migration was determined according to the manufacturer's instructions. In brief, data were generated for wells without chemoattractant, in addition to wells (bottom) incubated with either CCL2 or fMLF. Unless otherwise stated, data for each strain are shown as cells per field of view for treated cells following subtraction of cells per field of view in untreated wells. Chemokinesis controls were performed with equal amounts of CCL2 in the top and bottom wells.

Leukocyte adhesion molecule and chemokine receptor expression

Bone marrow derived-macrophages were examined for expression of adhesion molecules and chemokine receptors using flow cytometry. Alternatively, whole blood from various mouse strains was lysed using NH_4Cl, and monocytes were identified via staining for CD115 and F4/80, as previously described (44). The following Abs used in these experiments were purchased from BD Biosciences (San Diego, CA) unless stated otherwise: anti–CD45-allophycocyanin, anti–CD11b-FITC, anti–CD115-PE (eBioscience, San Diego, CA), anti–CXCR2-allophycocyanin (R&D Systems, Minneapolis, MN), anti–LFA-1-Alexa 647 (BioLegend, San Diego, CA), and sheep anti-rabbit IgG-FITC (Silenus Laboratories, Heidelberg, VIC, Australia). Rabbit anti-mouse CCR2 was a gift from Prof. Shaun McColl (University of Adelaide). PS/2 (anti-α_4 integrin) was grown from hybridoma and conjugated to Alexa 488 (Invitrogen, Carlsbad, CA) in-house. Cells were labeled with appropriate Ab cocktails and then analyzed on a MoFlo flow cytometer (Dako, Fort Collins, CO). Cells were defined as positive relative to the staining levels achieved in cells stained with isotype control Abs.

Cell lysate preparation and Western blot analysis

Cells were cultured at various time points in RPMI 1640/0.1% FCS at 37°C, 5% CO_2 in the presence or absence of CCL2 (100 ng/ml). LPS (1 μg/ml, 30 min; Sigma-Aldrich) was used as a positive control. Cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing phosphatase and protease inhibitors (Roche Diagnostics, Indianapolis, IN) (45). Immunoblotting was performed using Abs directed against phosphorylated and total p38, ERK1/2, and Akt (Cell Signaling Technology, Danvers, MA) as described elsewhere (45). Briefly, equal amounts of cellular proteins were fractionated on 10% SDS-polyacrylamide electrophoresis gels and transferred to Hybond-C extra nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked with blocking buffer and then incubated sequentially with appropriate primary and fluorescence-conjugated secondary Abs. Membrane blot densitometry was measured by scanning using the Odyssey system (LI-COR Biosciences, Lincoln, NE). Densitometry ratios were normalized to appropriate total protein content and results expressed as relative density.

RhoA GTPase activity assay

The activity of RhoA GTPase in cell lysates from control and CCL2-treated bone macrophages was assessed using a G-LISA assay to measure the GTP-bound form of RhoA (Cytoskeleton, Denver, CO). Briefly, bone marrow-derived macrophages from WT, $MIF^{-/-}$, and $CD74^{-/-}$ mice were incubated with CCL2 (100 ng/ml, 0–30 min). Cell lysates were incubated on RhoA GTPase affinity plates and color developed using HRP detection reagent mixture. Samples were read on a Dynatech MR7000 plate reader (Primera Scientific, Princeton, NJ) and results expressed as a ratio of OD of treated cells to that of untreated cells.

Staining of F-actin fiber formation using rhodamine-phalloidin

Macrophages plated on eight-well chamber slides (Lab-Tek, Brendale, QLD, Australia) were grown for 24 h and then cultured in serum-free RPMI 1640 medium for 16 h. After incubating with CCL2 (100 ng/ml, 30 min) or 10% FCS (10 min), cells were washed once with PBS and fixed with 4% formaldehyde in PBS for 15 min. The fixed cells were washed twice with

PBS and permeabilized with 0.5% Triton X-100 in PBS. Cells were then stained with rhodamine-phalloidin (Cytoskeleton) in PBS and cell nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, CA). After washing three times with PBS, the stained cells were examined using fluorescence microscopy. In some experiments, the role of Rho in F-actin fiber formation was assessed by 4 h pretreatment of the cells with the cell-permeable Rho inhibitor CT04 (1 μg/ml) (Cytoskeleton) (46).

Measurement of MAPK phosphatase-1 mRNA via quantitative RT-PCR

Total RNA was extracted from cells using an RNeasy mini kit (Qiagen, Doncaster, VIC, Australia), and 0.5 μg RNA was reverse transcribed using oligo(dT)$_{20}$ and SuperScript III reverse transcriptase (Invitrogen). PCR amplification was performed on a Rotor-Gene 3000 (Corbett Research/Qiagen). Primers used were as follows: MAPK phosphatase-1 (MKP-1), 5′-TGTCGATAACTGAAAGCTACAA-3′ and 5′-AACTTCATGCTTGACACACC-3′, and β-actin, 5′-TGTCCCTGTATGCCTCTGGT-3′ and 5′-GATGTCACGCACGATTTCC-3′ (47, 48). Standard and sample cDNAs were diluted 1:20 and added to individual capillary tubes. Amplification (40 cycles) was conducted in a total volume of 20 μl containing primer concentrations of SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA). Melting curve analysis was performed at the end of PCR. Relative quantification of target mRNA expression was calculated and normalized to β-actin. The results are presented as the fold induction of mRNA expression relative to the amount present in control samples.

Statistical analysis

Results are expressed as the mean ± SEM. Data were analyzed using either a Student *t* test or one-way ANOVA when multiple comparisons were required. For each test, p values <0.05 were regarded as statistically significant.

Results

CCL2-induced leukocyte recruitment in vivo is impaired in MIF$^{-/-}$ and CD74$^{-/-}$ mice

We first examined the role of MIF and CD74 in leukocyte recruitment responses induced by the chemokine CCL2 (MCP-1, or JE in the murine system). As shown in Fig. 1, in WT mice, local injection of CCL2 adjacent to the cremaster muscle did not modify leukocyte rolling, but it induced substantial leukocyte adhesion and emigration within 4 h of administration. In contrast, in both MIF$^{-/-}$ and CD74$^{-/-}$ mice, CCL2-induced adhesion and emigration were significantly attenuated (Fig. 1*B*, 1*C*), indicating that both MIF and CD74 act in a previously unappreciated fashion to facilitate chemokine-induced leukocyte recruitment.

MIF$^{-/-}$ and CD74$^{-/-}$ macrophages have impaired migratory responses in vitro

To determine whether deficiency of MIF or CD74 specifically affected leukocyte migratory function, we compared chemotactic responses of isolated leukocytes in vitro. As many of the cells recruited in response to CCL2 are monocyte/macrophages (41), we examined migration responses of peritoneal macrophages from WT, MIF$^{-/-}$, and CD74$^{-/-}$ mice in a modified Boyden chamber assay. In this assay, CCL2 induced dose-dependent macrophage migration, which was eliminated by addition of CCL2 to the upper chamber (data not shown), indicating induction of directed migration (chemotaxis). Macrophages from MIF$^{-/-}$ mice showed a significant reduction in CCL2-induced migration relative to WT macrophages (Fig. 2*B*), whereas the level of random migration, as shown by the number of macrophages that migrated in the absence of CCL2, did not differ between WT and MIF$^{-/-}$ cells (Fig. 2*A*). The phenotype of CD74$^{-/-}$ cells was markedly different in that they showed significantly increased random migration (Fig. 2*D*), whereas following exposure to the CCL2 gradient, migration was significantly lower relative to that of WT cells and was also markedly reduced relative to CD74$^{-/-}$ cells not exposed to chemokine (0 value on graph) (Fig. 2*E*). A similar phenotype was observed using fMLF as a chemotactic stimulus, in that MIF$^{-/-}$ cells migrated poorly compared with WT cells (Fig. 2*C*), and fMLF-exposed CD74$^{-/-}$ cells migrated less effectively than did unstimulated cells (Fig. 2*F*). These data indicate that deficiency of either MIF or CD74 is associated with a leukocyte-intrinsic defect in chemoattractant-induced migration, but they also show a role for CD74 in inhibiting random migration of macrophages, as previously reported for dendritic cells (49). Addition of exogenous MIF (100 ng/ml) to both wells was unable to restore macrophage migration to WT levels in either MIF$^{-/-}$ or CD74$^{-/-}$ cells (data not shown).

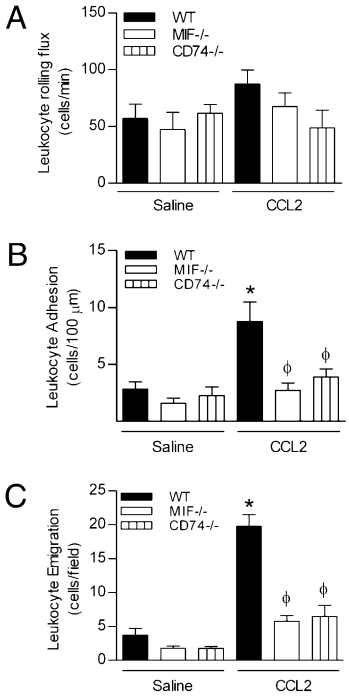

FIGURE 1. CCL2-induced leukocyte recruitment is reduced in mice lacking either MIF or CD74. Mice underwent intrascrotal injection of either saline or CCL2 (345 ng) and 4 h later leukocyte rolling (*A*), adhesion (*B*), and emigration (*C*) in postcapillary venules were assessed via intravital microscopy. Data are shown for the following mice: WT, $n = 4$ saline/$n = 12$ CCL2; MIF$^{-/-}$, $n = 4$ saline/$n = 6$ CCL2; CD74$^{-/-}$, $n = 4$ saline/$n = 10$ CCL2. *$p < 0.05$ versus WT saline, $^{\Phi}p < 0.05$ versus WT plus CCL2.

MIF$^{-/-}$ and CD74$^{-/-}$ macrophages have normal expression of adhesion molecules and chemokine receptors

Reduced cytokine responses in MIF$^{-/-}$ cells have previously been associated with decreased cytokine receptor expression (31), raising the possibility that altered chemokine receptor expression may underlie the deficient migratory responses observed in leukocytes from MIF$^{-/-}$ and CD74$^{-/-}$ mice. Therefore, we compared expression of chemokine receptors and adhesion molecules on macrophages from WT, MIF$^{-/-}$, and CD74$^{-/-}$ mice. CCR2 expression was similar in WT, MIF$^{-/-}$, and CD74$^{-/-}$ macrophages (Table I). Fig. 2*G* shows flow cytometric analysis of CCR2 expression on macrophages from WT and MIF$^{-/-}$ mice, showing overlapping expression patterns. Macrophages from CD74$^{-/-}$ mice showed essentially identical CCR2 expression (data not shown). Similarly, macrophage expression of CD11a, CD11b, and CXCR2 were not altered in MIF$^{-/-}$ or CD74$^{-/-}$ cells (Table I). In contrast, monocytes from MIF$^{-/-}$ but not CD74$^{-/-}$ mice displayed a small but significant reduction in expression of the α_4 integrin relative to that of WT cells (Table I).

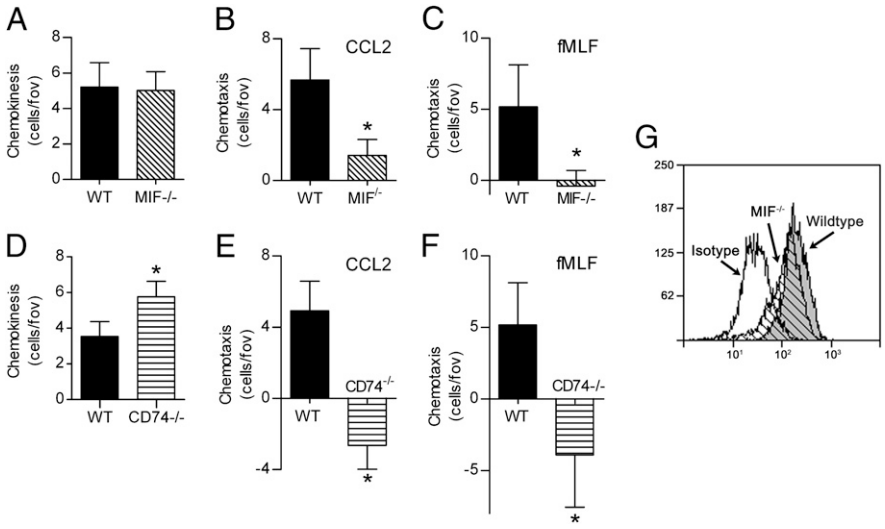

FIGURE 2. Macrophages from MIF$^{-/-}$ and CD74$^{-/-}$ mice have impaired chemoattractant-induced migration. *A–D*, Migratory responses of thioglycollate-elicited macrophages from MIF$^{-/-}$ and CD74$^{-/-}$ mice were examined in vitro in a modified Boyden chamber migration assay. *A* and *C*, Chemokinesis (random migration) of macrophages. No chemoattractant was present in either the upper or lower wells. Macrophages that had migrated to the lower side of the membrane were defined as having undergone "chemokinesis" with data expressed as cells per field of view (fov). *B* and *D*, CCL2-induced migration (chemotaxis). In these experiments, CCL2 was added to the lower well and cell migration was assessed. Data are expressed as cells per field of view, following subtraction of the data from randomly migrating cells of the same genotype. *C* and *F*, fMLF-induced migration (chemotaxis). fMLF was added to the lower well and cell migration assessed as for CCL2. Data are shown for chemokinesis and chemotaxis for WT versus MIF$^{-/-}$ cells (*A–C*) and WT versus CD74$^{-/-}$ cells (*C*, *D*, *F*). Data for cells from gene-deficient mice are compared with cells from WT mice examined in assays performed at the same time. Data represent mean ± SEM of 12–14 independent assays. *$p < 0.05$ versus WT. *G*, Flow cytometric analysis of CCR2 expression on macrophages from WT and MIF$^{-/-}$ mice. Data are shown for CCR2-stained cells from WT (filled) and MIF$^{-/-}$ mice (hatched) versus cells stained with a nonbinding isotype control Ab. Data are representative of $n = 4$ experiments (data shown in Table I).

CCL2-induced MAPK phosphorylation is reduced in MIF$^{-/-}$ and CD74$^{-/-}$ macrophages

Impaired chemotactic responses to chemokines in MIF$^{-/-}$ and CD74$^{-/-}$ leukocytes despite normal chemokine receptor expression suggested that MIF and CD74 modulate chemokine-induced intracellular signaling. We therefore studied signaling pathways (ERK and p38 MAPKs, and PI3K-Akt) that have been implicated in chemokine-induced leukocyte recruitment in other systems (13, 50–52). CCL2 did not induce phosphorylation of Akt in macrophages (data not shown), and therefore we focused on MAPK phosphorylation. CCL2 treatment of WT macrophages induced phosphorylation of ERK and p38 MAPK (Fig. 3). In macrophages from both MIF$^{-/-}$ and CD74$^{-/-}$ mice, CCL2 failed to induce an increase in ERK or p38 MAPK phosphorylation (Fig. 3), suggesting a requirement for MIF and CD74 in chemokine-induced ERK and p38 activation in this cell type. MAPK phosphorylation induced by LPS did not differ between WT, MIF$^{-/-}$, and CD74$^{-/-}$ macrophages, indicating that the impact of MIF or CD74 deficiency on MAPK phosphorylation was stimulus-specific.

Reduced CCL2-induced Rho GTPase activity in MIF$^{-/-}$ and CD74$^{-/-}$ macrophages

Swant et al. (53) have reported the facilitation by MIF of Rho GTPase activity as an upstream step in the regulation of the ERK MAPK pathway in response to growth factors. Additionally, Rho GTPases have been reported to be involved in chemotaxis induced by CCL2 (12), although the roles of MIF and CD74 in regulation of this pathway in leukocytes have not been assessed. To investigate whether CCL2-induced Rho GTPase activity was impaired in the absence of MIF or CD74, we examined RhoA GTPase activity in stimulated macrophages. In WT cells, CCL2 induced activation of RhoA GTPase within 10 min, persisting for at least 30 min (Fig. 4*A*), and actin polymerization, as shown by phalloidin staining (Fig. 4*B*, 4*C*). Actin polymerization was dependent on Rho activity as it was inhibited by the cell-permeable Rho inhibitor, CT04 (data not shown). In MIF$^{-/-}$ and CD74$^{-/-}$ macrophages, RhoA GTPase activation and actin polymerization were significantly reduced (Fig. 4*B*, 4*C*) in response to both a chemotactic stimulus (CCL2) and FCS (as a positive control)

Table I. Expression of adhesion molecules and chemokine receptors on macrophages from WT, MIF$^{-/-}$, and CD74$^{-/-}$ mice

Strain	CD11a (MFI)	CD11b (MFI)	α_4 Integrin (MFI)[a]	CCR2 (% Positive)	CXCR2 (% Positive)
WT ($n = 3$)	54 ± 7	79 ± 6	50 ± 1	66 ± 9	12 ± 4
MIF$^{-/-}$ ($n = 3$)	75 ± 7	81 ± 7	43 ± 1**	57 ± 26	15 ± 7
CD74$^{-/-}$ ($n = 5$)	59 ± 11	86 ± 5	50 ± 1	53 ± 21	13 ± 5

Data are derived from analysis of bone marrow-derived macrophages as described in *Materials and Methods* and represent MFI of staining for CD11a and CD11b, and percentage positive for CCR2 and CXCR2, relative to isotype control.

[a]Data for α_4 integrin were derived from blood monocytes, as defined by expression of CD115 and F4/80 ($n = 5$ mice for all strains). Data are shown as mean ± SEM.

**$p < 0.05$ versus WT cells.

MFI, mean fluorescence intensity.

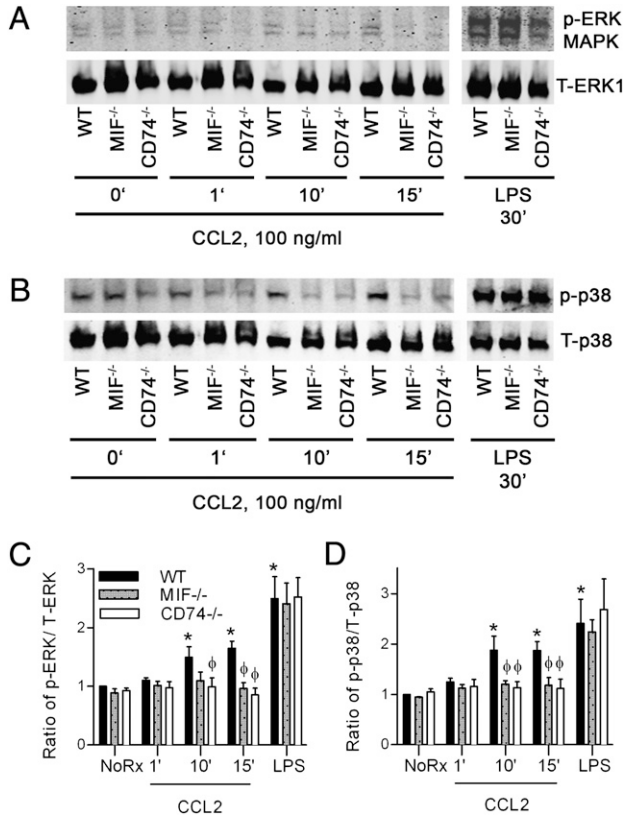

FIGURE 3. CCL2-induced MAPK activation is reduced in macrophages from MIF$^{-/-}$ and CD74$^{-/-}$ mice. Analysis of ERK and p38 phosphorylation in bone marrow-derived macrophages from WT, MIF$^{-/-}$, and CD74$^{-/-}$ mice. Macrophages were exposed to CCL2 (100 ng/ml) for 0–15 min. Subsequently, cell lysates were examined for total ERK (T-ERK1) and p-ERK (*A*), or total p38 MAPK (T-p38) and phosphorylated p38 MAPK (p-p38) (*B*) via Western blot. LPS (1 μg/ml, 30 min) was used as a positive control (shown in separate gels due to space limitations). *A* and *B*, Representative blots from at least $n = 3$ independent experiments. *C* and *D*, Densitometric analysis showing ratio of p-ERK to total ERK (*C*) or p-p38 to total p38 (*D*). Data are shown as mean ± SEM. $^{*}p < 0.05$ versus untreated (No Rx), $^{\Phi}p < 0.05$ versus WT at same time point.

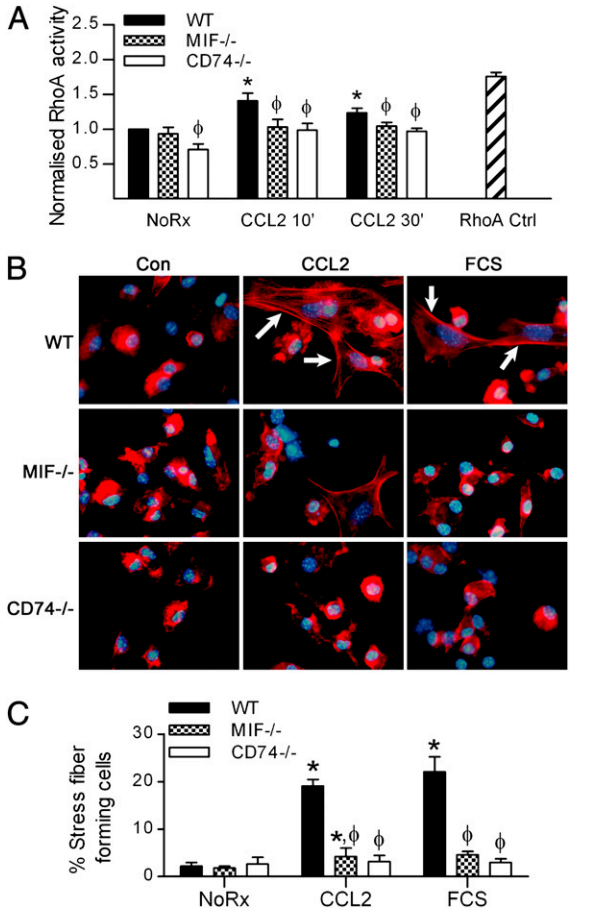

FIGURE 4. Chemokine-induced RhoA activation and F-actin fiber formation are reduced in macrophages from MIF$^{-/-}$ and CD74$^{-/-}$ mice. *A*, RhoA GTPase activation in WT, MIF$^{-/-}$, and CD74$^{-/-}$ macrophages was assessed using the G-LISA assay. Cells were untreated (No Rx) or exposed to CCL2 for either 10 or 30 min. Active RhoA was used as a positive control. Data are shown as mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus No Rx, $^{\Phi}p < 0.05$ versus WT at same time point. *B* and *C*, Comparison of actin polymerization in WT, MIF$^{-/-}$, and CD74$^{-/-}$ macrophages. Cells were either untreated (Con/No Rx) or treated with CCL2 (100 ng/ml, 30 min) or with 10% FCS (10 min) as a positive control. Actin filaments were stained with rhodamine-phalloidin (red) and nuclei were stained with DAPI (blue). Arrows indicate prominent actin polymerization in treated cells. Data are representative of at least three independent experiments. *C*, Percentage of cells with prominent actin polymerization. Data are shown as mean ± SEM ($n = 3$). $^{*}p < 0.05$ versus No Rx, $^{\Phi}p < 0.05$ versus WT at same time point.

(Fig. 4*C*). These data indicate that MIF and CD74 facilitate MAPK activation via the upstream Rho GTPase.

MIF can promote leukocyte recruitment in the absence of CD74

The current data demonstrate similar deficiencies in MIF$^{-/-}$ and CD74$^{-/-}$ macrophages, observations consistent with the hypothesis that CD74 participates in MIF-induced cellular responses (36). To test this hypothesis, we next examined the ability of exogenous MIF, injected locally adjacent to the cremaster muscle, to restore leukocyte recruitment in CCL2-treated mice lacking either MIF or CD74. Replenishment of MIF in CCL2-treated MIF$^{-/-}$ mice in this fashion restored leukocyte adhesion to levels similar to those in WT mice (Fig. 5*A*). However, this effect was also seen in CD74$^{-/-}$ mice, demonstrating that CD74 is not required for this effect of MIF (Fig. 5*A*). Moreover, MIF was also able to increase leukocyte adhesion in CCL2-treated WT mice, consistent with its ability to independently induce leukocyte recruitment (25). Notably, MIF administered in this fashion did not increase CCL2-induced leukocyte transmigration, irrespective of the presence or absence of endogenous MIF or CD74 (Fig. 5*B*).

To examine the role of CD74 in MIF-induced recruitment more directly, we assessed CD74$^{-/-}$ mice in a model of MIF-induced leukocyte recruitment we have previously characterized (25, 26). MIF treatment induced a robust leukocyte adhesion and emigration response in 4 h in WT mice (Fig. 6). This response did not occur when the MIF was first denatured by boiling, confirming that the response was due to MIF bioactivity. CD74$^{-/-}$ mice displayed significantly attenuated MIF-induced adhesion and emigration (Fig. 6*A*), although both parameters remained significantly elevated in these mice relative to saline-treated controls. Taken together, these data indicate that the roles of MIF and CD74 in promotion of leukocyte recruitment are at least partially independent.

MIF-deficient but not CD74-deficient macrophages show reduced MKP-1 expression

Previous studies have shown that expression of the MAPK-regulating protein MKP-1 is inhibited by MIF (32, 54, 55),

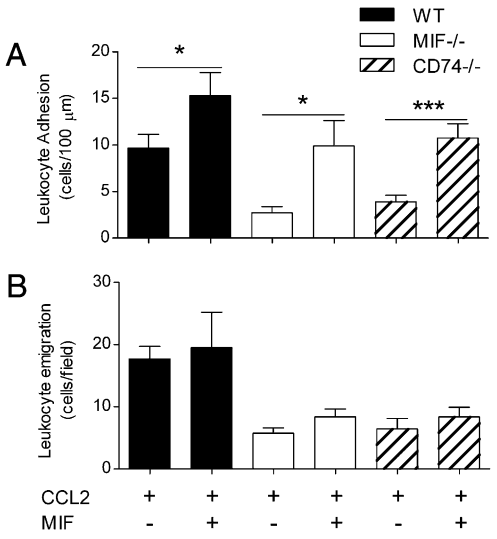

FIGURE 5. MIF increases CCL2-induced adhesion in WT, MIF$^{-/-}$, and CD74$^{-/-}$ mice. Intravital microscopic assessment of leukocyte adhesion (*A*) and emigration (*B*) in mice treated with either CCL2 alone or with CCL2 and MIF (1 μg, intrascrotally, at the same time as CCL2). Data are shown for $n = 6$ in all groups. *$p < 0.05$, ***$p < 0.001$, for the effect of addition of MIF on mice of the same strain.

providing a potential mechanism to explain the effect of MIF deficiency on MAPK phosphorylation. In the current study, MKP-1 mRNA expression in unstimulated MIF$^{-/-}$ macrophages was significantly elevated relative to that in WT cells, an effect that could be eliminated by exposing the cells to recombinant MIF (Fig. 7*A*). In contrast, MKP-1 mRNA expression in resting CD74$^{-/-}$ macrophages was not different to that in WT cells (Fig. 7*B*), providing further evidence of divergent mechanisms underlying alterations in chemokine responses in MIF$^{-/-}$ and CD74$^{-/-}$ macrophages.

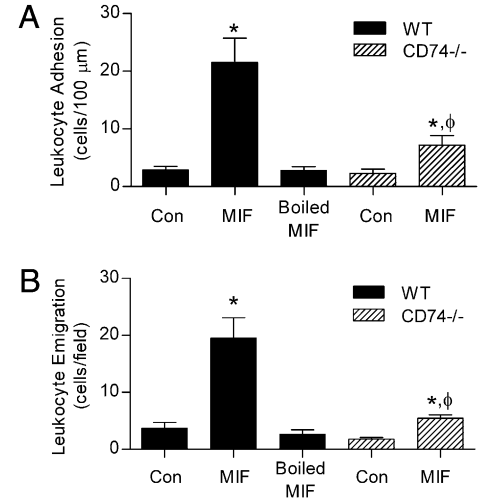

FIGURE 6. MIF-induced leukocyte recruitment is partially abrogated in CD74$^{-/-}$ mice. Intravital microscopic assessment of leukocyte adhesion (*A*) and emigration (*B*) in cremasteric postcapillary venules of WT and CD74$^{-/-}$ mice induced by local injection of either saline (Con, $n = 3$–4), MIF (1 μg, 4 h, $n = 6$), or MIF denatured by boiling (Boiled MIF, $n = 4$ in WT mice) as determined by intravital microscopy. *$p < 0.05$ versus saline control of same strain, $^{\phi}p < 0.05$ versus WT plus MIF.

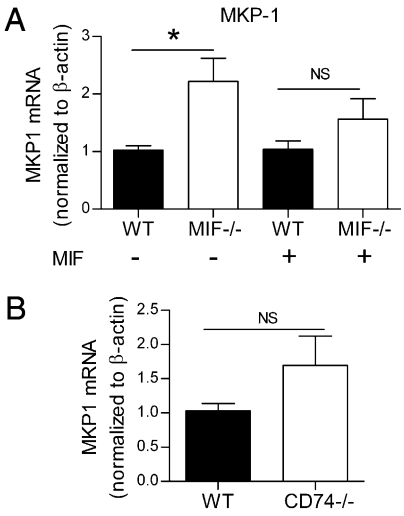

FIGURE 7. MKP-1 expression is elevated in MIF-deficient, but not CD74-deficient, macrophages. MKP-1 mRNA in bone marrow-derived macrophages as assessed using real-time PCR and expressed relative to β-actin mRNA. *A*, Comparison of MKP-1 mRNA in untreated WT and MIF$^{-/-}$ macrophages ($n = 8$) and in WT and MIF$^{-/-}$ cells exposed to MIF (100 ng/ml, 2 h, $n = 4$). Cells were isolated from WT and MIF$^{-/-}$ mice on the same day and isolated and cultured in identical conditions. *$p < 0.05$ versus WT. *B*, Comparison of MKP-1 mRNA in WT and CD74$^{-/-}$ macrophages in parallel experiments ($n = 6$–7).

Discussion

MIF has been shown to facilitate MAPK and PI3K/Akt signaling entrained by diverse stimuli (31–33, 35, 38, 56, 57), although whether MIF acts in this fashion within leukocytes to promote leukocyte recruitment is unknown (25, 28). In parallel, CD74 has been reported to transduce signals initiated by MIF in activation of ERK MAPK and Akt (34–36, 38), raising the possibility that MIF and CD74 regulate signaling events that are critical for leukocyte migration to sites of inflammation. In this study, we show that the absence of either MIF or CD74 markedly reduces macrophage chemotactic responses to CCL2, both in vivo and in vitro, concurrent with deficient CCL2-induced activation of ERK and p38 MAPK, RhoA, and actin polymerization. These observations indicate that MIF and CD74 each play important roles in promoting CCL2-induced leukocyte migration and are consistent with reports showing that CD74 acts as a key participant in MIF-mediated responses (36, 58). However, the findings that MIF could increase CCL2-induced leukocyte adhesion in vivo in CD74-deficient mice, and that MIF itself was able to promote leukocyte recruitment to some extent in CD74-deficient mice, demonstrate that CD74 is not required for all of the effects of MIF on leukocyte adhesion and migration. Moreover, findings that deficiency of CD74 but not MIF enhanced random migration of macrophages, and that expression of MKP-1 and the α_4 integrin were altered in MIF-deficient but not CD74-deficient cells, provide evidence that MIF and CD74 regulate leukocyte migration via distinct mechanisms. Nevertheless, to the best of our knowledge, these findings provide the first evidence that MIF and CD74 both promote CCL2-induced macrophage migration via facilitation of intracellular signaling processes crucial to chemoattractant responses.

During chemotaxis, the major signaling pathways activated by chemoattractants are p38 MAPK and PI3K/Akt (13, 50, 51), with emerging evidence of a role for ERK MAPK (17, 52, 59). For example, stimulation of neutrophils by chemotactic factors such as fMLP and KC has been shown to result in the activation of p38

MAPK (13, 60). We and others have shown MIF-dependent activation of ERK, p38, JNK, and Akt (31, 45, 61, 62). Moreover, activation of MAPKs by other proinflammatory stimuli has been shown to be impaired in the absence of MIF (31, 45). The latter observations raise the possibility that endogenous MIF may promote leukocyte migration via facilitation of the effects of chemokines on these signaling pathways. In this study, we observed that CCL2-induced p38 and ERK phosphorylation in macrophages was significantly reduced in the setting of either MIF or CD74 deficiency. These findings provide a mechanism explaining the altered in vitro and in vivo migration responses to CCL2 observed in macrophages lacking MIF or CD74. In contrast, MAPK phosphorylation did not differ between WT, MIF$^{-/-}$, and CD74$^{-/-}$ cells treated with LPS, indicating that more powerful inflammatory stimulation may override the ability of MIF to modulate this pathway, or that the effects of MIF and CD74 on MAPK signaling entrained by TLR activation differ from those following CCR2 activation.

The mechanism through which MIF modulates CCL2-induced MAPK activation in myeloid cells is not well understood. Swant et al. (53) reported activation of the RhoA GTPase, and Rho-dependent actin polymerization, during MIF-mediated ERK MAPK activation in a murine fibroblast cell line. In this study, we demonstrate that CCL2-induced activation of macrophage MAPK is accompanied by activation of RhoA GTPase activity and actin polymerization. Moreover, we demonstrate reductions in CCL2-induced RhoA GTPase activity and actin polymerization in the absence of MIF or CD74. These findings are consistent with a powerful enhancing role of MIF on RhoA-dependent signaling during activation of MAPKs by CCL2, in keeping with its effects on CCL2-induced chemotaxis. However, the effects of MIF on CCL2-induced signaling events may not be limited to effects on RhoA GTPase activation (63).

An additional mechanism for the MAPK-enhancing effect of MIF, particularly on p38 MAPK, may relate to its effects on MKP-1, also known as dual-specificity phosphatase-1, an enzyme that dephosphorylates activated MAPK proteins (32). MIF has previously been shown to inhibit the expression of MKP-1 in macrophages, and human rheumatoid arthritis fibroblast-like synoviocytes (31, 32, 64). Consistent with these findings, we observed increased MKP-1 expression in untreated MIF$^{-/-}$ bone marrow-derived macrophages. Given that responses to chemokines involve rapid signaling mechanisms, the constitutive elevation of MKP-1 in MIF$^{-/-}$ cells is a plausible explanation for the inhibitory effect of MIF deficiency on MAPK phosphorylation observed during rapid responses to CCL2. As such, the inhibition of MKP-1 expression by endogenous MIF in WT cells may be central to its facilitation of CCL2-induced macrophage activation and migration. MIF regulation of MKP-1 in rheumatoid arthritis fibroblast-like synoviocytes is mediated via the nuclear orphan receptor NURR1, the expression of which is upregulated in response to MIF and that in turn exerts inhibitory effects on MKP-1 transcription via a specific NURR1-responsive site in the MKP-1 promoter (64). It has not been established whether this mechanism pertains to macrophages. It is also noteworthy that the effects of MIF on MKP-1 and Rho GTPases may not be independent. The small Rho GTPase Rac has been found to be necessary for the modulation of MKP-1 expression by atrial natriuretic peptide in endothelial cells (65). In contrast, MKP-1 was not significantly increased in CD74-deficient cells, despite a similar reduction in MAPK phosphorylation. This suggests that alternative mediators of MAPK dephosphorylation, such as other members of the dual-specificity phosphatase family (66), may be upregulated in the absence of CD74, or alternately that CD74 facilitates MAPK activation through entirely distinct mechanisms.

To further understand the role of MIF in promoting monocyte migration, we examined the effect of addition of exogenous MIF to MIF-deficient cells. In vitro, addition of MIF had no effect on CCL2-induced monocyte migration. Similarly, in vivo, local injection of exogenous MIF did not increase CCL2-induced leukocyte migration, although it did increase leukocyte arrest in postcapillary venules. One interpretation of these findings is that MIF applied extracellularly cannot induce effects on leukocyte migration that mimic those induced by MIF present within cells. Notably, macrophages in particular have been shown to express large amounts of intracellular MIF constitutively (67). In contrast, exogenous MIF retained the ability to induce leukocyte arrest, both in WT mice and in mice lacking either MIF or CD74. This suggests that the mechanisms whereby MIF mediates leukocyte arrest differ from those whereby it promotes leukocyte migration. As this response was only seen in the in vivo experiments, this invokes possible roles for other cell types in the microvasculature, such as endothelial cells. Indeed, we have previously found that exogenous MIF promotes CCL2 release from endothelial cells (25), a response that may have contributed to the increased adhesion induced by exogenous MIF in the current study.

Following the original descriptions of CD74 as a MIF-binding protein capable of transducing MIF-induced signals (36, 58), CD74 has been shown to facilitate MIF-induced proliferation and survival of B cells, fibroblasts, and gastric epithelial cells (34, 68–70). These studies are supported by in vivo experiments in which anti-CD74 Ab treatment inhibited MIF-induced neutrophil recruitment into the airway and MIF-dependent bladder inflammation (37, 71). Taken together, these studies have been interpreted to mean that CD74 directly mediates the effects of MIF. In this study, we observed that MIF-induced leukocyte adhesion and emigration in the microcirculation were reduced in CD74$^{-/-}$ mice. Similarly, deficiency of CD74 and MIF had similar inhibitory effects on CCL2-induced leukocyte adhesion and migration in vivo, in vitro migration, and cellular responses such as MAPK activation and actin polymerization. These findings are each consistent with a role for CD74 in mediating the pro-recruitment effects of MIF. However, an alternative interpretation of these findings is also possible; that is, that CD74 promotes monocyte chemotactic responses to diverse stimuli independently of MIF. In addition to the phenotype of CD74$^{-/-}$ mice of attenuated recruitment responses, this possibility is supported by our observations of differences between CD74$^{-/-}$ and MIF$^{-/-}$ cells. These include increased MKP-1 and decreased α_4 integrin in MIF$^{-/-}$ but not CD74$^{-/-}$ cells, increased random migration in CD74$^{-/-}$ but not MIF$^{-/-}$ cells, and the retention of some leukocyte recruitment responses to MIF in CD74$^{-/-}$ mice. These findings provide clear evidence that MIF has CD74-independent effects on macrophage migration. However, irrespective of these divergences, to the best of our knowledge, these data provide the first evidence that both MIF and CD74 have important functions in promoting monocyte chemotactic responses.

Interpretation of these data are complicated further by studies that have demonstrated roles for CXCR2 and CXCR4 in binding MIF and mediating MIF-induced migration responses (26, 72, 73). The results of these studies suggest that CXCR2 and CXCR4 form complexes with CD74 and facilitate CD74-dependent responses to MIF. However, these molecules also were shown to bind MIF in cells lacking CD74 (26), suggesting that CD74 may not be strictly required for CXCR2 and CXCR4 to mediate this MIF-dependent function. As such, it is conceivable that either CXCR2 or CXCR4 may have been responsible for the MIF-induced responses ob-

served in CD74$^{-/-}$ mice in the current study. In vivo studies in which different MIF receptor components are targeted individually and the dynamics of the signaling complex are studied will be required to clarify the roles of these various MIF-binding proteins.

Recent studies have reported opposing functions for CD74 in cell migration. In dendritic cells, Faure-André et al. (49) demonstrated that absence of CD74 was associated with increased migratory function, both in vitro in collagen-coated transwells, and in vivo, indicating that CD74 acted as a "brake" on cell migration. CD74 was shown to associate with the motor protein, myosin II, as a potential mechanism underlying this effect. Similarly, recent work shows that activation of CD74, either by an activating Ab or via exposure to MIF, inhibits random migration of mesenchymal stem cells (74). The present observation of increased random migration of macrophages from CD74$^{-/-}$ mice supports these data and indicates that it also applies in macrophages in the absence of a chemotactic gradient. However, these findings are contrasted by recent studies of chronic lymphocytic leukemia cells in which MIF and CD74 facilitate CXCL12-induced migration and cell homing into the bone marrow, in part via upregulation of the α_4 integrin (75). Moreover, our findings of markedly reduced CCL2-induced macrophage migration in CD74-deficient macrophages, both in vitro and in vivo, also support the latter findings. Additionally, we show that the absence of CD74 results in reductions in CCL2-induced MAPK phosphorylation, RhoA activity, and actin polymerization, providing clear evidence that CD74 promotes chemotactic responses via facilitation of migration-associated intracellular signaling. The reasons underlying these divergent roles for CD74 are unclear, but cell type-specific effects relating to the level of CD74 expression in dendritic cells, versus in other nonprofessional APCs, may contribute.

In conclusion, these data indicate that both MIF and CD74 promote MAPK activation and RhoA GTPase-dependent actin polymerization in the context of promoting CCL2-induced leukocyte migration. These previously unrecognized actions of MIF add to the evidence implicating it as a key facilitator of inflammatory responses in vivo. The ability of MIF to promote inflammatory events entrained by multiple stimuli, now including CCL2, is in keeping with numerous studies demonstrating reductions in inflammatory responses in mice or cells deficient in MIF or treated with anti-MIF Abs. The multiple aspects of the immune-inflammatory response on which MIF impacts suggest that therapeutic targeting of MIF could be a powerful means of limiting the harmful effects of chronic inflammatory disease. Reduced responses of CD74$^{-/-}$ mice and cells to CCL2 clearly indicate that CD74 is also a critical regulatory molecule in these phenomena. Although the similarity of the effects of CD74 deficiency to those of MIF deficiency suggests CD74 as an essential participant in the actions of MIF, the participation of CD74 in responses to non-MIF stimulation in the form of CCL2, as well as the differences in in vitro responses between MIF$^{-/-}$ and CD74$^{-/-}$ cells, suggests the possibility that whereas the effects of CD74 overlap with those of MIF, they are independent.

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Disclosures

E.F.M. is a consultant to Cortical Pty Ltd., a biotech company involved in the development of anti-MIF therapies. R.B. is a consultant to Carolus Therapeutics and an inventor on patents for MIF therapies. The other authors have no financial conflicts of interest.

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