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J Immunol 2006; 177:8072-8079; ;
doi: 10.4049/jimmunol.177.11.8072
<http://www.jimmunol.org/content/177/11/8072>

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Macrophage Migration Inhibitory Factor Induces Macrophage Recruitment via CC Chemokine Ligand 2¹

Julia L. Gregory,* Eric F. Morand,* Sonja J. McKeown,* Jennifer A. Ralph,* Pamela Hall,* Yuan H. Yang,* Shaun R. McColl,[†] and Michael J. Hickey^{2*}

Macrophage migration inhibitory factor (MIF) was originally identified for its ability to inhibit the random migration of macrophages *in vitro*. MIF is now recognized as an important mediator in a range of inflammatory disorders. We recently observed that the absence of MIF is associated with a reduction in leukocyte-endothelial cell interactions induced by a range of inflammatory mediators, suggesting that one mechanism whereby MIF acts during inflammatory responses is by promoting leukocyte recruitment. However, it is unknown whether MIF is capable of inducing leukocyte recruitment independently of additional inflammatory stimuli. In this study, we report that MIF is capable of inducing leukocyte adhesion and transmigration in postcapillary venules *in vivo*. Moreover, leukocytes recruited in response to MIF were predominantly CD68⁺ cells of the monocyte/macrophage lineage. Abs against the monocyte-selective chemokine CCL2 (JE/MCP-1) and its receptor CCR2, but not CCL3 and CXCL2, significantly inhibited MIF-induced monocyte adhesion and transmigration. CCL2^{-/-} mice displayed a similar reduction in MIF-induced recruitment indicating a critical role of CCL2 in the MIF-induced response. This hypothesis was supported by findings that MIF induced CCL2 release from primary microvascular endothelial cells. These data demonstrate a previously unrecognized function of this pleiotropic cytokine: induction of monocyte migration into tissues. This function may be critical to the ability of MIF to promote diseases such as atherosclerosis and rheumatoid arthritis, in which macrophages are key participants. *The Journal of Immunology*, 2006, 177: 8072–8079.

In inflammatory diseases such as atherosclerosis, rheumatoid arthritis, and glomerulonephritis, monocyte/macrophages infiltrate inflamed tissues and play central roles in mediating tissue injury (1–4). To enter target sites from the circulation, monocytes must interact with and adhere to the endothelial lining, then respond to chemotactic cues to exit the vasculature and enter the affected site (5). A number of mediators have been identified which promote monocyte recruitment, including cytokines TNF, IL-1, and IFN- γ , and chemokines CCL2 (MCP-1) and CCL3 (MIP-1 α) (6–12). These mediators promote monocyte accumulation by increasing expression of endothelial adhesion molecules used by monocytes to interact with the endothelial lining and by providing chemotactic signals which arrest the monocyte on the endothelium and guide it out of the vasculature. An additional mechanism whereby accumulation of monocytes in tissues may be promoted is via the inhibition of migration of monocyte/macrophages out of the tissue. Indeed, the proinflammatory cytokine macrophage migration inhibitory factor (MIF)³ was originally identified due to its ability to inhibit random macrophage migra-

tion *in vitro* (13) raising the possibility that this cytokine could promote retention of macrophages at inflammatory sites. However, little is known regarding the ability of this cytokine to regulate leukocyte trafficking *in vivo*.

A growing body of evidence now indicates that MIF is a key player in inflammatory responses (14). In models of inflammatory diseases such as sepsis, atherosclerosis, arthritis, colitis, and experimental autoimmune encephalomyelitis, blockade or deletion of MIF attenuates inflammatory responses (15–19). The pleiotropic nature of this cytokine is illustrated by the variety of mechanisms implicated as responsible for these effects: activation of MAPK signaling (20), up-regulation of TLR4 (21), promotion of expression of proinflammatory mediators (14), counterregulation of endogenous glucocorticoids (22, 23), and inhibition of apoptosis (24). Evidence now suggests these functions also include regulation of leukocyte recruitment. MIF^{-/-} mice display reduced leukocyte rolling, adhesion, and transmigration in response to a range of mediators including LPS (25). In addition, blockade or absence of MIF results in reduced leukocyte accumulation in models of infection/endotoxemia, arthritis, and atherogenesis (17, 26–29). These findings suggest that MIF regulates leukocyte recruitment in the vasculature. However, it is unclear from these experiments whether this function of MIF occurs via an indirect pathway, involving downstream mediators regulated by MIF, or if MIF is capable of inducing leukocyte recruitment independently of other mediators.

Recent *in vitro* experiments have provided initial evidence suggesting that MIF is capable of inducing rapid recruitment responses (28). Schober et al. (28) reported that application of MIF to the apical surface of cultured endothelial cells induced MIF-dependent arrest of monocytes under flow conditions. However, it remains unknown whether MIF mediates comparable responses *in vivo*. Therefore, the aim of these experiments was to investigate the effects of MIF on the intact microvasculature, independent of

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Received for publication March 20, 2006. Accepted for publication September 14, 2006.

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¹ This work was supported by the National Health and Medical Research Council, Australia (Program Grant 334067 (to M.J.H. and E.F.M.) and Project Grant 349533 (to S.R.M.)) and the National Institutes of Health (Grant AR51807-01 (to M.J.H.)).

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³ Abbreviations used in this paper: MIF, macrophage migration inhibitory factor; PTX, pertussis toxin.

additional inflammatory stimulation. We used intravital microscopy to assess the microvasculature after exposure to MIF. These experiments reveal that MIF induces leukocyte adhesion and emigration in postcapillary venules, and that monocyte-lineage cells form the predominant population attracted into tissues by MIF.

Materials and Methods

rMIF and Abs

rMIF was produced in an *Escherichia coli* expression system (30) and provided by Cortical. The following mAbs used were purified from hybridoma supernatants: RB6-8C5/anti-Gr-1; FA-11/anti-CD68, KT3/anti-CD3; 2E6/anti-CD18 (used in vivo at 100 $\mu\text{g}/\text{animal}$); PS/2/anti- α_4 integrin (60 $\mu\text{g}/\text{animal}$); 6C7.1/anti-VCAM-1 (90 $\mu\text{g}/\text{animal}$) (hybridoma provided by Dr. D. Vestweber (Max-Planck-Institut, Meunster, Germany) and Dr. B. Engelhardt (Theodor Kocher Institute, University of Bern, Bern Switzerland)). For Abs used in vivo, rat IgG (Sigma-Aldrich) was used in control animals, except in the case of 2E6, in which hamster IgG (Auspep) served as control. Polyclonal Abs against murine CCL2, CCL3, CXCL2, CCR2, and CCL5 were generated in rabbits as described (31). In these experiments, purified rabbit IgG (Sigma-Aldrich) served as control.

Mice

Wild-type C57BL/6 mice, CCL2^{-/-} mice (32) (provided by Dr. B. Rollins, Dana-Farber Cancer Institute, Boston, MA), and P-selectin^{-/-} mice (The Jackson Laboratory) on a C57BL/6 background were all bred in-house. All animal experiments were reviewed and approved by the Monash University Animal Ethics Committee.

Intravital microscopy

Intravital microscopy of the cremaster muscle was performed as described previously (25). Briefly, mice were anesthetized with ketamine/xylazine, and the cremaster muscle was exteriorized onto an optically clear viewing pedestal. The cremasteric microcirculation was visualized using an intravital microscope (Axioplan 2 Imaging; Carl Zeiss) with a $\times 20$ objective lens (LD Achromplan 20X/0.40 NA; Carl Zeiss) and a $\times 10$ eyepiece. Three to five postcapillary venules (25–40 μm in diameter) were examined for each experiment. Images were visualized using a video camera and recorded on video tape for subsequent playback analysis. Leukocyte-endothelial cell interactions (rolling flux, rolling velocity, adhesion, emigration) were assessed as described previously (25). To exclude a role for residual endotoxin from the bacterial expression system used for rMIF production, in some experiments, MIF was denatured by boiling for 30 min. For experiments examining MIF-induced effects 2, 4, or 24 h after administration, MIF was injected intrascrotally in 150 μl of saline, before intravital microscopy. To assess acute effects of MIF, the cremaster muscle was first exteriorized, and then MIF was added to the superfusion buffer at the concentrations shown. In experiments assessing intra-arterial administration of MIF, the left femoral artery was cannulated in a retrograde direction and varying amounts of MIF were administered into the arterial microcirculation in 50- μl boluses. In experiments using neutralizing Abs, mice were pretreated with protein A-purified IgG (100 μg , i.p.) 1 h before MIF injection. In experiments using rabbit-derived Abs, purified rabbit IgG (Sigma-Aldrich) was used in control mice. To assess a role for G protein-mediated responses, some animals received pertussis toxin (PTX; 4 μg , i.p.) (List Biological Laboratories) 1 h before rMIF injection (33).

Immunohistochemistry

Cryostat sections were prepared from periodate/lysine/paraformaldehyde-fixed cremaster muscles and stained for total leukocytes using the pan-leukocyte marker CD45 (30-F11), neutrophils (RB6-8C5), macrophages (CD68/FA-11 and F4/80), CD11c⁺ dendritic cells (HL3), T lymphocytes (CD3/KT3), and B lymphocytes (B220/RA3-6B2) and using a three-layer immunoperoxidase technique as previously described (34). To quantitate infiltration of the various leukocyte subsets, the number of positively stained cells in the entire section were counted, and expressed relative to the area of tissue in the section, as determined by image analysis (Scion Image).

VCAM-1 expression

Quantitation of endothelial VCAM-1 expression in vivo was performed using Alexa 488 (Molecular Probes)-conjugated anti-VCAM-1, exactly as previously described (35). To determine whether the staining detected in unstimulated tissue was specific to VCAM-1 protein expression, in one group of experiments, mice were pretreated with a 2-fold excess dose of

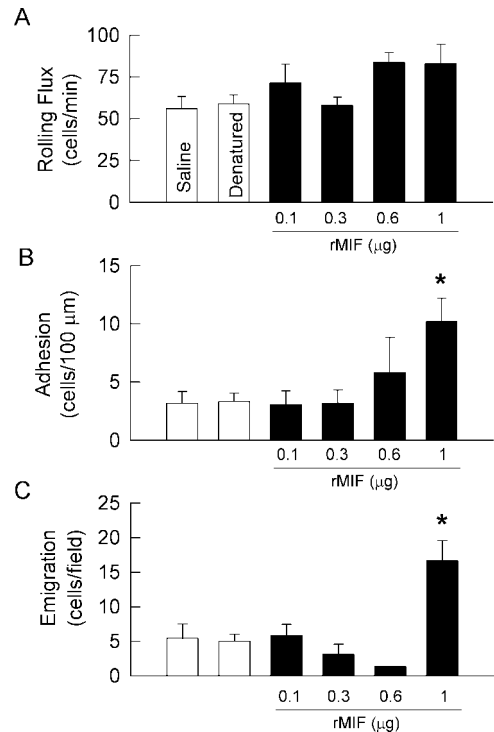


FIGURE 1. MIF-induced alterations in leukocyte endothelial cell interactions 4 h after MIF administration. Varying doses of rMIF were injected intrascrotally, and the cremasteric microvasculature was assessed 4 h later (0.1 μg , $n = 6$; 0.3/0.6 μg , $n = 3$; 1.0 μg , $n = 16$). Parameters measured were leukocyte rolling flux (A), adhesion (B), and emigration (C). Also shown are saline-injected mice ($n = 7$) and mice treated with 1 μg of denatured MIF ($n = 6$). Data are shown as mean \pm SEM. *, $p < 0.05$ vs saline-treated mice.

nonconjugated anti-VCAM-1. Five minutes later, Alexa 488-conjugated anti-VCAM-1 was administered and the normal protocol was followed for quantitation of VCAM-1 expression. Final data are shown as arbitrary units, representing the product of the average Alexa 488-associated fluorescence intensity multiplied by the length of positive vessel (35).

Real-time PCR analysis of CCL2 mRNA

Total RNA was extracted from cremaster muscles using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's protocol. A total of 0.5–1 μg of total RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen Life Technologies) and oligo(dT)₂₀-PCR amplification was performed on a Rotor-Gene 3000 (Corbett Research). The following primer-specific nucleotide sequences of murine MCP-1 (CCL2) were used: 5'-CCCCAAGAAGGAATGGGTCC-3' and 5'-GGTTGTGAAAAGGTAGTGG-3' and β -actin (5'-TGCCCTG TATGCCTCTGGT-3' and 5'-GATGTCACGCACGATTTC-3' (36, 37). Amplification (40 cycles) was conducted in a total volume of 10 μl containing cDNA dilutions, primers (3 pM), and the DNA Master SYBR Green I kit (Roche Diagnostics). Control reactions for product identification consisted of analyzing the melting peaks ($^{\circ}\text{C}$) and determining the length of the PCR products (base pair) by agarose gel electrophoresis. Amplification efficiency was controlled by the use of an internal control (housekeeping gene) and external standards. The amount of target mRNA expression was calculated and normalized to β -actin expression.

CCL2 production by endothelial cells

Lung microvascular endothelial cells were isolated using a method adapted from Bowden et al. (38). Briefly, lungs were dissected from 5- to 7-day-old mice, minced, and digested in Liberase Blendzyme 3 (Roche) for 1 h. Following filtration, cells were labeled with anti-ICAM-2 (BD Biosciences) followed by secondary labeling with goat anti-rat IgG microbeads (Miltenyi Biotec). ICAM-2-positive cells were immunomagnetically isolated using a mini-MACS separation unit (Miltenyi Biotec). Isolated cells were cultured in laminin-coated dishes in DMEM with 20% FCS, 25 $\mu\text{g}/\text{ml}$ endothelial mitogen (Biomedical Technologies), 100 $\mu\text{g}/\text{ml}$

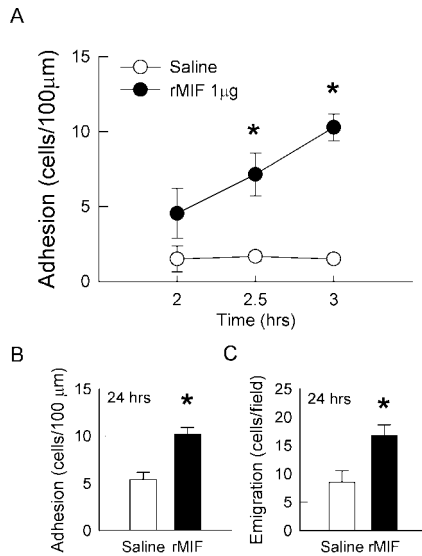


FIGURE 2. Time course of development of MIF-induced adhesion and transmigration. *A*, Leukocyte adhesion in postcapillary venules 2–3 h after intrascrotal injection of 1 µg of MIF ($n = 6$), compared with that in saline-injected mice ($n = 3$). *B* and *C*, Leukocyte adhesion (*B*) and emigration (*C*) in postcapillary venules 24 h after local injection of 1 µg of MIF ($n = 6$). Also shown are saline-injected mice (2–3 h, $n = 3$; 24 h, $n = 5$). Data are shown as mean \pm SEM. *, $p < 0.05$ vs saline-treated mice.

porcine heparin (Sigma-Aldrich), 1% nonessential amino acids, sodium pyruvate, L-glutamine, and antibiotics at standard concentrations (Invitrogen Life Technologies). After 5 days in culture, cells were passaged into 24-well laminin-coated plates and cultured for a further 1–2 days until confluent. Endothelial cell purity was assessed by staining with DiO-Ac-LDL (Biomedical Technologies) for 4 h and analysis via flow cytometry. Cultures routinely contained >92% Ac-LDL-positive cells.

To assess CCL2 release, endothelial monolayers were incubated in 5% FCS DMEM for 1 h then treated for 4 h in 5% FCS DMEM with or without MIF (100 ng/ml). The level of CCL2 in the supernatant was quantitated using a sandwich ELISA (BD Biosciences). Samples were run in duplicate.

Statistics

Data are displayed as mean \pm SEM. Data were analyzed using unpaired Student's *t* tests unless indicated otherwise. Significance was defined as $p < 0.05$.

Results

MIF induces leukocyte adhesion and transmigration in postcapillary venules

We first assessed the effect of MIF 4 h after administration (Fig. 1). Local injection of 0.1–1.0 µg of MIF adjacent to the cremaster muscle did not alter leukocyte rolling flux (Fig. 1*A*) or rolling velocity (data not shown) in postcapillary venules. In contrast, 1.0 µg of MIF induced a significant increase in leukocyte adhesion (Fig. 1*B*) and emigration (Fig. 1*C*). The microvascular shear rate did not differ significantly between mice treated with saline ($581 \pm 29 \text{ s}^{-1}$, $n = 7$) or MIF ($467 \pm 44 \text{ s}^{-1}$, $n = 16$), indicating that the MIF-induced response was not due to an alteration in microvascular shear forces. Also, the MIF-induced response was abolished by boiling the protein before administration, indicating that the response was not due to residual endotoxin from the bacterial expression system used for rMIF production. Analysis of the time course of the response revealed that leukocyte adhesion increased within 2.5 h of MIF administration (Fig. 2*A*). Furthermore, leukocyte adhesion and emigration persisted in venules examined 24 h after MIF administration (Fig. 2, *B* and *C*), indicating that MIF induced a prolonged recruitment response. However, the numbers

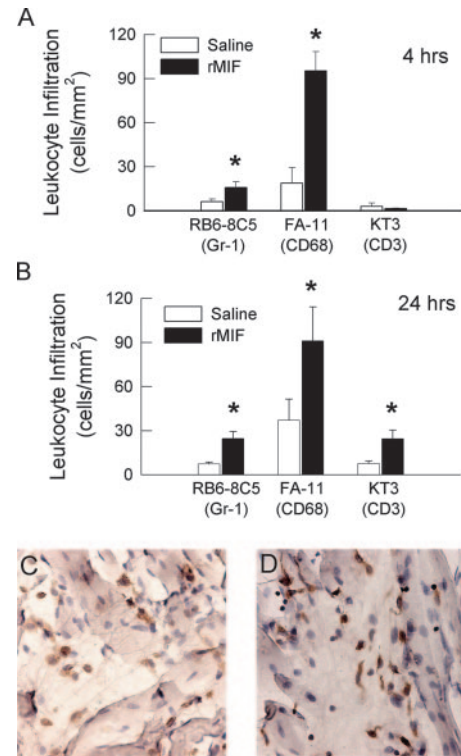


FIGURE 3. Immunohistochemical characterization of the leukocyte infiltrate in MIF-treated mice 4 (*A*) and 24 (*B*) h after intrascrotal administration of MIF (1 µg). Leukocytes were classified as neutrophils (anti-Gr-1⁺), monocytes (anti-CD68⁺), or T cells (anti-CD3⁺) and expressed as cells per mm² of muscle, using image analysis to determine cross-sectional area. Data were compared in mice treated with either saline ($n = 4$) or MIF ($n = 4$) for both 4 and 24 h. *, $p < 0.05$ vs saline-treated mice. *C* and *D*, Examples of immunohistochemical staining for CD45 (*C*) and CD68 (*D*) (brown staining) on leukocytes in MIF-treated cremaster muscles.

of cells observed at 4 and 24 h were similar, suggesting either that most recruitment occurred during the first 4 h, or that by 24 h, the rate of leukocyte entry and departure were similar.

MIF-induced leukocyte recruitment is monocyte selective

Examination of tissues following exposure to MIF revealed that the majority of leukocytes recruited in response to MIF expressed the monocyte-lineage marker, CD68 (Fig. 3). This applied equally in tissues examined 4 and 24 h after MIF administration. In contrast, these cells did not express the macrophage maturation marker, F4/80 (data not shown). We also observed a small increase in the number of extravascular Gr-1⁺ cells (Fig. 3, *A* and *B*). MIF did not induce recruitment of CD3⁺ (Fig. 3*A*) or B220⁺ (data not shown) lymphocytes at 4 h, although a small increase in CD3⁺ cells was observed at 24 h (Fig. 3*B*). CD11c⁺ cells were detectable at low and equivalent numbers (>5 cells/mm²) in both saline and MIF-treated mice.

To further clarify the monocyte selectivity of MIF-induced leukocyte recruitment, we performed additional 4-h MIF experiments and stained tissue-resident and recruited leukocytes with both the pan-leukocyte marker CD45 and CD68 (Fig. 3, *C* and *D*). MIF treatment induced a 270% increase in the number of CD45⁺ leukocytes in the muscle tissue relative to saline alone (data not shown). In saline-treated mice, CD68⁺ cells represented 22% of the CD45⁺ cells. In contrast, in MIF-treated mice, the number of CD68⁺ cells detected ($240 \pm 82 \text{ cells/mm}^2$) did not differ significantly from the number of CD45⁺ cells present ($213 \pm 58 \text{ cells/}$

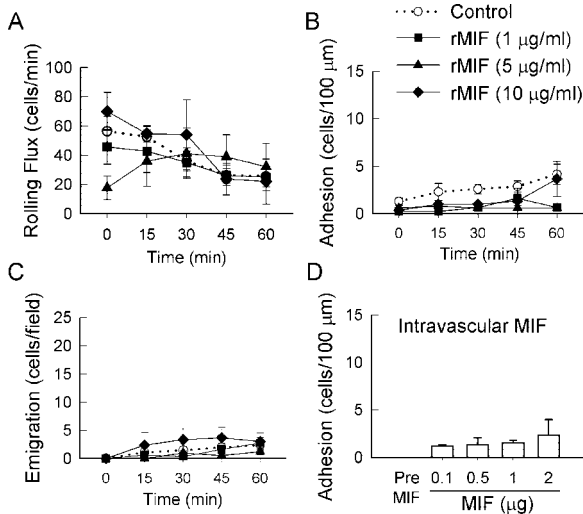


FIGURE 4. Acute effects of MIF on leukocyte-endothelial cell interactions. *A–C*, The cremasteric microvasculature was superfused with varying concentrations of MIF over 60 min, and leukocyte rolling flux (*A*), adhesion (*B*), and transmigration (*C*) quantified at 15-min intervals. Control, $n = 7$; rMIF, 1 $\mu\text{g}/\text{ml}$, $n = 4$; 5 $\mu\text{g}/\text{ml}$, $n = 4$; 10 $\mu\text{g}/\text{ml}$, $n = 3$. *D*, Effect of local intra-arterial administration of MIF on leukocyte adhesion. The cremaster muscle was exteriorized and MIF was administered into the arterial circulation directly upstream of the muscle at increasing doses ranging from 100 ng to 2 μg . The immediate effect on adhesion in multiple postcapillary venules assessed over the ensuing 10 min ($n = 3$ mice).

mm^2). Together, these findings indicate that MIF selectively recruits monocyte-lineage cells.

The kinetics of MIF-mediated leukocyte arrest differ from those of classical chemokines

One possible mechanism whereby MIF induces leukocyte recruitment is via a direct action on leukocytes, inducing arrest and transmigration in a manner similar to that of a classical chemokine. To assess the ability of MIF to induce a response of this type, we superfused the cremaster muscle with MIF over a 60-min period. MIF superfusion at concentrations similar to those used in the 4 h

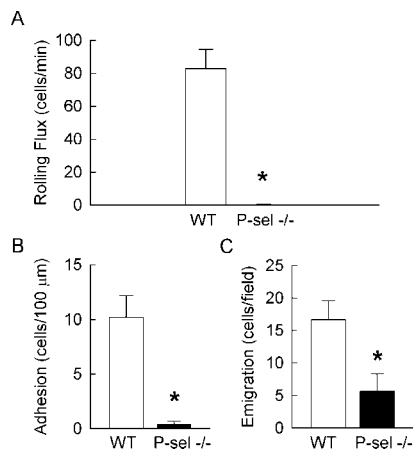


FIGURE 5. Role of P-selectin in MIF-mediated leukocyte recruitment. P-selectin^{-/-} mice ($n = 6$) were treated with MIF (1 μg , intrascrotal), and leukocyte rolling flux (*A*), adhesion (*B*), and emigration (*C*) assessed at 4 h. Also shown are the results from wild-type mice treated with MIF in the identical manner ($n = 16$). Data are shown as mean \pm SEM. *, $p < 0.05$ vs wild-type mice.

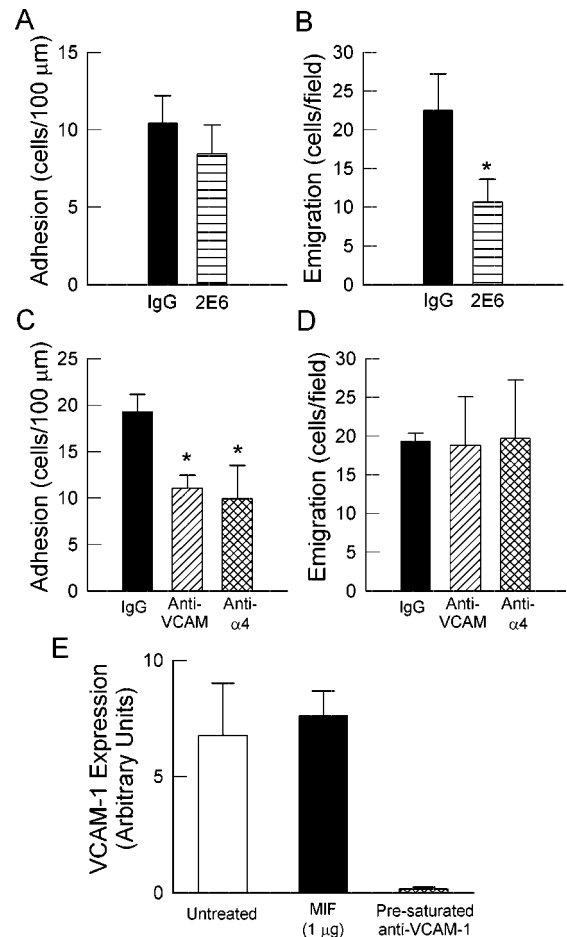


FIGURE 6. Role of β_2 integrins, VCAM-1, and the α_4 integrin in MIF-induced leukocyte recruitment. *A* and *B*, Effects of pretreatment with either the β_2 integrin-blocking mAb 2E6 ($n = 6$) or control hamster IgG ($n = 6$) on MIF-induced (1 μg , 4 h, intrascrotal) adhesion (*A*) and emigration (*B*). *C* and *D*, Effects of pretreatment with anti-VCAM-1 ($n = 4$), and anti- α_4 integrin ($n = 5$) vs control rat IgG ($n = 6$) on MIF-induced adhesion (*C*) and emigration (*D*). *E*, Effect of MIF on VCAM-1 expression. VCAM-1 expression was assessed in vivo via i.v. administration of Alexa 488-conjugated anti-VCAM-1 and image analysis of fluorescence intravital microscopy images of VCAM-1 staining. Mice examined were untreated ($n = 4$) or MIF-treated (1 μg , 4 h, intrascrotal, $n = 4$). An additional group of nonstimulated mice were assessed following pretreatment with a saturating dose of nonconjugated anti-VCAM-1 ($n = 3$) to assess specificity of VCAM-1 staining. Data are shown as mean \pm SEM. *, $p < 0.05$ vs control IgG-treated mice.

experiments did not cause increases in leukocyte rolling, adhesion, or emigration (Fig. 4, *A–C*), indicating that MIF does not induce leukocyte arrest and transmigration within this time course in cremasteric postcapillary venules. This contrasts with our previous observation of rapid increases in adhesion and transmigration induced by the chemokine CXCL1/KC applied in an identical manner (39).

Application of MIF to the apical surface of endothelial cells has been shown to induce MIF-dependent monocyte arrest (28). However, administration of a range of doses of MIF into the arterial circulation immediately upstream of the cremaster muscle did not cause substantial arrest of rolling leukocytes in cremasteric postcapillary venules (Fig. 4*D*), indicating that the presence of MIF within the microvasculature is insufficient to cause arrest of rolling leukocytes.

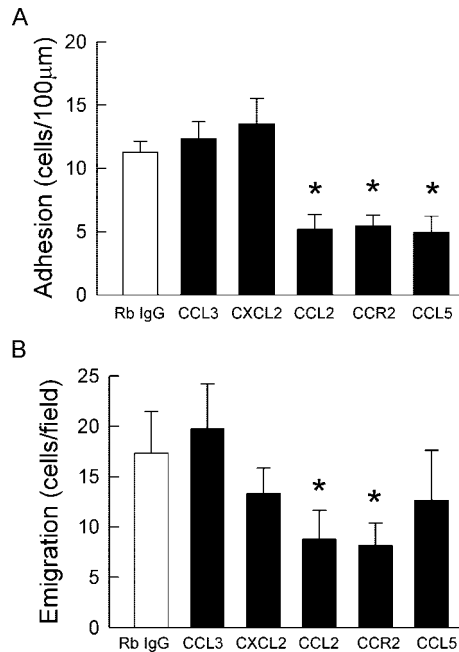


FIGURE 7. Roles of various chemokines and chemokine receptors in MIF-induced monocyte recruitment. Mice were pretreated with Abs against CCL3 (MIP-1 α), CXCL2 (MIP-2), CCL2 (JE/murine MCP-1), CCR2, and CCL5 (RANTES) and then treated with MIF (1 μ g, 4 h, intrascrotal). Intravital microscopy was used to assess the effects on MIF-induced leukocyte adhesion (A) and emigration (B), via comparison with mice treated with nonspecific rabbit IgG (Rb IgG, $n = 8$). Anti-CCL2 ($n = 10$) and anti-CCR2 ($n = 8$) treatments significantly reduced MIF-induced adhesion and emigration whereas anti-CCL3 ($n = 4$) and CXCL2 ($n = 3$) were without effect. Anti-CCL5 ($n = 6$) reduced adhesion but not emigration. Data are shown as mean \pm SEM. *, $p < 0.05$ vs control IgG-treated mice.

Adhesion molecule requirements of MIF-induced recruitment

The observation that leukocyte rolling flux and velocity were not altered in MIF-treated mice (Fig. 1A) suggested that expression of molecules which mediate leukocyte rolling was not affected by this treatment. However, in the cremaster muscle, leukocyte rolling is supported by constitutive expression of P-selectin (40). Therefore, we examined MIF-induced leukocyte recruitment in MIF-treated P-selectin^{-/-} mice. In these mice, leukocytes were rarely observed to undergo rolling interactions (Fig. 5A), and MIF failed to induce leukocyte adhesion or emigration (Fig. 5, B and C). Blockade of the leukocyte β_2 -integrin reduced leukocyte emigration but not adhesion suggesting that the β_2 -integrin was not necessary for leukocyte adhesion to the endothelium but contributed to the process of leukocyte egress from the vasculature (Fig. 6, A and B). In contrast, blockade of either VCAM-1 or the α_4 integrin reduced adhesion by ~50%, without altering emigration (Fig. 6, C and D).

Given the effect of VCAM-1 blockade, we next investigated whether vascular VCAM-1 expression was altered by MIF treatment, using fluorochrome-conjugated anti-VCAM-1 *in vivo* (Fig. 6E). VCAM-1 was constitutively expressed in the cremasteric microvasculature of untreated mice. Pretreatment with nonconjugated anti-VCAM-1 eliminated staining in these animals, demonstrating that this staining was not due to nonspecific Ab accumulation. However, the level of VCAM-1 expression in the vasculature of MIF-treated mice did not differ from that in untreated mice (Fig. 6E).

MIF-induced monocyte recruitment involves the CCL2/CCR2 pathway

The selectivity of MIF for recruitment of monocyte-lineage cells suggested the involvement of a monocyte-specific chemoattractant

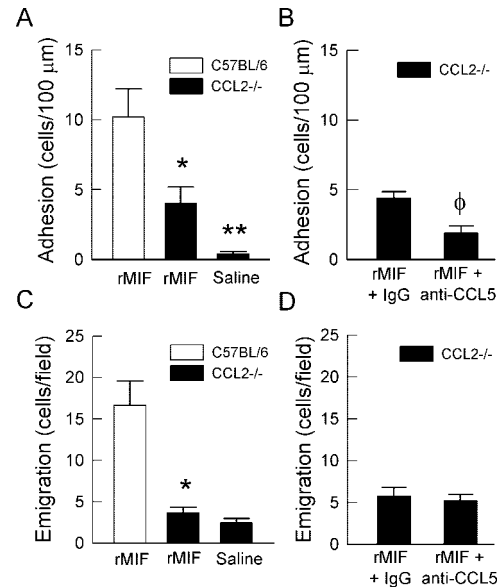


FIGURE 8. Assessment of basal and MIF-induced adhesion and emigration in CCL2^{-/-} mice. Data are shown for leukocyte adhesion (A) and emigration (C) in MIF-treated (1 μ g, 4 h) wild-type mice ($n = 16$), and CCL2^{-/-} mice either injected with MIF (1 μ g) or saline ($n = 6$ for both groups). In addition, to examine the role of CCL5 in residual leukocyte recruitment in CCL2^{-/-} mice, CCL2^{-/-} mice were treated with either control IgG or anti-CCL5 Ab. Data are shown for leukocyte adhesion (B) and emigration (D) in control IgG-treated ($n = 6$) and anti-CCL2-treated mice ($n = 6$). Data are shown as mean \pm SEM, relative to rMIF-treated wild-type mice. *, $p < 0.05$ vs MIF-treated wild-type mice. **, $p < 0.05$ vs MIF-treated CCL2^{-/-} mice. ϕ , $p < 0.05$ vs MIF-treated CCL2^{-/-} mice pretreated with control IgG.

pathway. To test this possibility, we first assessed the effects of blocking G protein-mediated signaling using PTX. Pretreatment of mice with PTX significantly reduced leukocyte adhesion (MIF alone: 10.2 ± 2.0 cells/100 μ m ($n = 16$) vs MIF + PTX: 3.7 ± 1.0 ($n = 8$), $p < 0.05$), indicating that the MIF-induced response was G protein dependent, suggesting the involvement of a G protein-coupled chemoattractant receptor. Therefore, we investigated the roles of various monocyte-specific chemokines and their receptors. Blockade of CCL2 (JE/murine MCP-1) and its receptor CCR2 each reduced adhesion and emigration by ~50% (Fig. 7). In contrast, inhibition of CCL3 (MIP-1 α) and CXCL2 (MIP-2) had no effect on adhesion or emigration (Fig. 7). Finally, anti-CCL5 (RANTES) treatment reduced MIF-induced adhesion but not emigration.

The findings of a key role for the CCL2/CCR2 pathway were supported by experiments in CCL2^{-/-} mice, in which MIF-induced leukocyte adhesion and emigration were both significantly reduced relative to MIF-treated wild-type mice (Fig. 8, A and C). Comparison with CCL2^{-/-} mice treated with saline alone, however, revealed that MIF was capable of inducing a small increase in leukocyte adhesion in the absence of CCL2 (Fig. 8A). However, this did not translate to an increase in extravascular leukocytes, indicating that CCL2 was a requirement for MIF-induced transmigration (Fig. 8C). To identify the chemokine responsible for the residual MIF-induced adhesion in CCL2^{-/-} mice, and given the effect of anti-CCL5 treatment in wild-type mice, we assessed the effect of this treatment in CCL2^{-/-} mice (Fig. 8, B and D). CCL5 blockade caused a further reduction in MIF-induced adhesion in CCL2^{-/-} mice, but did not alter emigration, indicating that CCL5 contributes to leukocyte adhesion only.

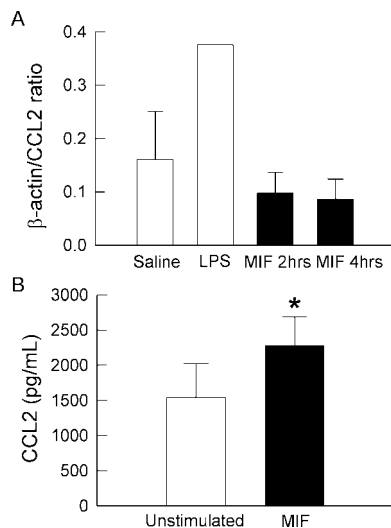


FIGURE 9. Effect of rMIF on CCL2 mRNA expression in the cremaster muscle and endothelial release of CCL2. *A*, Mice were injected with MIF and cremaster muscles harvested 2 and 4 h after MIF administration ($n = 3$ each). CCL2 mRNA was quantitated using real-time PCR. Also shown are data for mice injected intrascrotally with saline alone, or LPS (100 ng) alone as a positive control for CCL2 induction. *B*, Effect of rMIF on CCL2 release from murine microvascular endothelial cells. Monolayers of microvascular endothelial cells isolated from lungs of wild-type mice were left untreated or treated with MIF (100 ng/ml) for 4 h, and release of CCL2 into the culture supernatant assessed. Data are shown as the mean of paired samples with or without MIF treatment ($n = 3$). CCL2 was undetectable in untreated medium. *, $p < 0.05$ vs untreated cells, via paired t test.

Immunohistochemical analysis of tissues from MIF-treated $CCL2^{-/-}$ mice revealed that the reduction in recruitment was solely as a result of a reduction in the number of $CD68^{+}$ cells (wild type: 95.1 ± 13.3 vs $CCL2^{-/-}$: 19.0 ± 5.3 cells/mm 2 , $p < 0.05$), whereas the number of neutrophils present was unchanged from that in wild-type mice (wild-type: 15.7 ± 4.1 vs $CCL2^{-/-}$: 15.6 ± 2.7 cells/mm 2 , NS).

MIF induces endothelial release of CCL2

To investigate whether MIF altered CCL2 expression, we examined expression of CCL2 mRNA in MIF-treated mice. As a positive control, local injection of LPS alone induced a marked increase in CCL2 mRNA. In contrast, CCL2 mRNA was not increased by treatment with MIF at either 2 or 4 h (Fig. 9A). Murine microvascular endothelial cells have been reported to constitutively express substantial CCL2 mRNA, and not increase transcription in response to TNF (41). This raises the possibility that CCL2 may be rapidly released by endothelial cells in response to inflammatory stimuli, in the absence of alterations in mRNA expression. Therefore, we examined the ability of primary endothelial cells to release CCL2 in response to MIF (Fig. 9B). Endothelial cells from wild-type mice constitutively released CCL2 during a 4-h culture period. However, exposure to MIF induced a significant increase in CCL2 release within 4 h ($p < 0.05$).

Discussion

Reduced inflammatory responses associated with the blockade or absence of MIF have been shown in a wide range of inflammatory models (15–19). Although MIF impacts upon a wide range of cellular pathways, the observation that the absence of MIF is associated with reduced leukocyte infiltration in several of these models suggests that one of the key proinflammatory pathways affected by MIF is recruitment of leukocytes (17, 26, 27). This is supported by

our recent observation of reduced rolling and adhesive interactions in postcapillary venules of inflamed $MIF^{-/-}$ mice (25). It is possible that this effect results from the ability of MIF to enhance the actions of alternative mediators such as proinflammatory cytokines. However, an alternative explanation is that MIF alone may be sufficient to induce leukocyte recruitment. The present findings show that application of MIF to the microvasculature does induce leukocyte adhesion and transmigration. Although exogenous MIF has been shown to augment inflammatory responses in whole animals and at a cellular level, this is the first report that this cytokine is capable of inducing leukocyte-endothelial cell interactions and leukocyte recruitment in vivo, in the absence of additional inflammatory stimuli (15, 22).

The leukocytes recruited in response to MIF were predominantly monocyte-lineage cells. This was an unexpected finding inasmuch as MIF has been consistently associated with, and indeed named for, the ability to inhibit random macrophage migration in vitro (13, 42). Given this, the finding that MIF selectively promotes the entry of monocyte-lineage cells into tissues was unexpected. Despite this, results of some studies have raised the possibility that MIF may promote macrophage recruitment specifically. In models of atherosclerosis, MIF blockade has been shown to inhibit intimal accumulation of $CD68^{+}$ monocytes (28, 29). It is well-established that monocytes are the major leukocyte subgroup recruited to the vascular wall in these models. Given that macrophage recruitment to the vascular wall continues in the absence of MIF, albeit to a lesser degree, these models do not provide information regarding the selectivity of MIF for recruitment of monocyte/macrophages. Further evidence suggesting that MIF has selective recruitment actions on monocytes comes from studies demonstrating that application of MIF to the endothelial surface induces monocyte adhesion under flow conditions in vitro. These findings led to the hypothesis that MIF can bind directly to rolling monocytes to induce their arrest (28). However, as these experiments were performed with purified monocytes, they did not examine the selectivity of MIF for this specific leukocyte population. In contrast, the monocyte selectivity observed in the present experiments occurred under in vivo conditions, where all circulating leukocytes were potentially able to respond to MIF. Moreover, MIF was the only inflammatory stimulus applied to the tissue. These findings provide strong evidence that one of the effects of MIF is to promote selective recruitment of cells of the monocyte/macrophage lineage.

Several lines of evidence indicate that the specificity of MIF for monocyte recruitment arose from a pathway involving the monocyte-attracting chemokine CCL2 and its monocyte-expressed receptor, CCR2. Blockade of either CCL2 or CCR2 resulted in significant and comparable reductions in leukocyte adhesion and transmigration. In addition, $CCL2^{-/-}$ mice treated with MIF displayed similar reductions in adhesion and transmigration compared with $CCL2^{+/+}$ mice, and immunohistochemical assessment revealed that the deficit in extravascular leukocytes in $CCL2^{-/-}$ mice stemmed entirely from a reduction in $CD68^{+}$ cells. To identify potential cellular sources of CCL2, we examined CCL2 release from isolated endothelial cells and observed that MIF induced the release of CCL2 from these cells. Taken together, these findings are consistent with a mechanism in which MIF induces release of CCL2 from endothelial cells, with CCL2 subsequently promoting adhesion and recruitment of monocytes. Previous studies have suggested a role for MIF in regulation of chemokine expression. MIF blockade has previously been shown to reduce CXCL2/MIP-2 expression in lungs of LPS-treated mice (26). Moreover, MIF has also been shown to increase expression of chemokines in neuroblastoma cell lines, human alveolar cells, and mouse lungs

(43–45). However, before this study, the effects of MIF on CCL2 expression were unknown. The present findings suggest that MIF induces release of CCL2 from endothelial cells. This raises the possibility that this function of MIF could play critical roles in macrophage-mediated inflammatory responses.

Despite the strong evidence of a role for CCL2, it is conceivable that MIF-induced leukocyte recruitment involves the actions of multiple chemokines, with leukocyte arrest and transmigration being regulated by different chemoattractants working in a sequential fashion (46). This is supported by the observation that CCL5 promotes adhesion but not transmigration in response to MIF. Furthermore, MIF also induced neutrophil recruitment, albeit to a lesser degree, suggesting the involvement of additional chemoattractants capable of inducing responses in neutrophils. To examine this further, we are currently investigating chemoattractants which function via CXCR2, which previous studies have shown to attract both neutrophils and monocytes (10, 47). The present data indicate that CXCL2 (MIP-2) is not responsible for this effect.

The observation that the MIF-induced response required several hours to develop is consistent with the induction of a response from an intermediary tissue-resident cell. However, Schober et al. (28) have reported that MIF applied to the endothelial surface induces arrest of purified monocytes in a manner similar to that of an immobilized chemokine. In addition, unpublished work indicates that MIF-mediated monocyte arrest can be induced on the endothelial surface of arteries from atherosclerosis-prone mice (J. Bernhagen and C. Weber, personal communication). Although the data from the present study support an indirect pathway whereby MIF induces release of secondary mediators such as CCL2, it is also possible that MIF may be expressed on the endothelial surface and induce monocyte recruitment via a direct pathway. Indeed, the mechanisms at work in the microvasculature, as assessed in the present study, may be distinct from those active in large atherosclerotic arteries where MIF expression in endothelial cells is markedly elevated (28, 29). Together, these findings suggest that MIF can induce monocyte recruitment via multiple mechanisms. Another possible explanation for these observations is that MIF has been reported to induce TNF production (44, 48). Therefore, in the present model it is conceivable that monocytes, either initially recruited in response to MIF or residing in the tissue, release TNF in response to MIF, which further promotes leukocyte recruitment to the affected area.

Assessment of the adhesion molecule pathways used in MIF-induced leukocyte recruitment revealed specific and nonoverlapping roles for the leukocyte integrins. The α_4 integrins contributed to adhesion (via VCAM-1) but not emigration, whereas the β_2 integrins were not required for adhesion but contributed to the process of transmigration. This suggests that adhesion molecule usage by leukocytes recruited in response to MIF is not characterized by redundancy, but involves specific roles for individual integrins in sequential steps in the recruitment process. There is growing evidence of specificity of leukocyte integrins for particular tasks. Using an in vitro chemotaxis assay, Heit et al. (49) recently demonstrated that integrin use required for directional migration varied according to the chemoattractant involved. The present data suggest that this specificity extends to differential roles in adhesion and transmigration, at least for the leukocyte population recruited in response to MIF. It was also noteworthy that the present results indicate that MIF does not induce up-regulation of endothelial selectins or VCAM-1. This is in contrast to a recent report describing MIF-induced up-regulation of VCAM-1 expression by monocytes (50). The functional importance of monocyte VCAM-1 expression requires further clarification. However, our finding that constitutively expressed VCAM-1 contrib-

uted to MIF-induced recruitment suggests that this process may be most effective in vascular beds in which VCAM-1 is already expressed.

The current data demonstrate a previously unrecognized action of MIF: stimulation of monocyte/macrophage recruitment via the CCL2/CCR2 pathway. In many of the diseases in which MIF is implicated, macrophages are key contributors. Moreover, previous data indicate that MIF participates in macrophage activation. The present results suggest an additional pathway whereby MIF promotes macrophage-mediated inflammatory responses via induction of the recruitment of monocytes into affected areas. By both attracting and activating monocyte/macrophages, MIF may contribute to the initiation and perpetuation of detrimental inflammation associated with diseases such as atherosclerosis and arthritis.

Acknowledgments

We thank Dr. Barrett Rollins (Dana-Farber Cancer Institute, Boston, MA) and Dr. Greg Tesch (Department of Nephrology, Monash Medical Centre, Victoria, Australia) for the CCL2^{-/-} mice, Dr. Dietmar Vestweber (Max-Planck-Institut, Meunster, Germany) and Dr. Britta Engelhardt (Theodor Kocher Institute, University of Bern, Switzerland) for the anti-VCAM-1 hybridoma, and Drs. Jurgen Bernhagen and Christian Weber (University Hospital, Rhine-Westphalia Technical University, Aachen, Germany) for helpful discussions.

Disclosures

The authors have no financial conflict of interest.

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