MAJOR ARTICLE

Macrophage Migration Inhibitory Factor Deficiency Is Associated With Impaired Killing of Gram-Negative Bacteria by Macrophages and Increased Susceptibility to *Klebsiella pneumoniae* Sepsis

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The cytokine macrophage migration inhibitory factor (MIF) is an important component of the early proinflammatory response of the innate immune system. However, the antimicrobial defense mechanisms mediated by MIF remain fairly mysterious. In the present study, we examined whether MIF controls bacterial uptake and clearance by professional phagocytes, using wild-type and MIF-deficient macrophages. MIF deficiency did not affect bacterial phagocytosis, but it strongly impaired the killing of gram-negative bacteria by macrophages and host defenses against gram-negative bacterial infection, as shown by increased mortality in a *Klebsiella* pneumonia model. Consistent with MIF's regulatory role of Toll-like 4 expression in macrophages, MIF-deficient cells stimulated with lipopolysaccharide or *Escherichia coli* exhibited reduced nuclear factor κB activity and tumor necrosis factor (TNF) production. Addition of recombinant MIF or TNF corrected the killing defect of MIF-deficient macrophages. Together, these data show that MIF is a key mediator of host responses against gram-negative bacteria, acting in part via a modulation of bacterial killing by macrophages.

Keywords. Macrophage migration inhibitory factor; Macrophage; Phagocytosis; Bacterial killing; Gram-negative Bacteria.

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine with broad cellular and tissue distribution. Initially described as an effector molecule of the adaptive immune system, MIF has now also been shown to be an integral part of the early cytokine response of the innate immune system. Constitutively expressed and rapidly released by innate immune cells (monocytes, macrophages, dendritic cells, and neutrophils) on exposure to danger signals, MIF promotes cell survival and proliferation and vigorous inflammatory and immune responses [1].

Cellular activation by MIF is mediated by a receptor complex consisting of CD74, acting primarily as the MIF binding moiety; CD44; CXCR2; and CXCR4 (the signal transducer components of the receptor complex) [2–5]. On receptor binding, MIF activates the extracellular signal-regulated kinase (ERK1/2), the p38 mitogen-activated protein kinase (MAPK), and the phosphoinositide-3-kinase/Akt (PI3K/Akt) signaling pathways [6–8]. At a molecular level, MIF mediates its biological effects via intracellular interactions with p53, CSN5/JAB-1, and ribosomal protein S19 [9–11].

MIF promotes proinflammatory and innate immune responses of myeloid cells through different mechanisms. In monocytes and macrophages, MIF sustains the expression of Toll-like receptor 4 (TLR4), and

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therefore the responsiveness to lipopolysaccharide (LPS) and gram-negative bacteria [12, 13]. It also functions as an inactivator of p53-mediated LPS-induced cell death [14] and counterregulates the antiinflammatory and immunosuppressive effects of glucocorticoids in part by inhibiting steroid-induced MAPK phosphatase-1 (MKP-1) expression [15]. Numerous preclinical and clinical studies have implicated MIF in host defenses against bacterial invasion and in the pathogenesis of sepsis [1, 16–22]. MIF blood levels are elevated in septic patients and are associated with dysregulated adrenal function, expression of proinflammatory cytokines, disease severity, and patient's outcome [20, 23, 24]. Consistent with an important role for MIF in human sepsis, functional polymorphisms of the *MIF* gene promoter have recently been shown to influence the susceptibility to, severity of, and outcome of pneumococcal and meningococcal sepsis and streptococcal pneumonia [25–27].

Despite a large body of evidence indicating that MIF is an important effector molecule of the host defense response against bacterial infections, very little is known in terms of the antimicrobial defense mechanisms mediated by MIF. In particular, no information is available regarding uptake and clearance of bacteria by phagocytic innate immune cells. To fill that gap, we studied the phagocytosis and killing of a large panel of gram-negative (*Escherichia coli* O11, O18, and J5; *Klebsiella pneumoniae*; and *Pseudomonas aeruginosa*) and gram-positive (*Streptococcus mitis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* AW7 and P8) bacterial strains by wild-type and MIF-deficient macrophages. We report that MIF deficiency did not affect bacterial phagocytosis but strongly impaired the killing of *E. coli* and *K. pneumoniae* by macrophages and host defenses against gram-negative infection, as shown by increased mortality in a *Klebsiella* pneumonia model. MIF-deficient macrophages exhibited impaired TLR4 signaling with reduced nuclear factor κB (NF-κB) activity and tumor necrosis factor (TNF) production.

MATERIALS AND METHODS

Mouse and Cells

RAW 264.7 macrophages were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2 mM glutamine (Invitrogen, Basel, Switzerland) and 10% (v/v) heat-inactivated fetal calf serum (FCS; Seromed, Berlin, Germany) [12]. RAW 264.7 macrophages were transfected with either empty or MIF antisense pBK plasmids (Stratagene, La Jolla, CA). Stably transfected clones were obtained by limited dilution and selection, using Geneticin (Invitrogen). Two clones of MIF-deficient RAW 264.7 macrophages (designated as AS 2.8 and AS 2.23) expressing markedly reduced levels of MIF and control RAW 264.7 macrophages (pBK1.1) were selected for the present studies (Figure 1*A*) [10, 12]. $MIF^{-/-}$ mice backcrossed 8 times on the BALB/c background were obtained from John David (Harvard Medical School, Boston, MA) [19]. Bone marrow–derived macrophages (BMDMs) were cultured as described [28]. Animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations 876.7 6 and 877.7) and were performed according to our institution's guidelines for animal experiments.

Bacteria, Reagents, and Cytokines

The following panel of laboratory and clinical gram-negative and gram-positive bacterial strains isolated from septic patients hospitalized at the Centre Hospitalier Universitaire Vaudois were used: *E. coli* O111:B4 (smooth LPS type), *E. coli* J5 (rough LPS type), *E. coli* O18:K1:H7 (capsule-producing strain), *K. pneumoniae* strain Caroli, *P. aeruginosa*, *S. pyogenes*, *S. mitis* 205, and *S. aureus* AW7 and P8. Bacteria were grown in tryptic soy broth (TSB; Becton Dickinson, Erembodegem, Belgium). *E. coli* O111:B4 LPS was from Sigma-Aldrich (St Louis, MO), and TNF was from Roche Diagnostics (Rotkreuz, Switzerland). Recombinant murine MIF was prepared as described [12] and contained <10 pg of endotoxin per microgram of protein, as determined by an endotoxin spectrophotometric assay (Endosafe-Portable Test System; Charles River Laboratories, Charleston, SC).

Assay for Bacterial Phagocytosis and Bacterial Killing

Bacteria were grown overnight in TSB at 37°C, washed in phosphate-buffered saline, and adjusted to approximately 10^7 CFU/mL in RPMI 1640 medium containing 10% FCS. Phagocytosis and killing assays were performed as previously described [29]. Briefly, macrophages were incubated with bacteria (20 bacteria/macrophage). After 1 hour, extracellular bacteria were washed away, and macrophages were incubated for 30 minutes (to assess phagocytosis) or 24 hours (to assess killing) in the presence of either gentamicin (for all bacterial strains except *S. aureus*) or lysostaphin (for *S. aureus*) to eliminate the remaining extracellular bacteria. Cells were lysed, and the number of intracellular bacteria was determined by plating serial dilutions of cell lysates on agar plates. Killing was expressed as the percentage of bacteria recovered after 24 hours, using the formula [(bacterial count at 24 hours)/(bacterial count at 1 hour)] × 100.

Nitric Oxide (NO) and TNF Measurements

A total of 4×10^4 macrophages were plated in 96-well cell-culture plates containing 200 μL of medium and were then stimulated with bacteria (10^6–10^7 colony-forming units [CFU]/mL) or with LPS (100 ng/mL). Concentrations of TNF and of nitrite/nitrate were measured in cell-culture supernatants, as described elsewhere [30].

RNA Analysis

Total RNA was isolated using the TriZol reagent (Invitrogen, Basel, Switzerland). Expression of TNF and glyceraldehyde-3-

phosphate dehydrogenase messenger RNA (mRNA) was assessed by Northern blotting, using specific complementary DNA probes [15].

Electrophoretic Mobility Shift Assay

A total of 2 μg of nuclear extracts were incubated for 15 minutes at room temperature with a radiolabeled consensus NF-κB probe (Santa Cruz, Santa Cruz, CA) and analyzed by an electrophoretic mobility shift assay, as described elsewhere [31].

Luciferase Assays

RAW 264.7 macrophages were transiently cotransfected with the NF-κB–pGL2 luciferase vector and the *Renilla* pRL-TK vector (Promega, Dübendorf, Switzerland). Cells were incubated for 18 hours with LPS (100 ng/mL) or *E. coli* O111 (10^8 CFU/mL). Luciferase and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) [32]. Results were expressed as relative luciferase activity (ratio of luciferase to *Renilla* luciferase activity).

Klebsiella Pneumoniae Pneumonia Model

K. pneumoniae (150–350 CFUs) were injected intranasally into $MIF^{+/+}$ and $MIF^{-/-}$ BALB/c mice [30]. Severity scores, body weight, and survival were monitored once daily up to day 30. The severity score was graded as follows: grade 1, ruffled fur; grade 2, ruffled fur plus mobility disturbance, conjunctivitis, or diarrhea; grade 3, ruffled fur, plus mobility disturbance, plus either conjunctivitis or diarrhea; grade 4, moribund; and grade 5, death. In selected experiments, mice were euthanized 3 days after bacterial challenge. Lungs were collected, and bacterial loads were determined by plating serial dilutions of lung homogenates onto agar plates.

Statistical Analysis

Comparisons between groups were assessed using analysis of variance and the Student *t* test. Survival data were presented as Kaplan-Meier curves and time to death in the treatment groups were compared using the log-rank sum test. Two-sided *P* values of less than .05 were considered to indicate statistical significance. Analyses were performed using PRISM version 5.0 (GraphPad Software, La Jolla, CA).

RESULTS

Impaired Killing of Gram-Negative Bacteria in MIF-Deficient Macrophages

To examine whether MIF affected the phagocytosis and killing of bacteria by macrophages, control and MIF-deficient RAW 264.7 macrophages were exposed to a panel of gram-negative bacteria (*E. coli* strains O111, O18, and J5; *K. pneumoniae*;

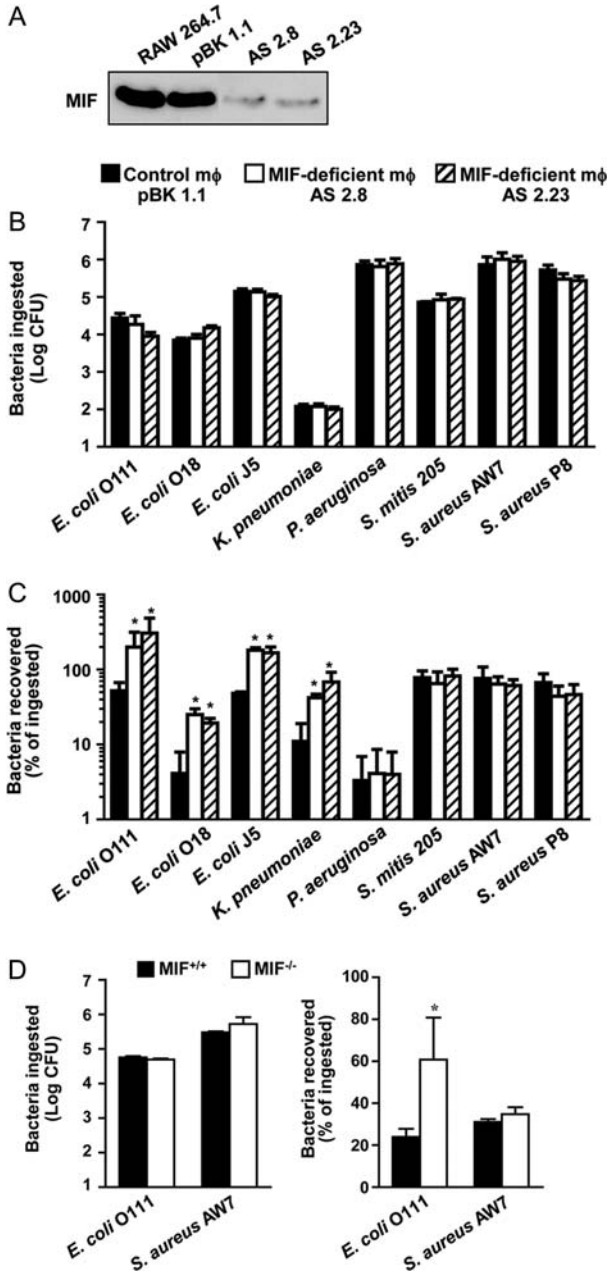

Figure 1. Impaired killing of gram-negative bacteria in macrophage migration inhibitory factor (MIF)–deficient macrophages. Western blot analysis of MIF content in control (pBK1.1) and MIF-deficient (AS 2.8 and AS 2.23) RAW 264.7 macrophages obtained as described in Materials and Methods (*A*). Control (black bars) and MIF-deficient (open and hatched bars) macrophages (*B* and *C*) and $MIF^{+/+}$ (black bars) and $MIF^{-/-}$ (white bars) bone marrow–derived macrophages (BMDMs; *D*) were exposed to *Escherichia coli* O111, *E. coli* O18, *E. coli* J5, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus mitis* 205, *Staphylococcus aureus* AW7, or *S. aureus* P8. Phagocytosis (*B* and *D*) and killing (*C* and *D*) of bacteria were quantified as described in Materials and Methods. Data are mean ± SD of 4 determinations from 1 representative experiment out of 3. **E. coli* O111: $P = .04$ and 0.03; *E. coli* O18, *E. coli* J5, and *K. pneumoniae*: $P < .005$ for AS 2.8 and AS 2.23 versus pBK 1.1, respectively (*C*). *$P = .007$ versus $MIF^{+/+}$ BMDMs (*D*).

and *P. aeruginosa*) and gram-positive bacteria (*S. mitis* and *S. aureus* strains AW7 and P8). After 1 hour, the mean number of bacteria taken up by control RAW 264.7 macrophages (5×10^5 cells) ranged between 10^4 and 10^6 CFU for all bacterial strains except *K. pneumoniae* (10^2 CFU). As shown in Figure 1*B*, the numbers of bacteria phagocytosed by MIF-deficient (AS 2.8 and AS 2.23) and control RAW 264.7 macrophages were similar for all the bacterial strains tested.

Bacterial killing by macrophages was assessed after 24 hours of incubation. Control macrophages killed bacteria with various degrees of efficiency. The proportion of ingested bacteria killed by macrophages was much higher for gram-negative organisms (*P. aeruginosa*, 97%; *E. coli* O18, 95%; *K. pneumoniae*, 89%; *E. coli* O111, 48%; and *E. coli* J5, 52%) than for gram-positive organisms (*S. aureus* P8, 33%; *S. mitis*, 33%; and *S. aureus* AW7, 24%; Figure 1*C*). MIF-deficient RAW 264.7 macrophages exhibited a severe impairment of the capacity to kill enteric gram-negative bacteria. The numbers of recovered bacteria were 4–6-fold higher in MIF-deficient than control RAW 264.7 macrophages (*E. coli* O111: $P = .04$ and $P = .03$ for MIF-deficient AS 2.8 and AS 2.23 macrophages, respectively, vs control macrophages; *E. coli* O18, *E. coli* J5, and *K. pneumoniae*: $P < .005$). In contrast, MIF-deficient macrophages did not exhibit defective killing capacity toward gram-negative nonfermenters (*P. aeruginosa*) or gram-positive cocci (*S. mitis* and *S. aureus*; Figure 1*C*). To confirm these findings, we performed similar experiments with $MIF^{-/-}$ BMDMs exposed to either *E. coli* or *S. aureus*. Like in RAW 264.7 macrophages, ingestion of *E. coli* and *S. aureus* did not differ between $MIF^{+/+}$ and $MIF^{-/-}$ BMDMs, yet killing of *E. coli* (but not *S. aureus*) was reduced 2.8-fold in $MIF^{-/-}$ BMDMs, compared with $MIF^{+/+}$ BMDMs ($P = .007$; Figure 1*D*).

We then performed add-back experiments to determine whether the addition of exogenous recombinant MIF protein would restore the bacterial killing capacity of MIF-deficient macrophages. MIF-deficient RAW 264.7 macrophages were incubated for 18 hours with recombinant MIF prior to exposure to *E. coli*. As anticipated, the phagocytosis of *E. coli* remained unchanged in cells preincubated with recombinant MIF (Figure 2*A*). In contrast, MIF supplementation in MIF-deficient macrophages increased the killing of *E. coli* by 2.5–4-fold ($P = .05$ and $P = .008$ for MIF-deficient AS 2.8 and AS 2.23 macrophages, respectively; Figure 2*B*). Together, these results indicated that MIF deficiency in macrophages did not affect the ingestion of either gram-negative or gram-positive bacteria but impaired the killing of enteric gram-negative bacteria (*E. coli* and *K. pneumoniae*).

Decreased TNF Production by MIF-Deficient Macrophages Stimulated With Gram-Negative Bacteria or LPS

On activation by microbes or microbial-derived products, macrophages produce and release a large panel of cytokines and antimicrobial molecules that play a critical role in the initiation of the innate and adaptive host defense responses. To investigate the mechanism underlying the defective bactericidal activity of MIF-deficient macrophages, we first looked at the production of NO, which is a critical component of the host microbicidal innate immune armamentarium. Control and MIF-deficient RAW 264.7 macrophages produced similar amounts of NO 2, 8, and 24 hours after stimulation with either *E. coli* O111 or *K. pneumoniae* (Figure 3). We then examined the production of TNF, which is a pivotal proinflammatory cytokine rapidly released by innate immune cells on microbial invasion. TNF production was massively downregulated in MIF-deficient RAW 264.7 macrophages after stimulation with gram-positive bacteria (*E. coli* O111, *E. coli* O18, *K. pneumoniae*, or *P. aeruginosa*) or LPS (Figure 4) but not after exposure to gram-positive bacteria (*S. pyogenes*, *S. mitis*, or *S. aureus*; data not shown). Addition of recombinant mouse TNF did not enhance phagocytosis, but it restored the bactericidal capacity of MIF-deficient AS 2.8 and AS 2.23 macrophages ($P = .01$ and $P = .0008$, respectively; Figure 5). Together, these results suggest that reduced TNF production was associated with and may have accounted for the impaired anti–gram-negative bactericidal activity of MIF-deficient macrophages.

Reduced Intracellular Signaling in MIF-Deficient Macrophages Exposed to Gram-Negative Bacteria

Given that MIF deficiency is associated with reduced expression of TLR4 in the macrophage [12, 13], we then studied the signal transduction of MIF-deficient macrophages on activation with gram-negative bacteria. In control RAW 264.7 macrophages, TNF mRNA expression peaked 2 hours after stimulation with *E. coli* or *K. pneumoniae* (Figure 6*A*). Consistent with the data on TNF production (Figure 4), the peak of TNF mRNA was delayed (reached only after 8 hours) and markedly reduced (5-fold) in MIF-deficient RAW 264.7 macrophages (Figure 6*A*). Induction of *Tnf* gene transcription on activation of TLRs by gram-negative bacteria requires nuclear translocation of NF-κB. We therefore quantified by electrophoretic mobility shift assay the NF-κB DNA binding in nuclear extracts of RAW 264.7 macrophages. Thirty minutes after stimulation with *E. coli*, NF-κB DNA-binding activity increased 9-fold in control macrophages but only 2.2–3.5-fold in MIF-deficient AS 2.8 and AS 2.23 macrophages (Figure 6*B*). To confirm that reduced nuclear localization of NF-κB affected NF-κB–dependent transcription, we quantified NF-κB–mediated transcriptional activity in RAW 264.7 macrophages transfected with a NF-κB–driven luciferase reporter plasmid. LPS and *E. coli* increased luciferase activity more strongly in control macrophages than in MIF-deficient macrophages (LPS: 10-fold vs 3.8-fold, $P = .0001$; *E. coli*: 5.7-fold vs 2.3-fold, $P = .02$; Figure 6*C*).

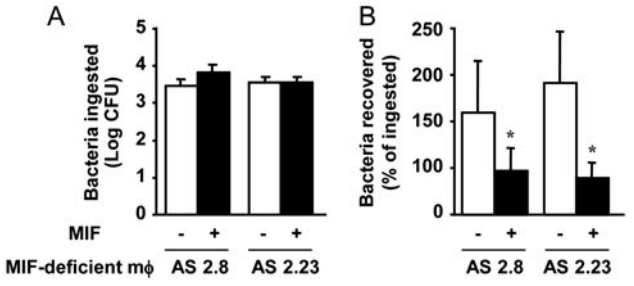

Figure 2. Recombinant macrophage migration inhibitory factor (MIF) protein restored the capacity of MIF-deficient macrophages to kill *Escherichia coli*. MIF-deficient macrophages were cultured 18 hours with (black bars) or without (open bars) recombinant MIF (100 ng/mL) and then exposed to *E. coli* O111 (MOI 20). Phagocytosis (*A*) and killing (*B*) of bacteria were quantified as described in Materials and Methods. Data are mean ± SD of 4 determinations from 1 representative experiment out of 2. **P* = .05 and *P* = .008 for AS 2.8 and AS 2.23, respectively.

Increased Mortality of Gram-Negative Pneumonia in MIF-Deficient Mice

Given that MIF deficiency impaired the killing of *E. coli* and *K. pneumoniae*, we thought to evaluate the outcome of $MIF^{+/+}$ and $MIF^{-/-}$ mice in a gram-negative pneumonia sepsis model induced by the intranasal inoculation of *K. pneumoniae*. The impact of MIF-targeted therapy in *E. coli* sepsis has been previously reported [20, 33]. Severity scores (Figure 7*A*), body weight losses (Figure 7*B*), bacterial loads (Figure 7*C*), and mortality (Figure 7*D*) were significantly higher in $MIF^{-/-}$ than in $MIF^{+/+}$ mice. These data showed that the defective bactericidal activity of MIF-deficient macrophages against gram-negatives in vitro translated into increased morbidity and mortality of gram-negative sepsis in vivo, supporting the idea that MIF is an important effector molecule of innate immunity.

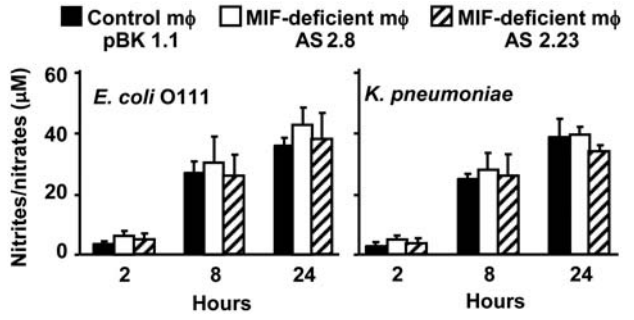

Figure 3. Normal nitric oxide production by macrophage migration inhibitory factor (MIF)–deficient macrophages. Control (pBK1.1, black bars) and MIF-deficient (AS 2.8 and AS 2.23, open and hatched bars) macrophages were cultured for 18 hours with 10^8 colony-forming units (CFU)/mL *Escherichia coli* O111 or *K. pneumoniae*. Concentrations of nitrites/nitrates in cell-culture supernatants were measured using the Griess reagent. Data are mean ± SD of triplicates from 1 representative experiment out of 2.

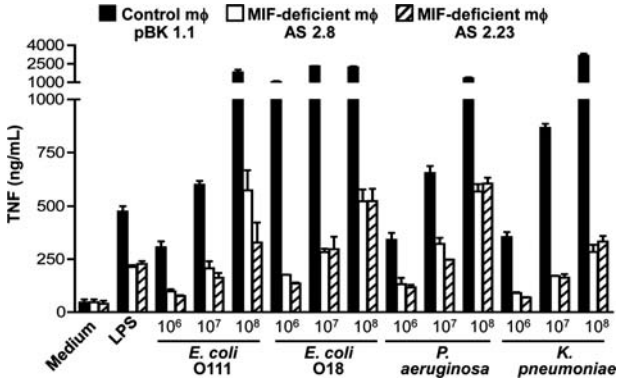

Figure 4. Decreased tumor necrosis factor (TNF) production by macrophage migration inhibitory factor (MIF)–deficient macrophages stimulated with lipopolysaccharide (LPS) or gram-negative bacteria. Control (pBK1.1, black bars) and MIF-deficient (AS 2.8 and AS 2.23, open and hatched bars) macrophages were stimulated with LPS (100 ng/mL) or with *Escherichia coli* O111, *E. coli* O18, *Pseudomonas aeruginosa,* or *Klebsiella pneumoniae* (10^6–10^8 colony-forming units (CFU)/mL). TNF concentrations were measured in cell-culture supernatants harvested 4 hours after stimulation. Data are mean ± SD of triplicates from 1 representative experiment out of 3.

DISCUSSION

Constitutive expression of MIF was found to be critical for the killing of enteric gram-negative bacteria by macrophages and for host defenses against gram-negative pneumonia. TLR4 signaling pathway was defective in MIF-deficient macrophages as shown by reduced NF-κB activity and production of proinflammatory cytokines. These observations provided a molecular basis for the innate immune defects of MIF-deficient cells or animals (present study) or associated with the use of neutralizing anti-MIF antibodies or MIF inhibitors in MIF

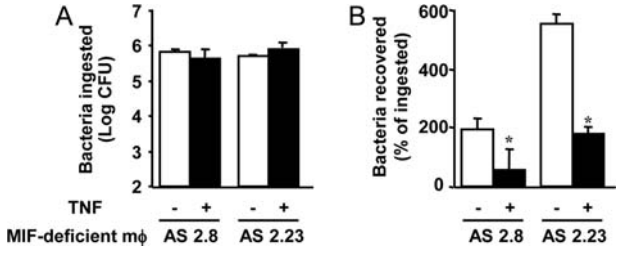

Figure 5. Tumor necrosis factor (TNF) complementation restored the bactericidal activity of macrophage migration inhibitory factor (MIF)–deficient macrophages. MIF-deficient macrophages were cultured for 18 hours with (black bars) or without (open bars) TNF (10 ng/mL) before exposure to *E. coli* O111. Phagocytosis (*A*) and killing (*B*) of bacteria were quantified as described in Material and Methods. Data are mean ± SD of 4 determinations from 1 representative experiment representative out of 2. **P* = .01 and *P* = .0008 for AS 2.8 and AS 2.23.

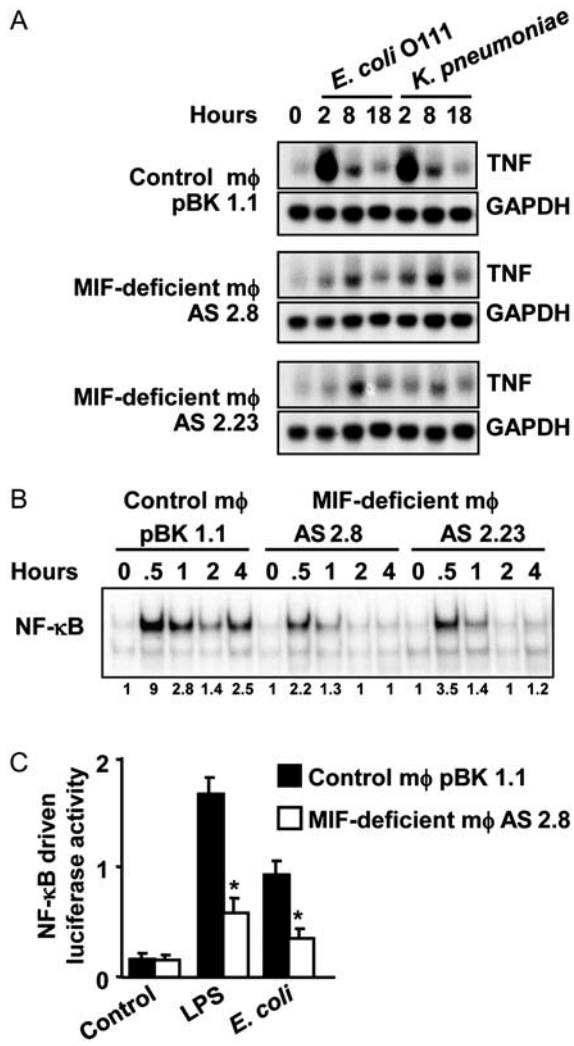

Figure 6. Impaired intracellular signaling in macrophage migration inhibitory factor (MIF)–deficient macrophages stimulated with gram-negative bacteria. *A*, Northern blot analyses of tumor necrosis factor (TNF) and glyceraldehyde-3-phosphate dehydrogenase messenger RNA expression in control and MIF-deficient macrophages cultured with 10^8 colony-forming units (CFU)/mL *Escherichia coli* O111 or *Klebsiella pneumoniae*. Results are representative of two experiments. *B*, Electrophoretic mobility shift assay of nuclear factor κB (NF-κB) DNA binding activity in nuclear extracts of control and MIF-deficient macrophages cultured with *E. coli* O111 (10^8 CFU/mL). *C*, NF-κB–driven luciferase activity in control (pBK1.1, black bars) and MIF-deficient (AS 2.8, open bars) macrophages cultured for 18 hours with lipopolysaccharide (LPS; 100 ng/mL) or *E. coli* O111 (10^8 CFU/mL). Data are mean ± SD of triplicates from 1 representative experiment out of 3 (*$P = .0001$ and $P = .02$).

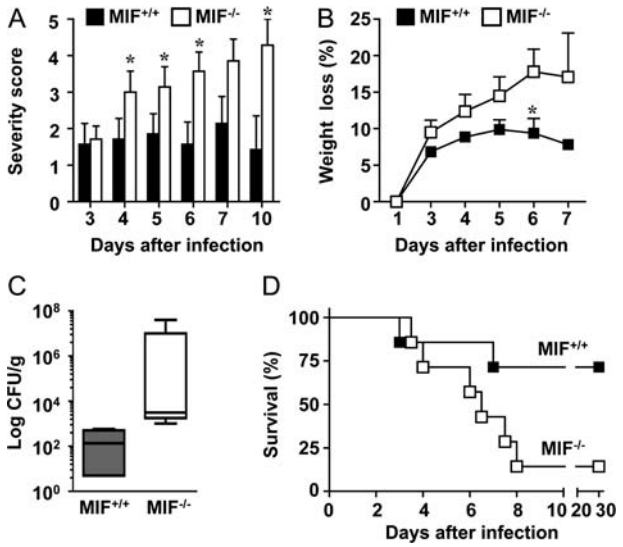

Figure 7. Severity scores, body weight losses, bacterial counts, and mortality in a *Klebsiella pneumoniae* pneumonia model. Macrophage migration inhibitory factor $(MIF)^{+/+}$ and $MIF^{-/-}$ mice were inoculated intranasally with 340 (*A*, *B*, and *D*; n = 7 mice per group) or 160 (*C*; n = 4 $MIF^{+/+}$ and 6 $MIF^{-/-}$ mice) colony-forming units (CFU) of *K. pneumoniae*. *A*, Severity scores (*$P < .05$). *B*, Percentage body weight losses (*$P = .04$). *C*, Bacterial counts (mean, 138 CFU/g [range, 5–580 CFU/g] vs 3080 CFU/g [range, 10^3–4×10^7 CFU/g]; $P = .01$) in lungs harvested 3 days after infection. *D*, Kaplan–Meier survival curves ($P = .02$).

sufficient cells or animals [12, 16, 20, 34]. These data are also in line with our previous findings that MIF-deficient macrophages displayed a phenotype characterized by hyporesponsiveness to LPS due to a reduced expression of TLR4 [12, 13].

The observation that MIF was dispensable for the phagocytosis of a broad range of bacteria by innate immune cells was surprising for 3 main reasons. First, considering that proinflammatory cytokines promote bacterial uptake by phagocytes, one might have expected MIF-deficient macrophages to exhibit impaired phagocytic capacity in the context of reduced proinflammatory autocrine activities. Yet, the present results are in line with the observation that TLR4-deficient BMDMs and dendritic cells or alveolar macrophages take up *Salmonella* Typhimurium and *E. coli* as efficiently as wild-type cells [35, 36]. Second, work by Swant et al showed that MIF promotes Rho GTPase-dependent signaling and stress fiber formation in fibroblasts [37]. Given that Rho-GTPase signaling controls actin polymerization and cytoskeleton reorganization during particle ingestion, one might have expected MIF-deficient macrophages to also exhibit phagocytosis defects. But this was not the case, a finding in agreement with the results published by Wiersinga et al that showed no impact of MIF on phagocytosis of gram-negative bacteria (ie, *Burkholderia pseudomallei*) by macrophages and neutrophils [38]. These results suggest that the impact of MIF on Rho-GTPase may be limited to certain members of the Rho family. Alternatively, MIF-mediated effects on Rho-GTPase signaling might be cell specific. Third, recombinant MIF has been shown previously to augment the uptake of fluorescent latex beads by RAW 264.7 macrophages [39]. However, there are inherent differences in

the sensing and uptake of inert particles or live bacteria by phagocytes [40]. Consistent with the present data, phagocytosis of inert microspheres was similar in wild-type, TLR2-TLR4 double-knockout or MyD88$^{-/-}$ macrophages [41].

TLRs are recruited to phagosomes and participate in the sensing of microbial pathogens [42]. The role played by TLRs in the maturation of phagosomes is controversial. On phagocytosis of *E. coli* or *S. aureus,* TLR2-, TLR4-, and MyD88-deficient macrophages acquire lysosomal markers at slower rates than wild-type macrophages, suggesting that TLR signaling was important for the maturation of phagosomes [41]. However, other investigators reported that phagosome maturation or phagosome-lysosome fusion were independent of TLR signaling [43]. The facts that MIF deficiency was associated with reduced TLR4 expression, NF-κB activity, cytokine production, and killing of gram-negative (but not gram-positive) bacteria support a role for TLR signaling in the control of phagolysosome formation or bactericidal activity.

Cytokine complementation with MIF (Figure 2), TNF (Figure 5), or interferon (IFN-γ; data not shown) increased the bactericidal activity of MIF-deficient macrophages without modification of the phagocytic activity, confirming that phagocytosis and bactericidal activity are regulated via different signaling pathways [44]. Importantly, these results suggest that MIF restored the bactericidal activity of macrophages via a MIF receptor–dependent autocrine/paracrine mechanism [2–5] and not via an intracellular JAB1-mediated pathway [10].

At first glance, the fact that MIF deficiency affected the killing of enteric (*E. coli* and *K. pneumoniae*) but not of nonfermentative (*P. aeruginosa*) gram-negative bacteria may appear surprising. However, we also observed that MIF$^{-/-}$ mice were highly susceptible to pneumonia caused by *K. pneumoniae*, whereas MIF$^{-/-}$ mice were reported to be more resistant than wild-type mice to *P. aeruginosa* lung infection [19]. Likewise, mice deficient in Toll/IL-1R domain-containing adaptor protein, a key adaptor molecule of TLR1, TLR2, TLR4, and TLR6 signaling, were shown to be resistant to *P. aeruginosa* pneumonia but highly susceptible to *K. pneumoniae* pneumonia [45]. Interestingly, MIF deficiency did not affect the killing of gram-positive bacteria. As previously reported, MIF deficiency did not impair TLR2 expression in macrophages [12]. The critical role played by TLR2 in the sensing of gram-positive bacteria and host responses to subcomponents of peptidoglycan may explain why innate immune responses to gram-positive bacteria were unaffected in MIF-deficient macrophages.

Previous studies have revealed cell-specific differences regarding the involvement of TLRs in host defenses against gram-negative bacteria. Indeed, TLR4 deficiency profoundly affected the killing of *S.* Typhimurium by bone marrow-deived dendritic cells whereas it did not impact the bactericidal activity of bone marrow-derived dendritic cells [35]. Similarly, we observed that the uptake and killing of *E. coli* were comparable in MIF$^{-/-}$ and wild-type BMDMs (data not shown). Moreover, the absence of MIF did not impact NO production following bacterial infection in vitro and in vivo ([21, 22] and present study), while it had a negative impact on NO production and susceptibility to parasitic infections caused by *Leishmania major* and *Taenia crassiceps* [46–48]. In a similar fashion, inducible nitric oxide synthase (NOS2) knockdown modestly affected the killing of *E. coli* by macrophages but had a major negative impact on parasitic infections [49, 50]. Interestingly, thioglycollate-elicited MIF$^{-/-}$ macrophages stimulated with LPS and IFN-γ had been shown previously to release higher levels of NO than MIF$^{+/+}$ macrophages [19]. The use of various subsets of macrophages, of stimuli engaging different sets of innate immune receptors, or of cells with different genetic backgrounds may explain these discrepancies. Together, these results nicely illustrate the high degree of specificity of the host innate immune defenses in terms of microbial sensing and effector responses even within a given class of microbial pathogens, such as extracellular gram-negative bacteria. Genetic studies have also revealed that polymorphisms of genes involved in antimicrobial defenses impact host defenses against infection. Consistent with an important role played by MIF in innate immunity, recent findings in humans indicate that *MIF* gene polymorphisms were associated with the susceptibility to and outcome of infectious diseases [25–27].

In conclusion, the present work has revealed an unsuspected role for MIF in the killing of extracellular gram-negative bacteria and argues in favor of an intrinsic contribution of MIF in innate immune defenses against bacterial infection. This observation confirms and extends previous studies strongly supporting the view that MIF helps preserving host integrity against infectious threats.

Notes

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References

1. Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol **2003**; 3:791–800.
2. Leng L, Metz CN, Fang Y, et al. MIF signal transduction initiated by binding to CD74. J Exp Med **2003**; 197:1467–76.
3. Shi X, Leng L, Wang T, et al. CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. Immunity **2006**; 25:595–606.

4. Bernhagen J, Krohn R, Lue H, et al. MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med **2007**; 13:587–96.
5. Schwartz V, Lue H, Kraemer S, et al. A functional heteromeric MIF receptor formed by CD74 and CXCR4. FEBS Lett **2009**; 583:2749–57.
6. Mitchell RA, Metz CN, Peng T, Bucala R. Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. J Biol Chem **1999**; 274:18100–6.
7. Santos LL, Lacey D, Yang Y, Leech M, Morand EF. Activation of synovial cell p38 MAP kinase by macrophage migration inhibitory factor. J Rheumatol **2004**; 31:1038–43.
8. Lue H, Thiele M, Franz J, et al. Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. Oncogene **2007**; 26:5046–59.
9. Hudson JD, Shoaibi MA, Maestro R, Carnero A, Hannon GJ, Beach DH. A proinflammatory cytokine inhibits p53 tumor suppressor activity. J Exp Med **1999**; 190:1375–82.
10. Kleemann R, Hausser A, Geiger G, et al. Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. Nature **2000**; 408:211–6.
11. Filip AM, Klug J, Cayli S, et al. Ribosomal protein S19 interacts with macrophage migration inhibitory factor and attenuates its pro-inflammatory function. J Biol Chem **2009**; 284:7977–85.
12. Roger T, David J, Glauser MP, Calandra T. MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nature **2001**; 414:920–4.
13. Roger T, Froidevaux C, Martin C, Calandra T. Macrophage migration inhibitory factor (MIF) regulates host responses to endotoxin through modulation of Toll-like receptor 4 (TLR4). J Endotoxin Res **2003**; 9:119–23.
14. Mitchell RA, Liao H, Chesney J, et al. Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. Proc Natl Acad Sci U S A **2002**; 99:345–50.
15. Roger T, Chanson AL, Knaup-Reymond M, Calandra T. Macrophage migration inhibitory factor promotes innate immune responses by suppressing glucocorticoid-induced expression of mitogen-activated protein kinase phosphatase-1. Eur J Immunol **2005**; 35:3405–13.
16. Bernhagen J, Calandra T, Mitchell RA, et al. MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature **1993**; 365:756–9.
17. Calandra T, Bernhagen J, Metz CN, et al. MIF as a glucocorticoid-induced modulator of cytokine production. Nature **1995**; 377:68–71.
18. Calandra T, Spiegel LA, Metz CN, Bucala R. Macrophage migration inhibitory factor is a critical mediator of the activation of immune cells by exotoxins of Gram-positive bacteria. Proc Natl Acad Sci U S A **1998**; 95:11383–8.
19. Bozza M, Satoskar AR, Lin G, et al. Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J Exp Med **1999**; 189:341–6.
20. Calandra T, Echtenacher B, Roy DL, et al. Protection from septic shock by neutralization of macrophage migration inhibitory factor. Nat Med **2000**; 6:164–70.
21. Koebernick H, Grode L, David JR, et al. Macrophage migration inhibitory factor (MIF) plays a pivotal role in immunity against *Salmonella typhimurium*. Proc Natl Acad Sci U S A **2002**; 99:13681–6.
22. Pollak N, Sterns T, Echtenacher B, Mannel DN. Improved resistance to bacterial superinfection in mice by treatment with macrophage migration inhibitory factor. Infect Immun **2005**; 73:6488–92.
23. Beishuizen A, Thijs LG, Haanen C, Vermes I. Macrophage migration inhibitory factor and hypothalamo-pituitary-adrenal function during critical illness. J Clin Endocrinol Metab **2001**; 86:2811–6.
24. Emonts M, Sweep FC, Grebenchtchikov N, et al. Association between high levels of blood macrophage migration inhibitory factor, inappropriate adrenal response, and early death in patients with severe sepsis. Clin Infect Dis **2007**; 44:1321–8.
25. Yende S, Angus DC, Kong L, et al. The influence of macrophage migration inhibitory factor gene polymorphisms on outcome from community-acquired pneumonia. FASEB J **2009**; 23:2403–11.
26. Renner P, Roger T, Bochud PY, et al. A functional microsatellite of the macrophage migration inhibitory factor gene associated with meningococcal disease. FASEB J **2012**; 26:907–16.
27. Doernberg S, Schaaf B, Dalhoff K, et al. Association of macrophage migration inhibitory factor (MIF) polymorphisms with risk of meningitis from *Streptococcus pneumoniae*. Cytokine **2011**; 53:292–4.
28. Roger T, Froidevaux C, Le Roy D, et al. Protection from lethal gram-negative bacterial sepsis by targeting Toll-like receptor 4. Proc Natl Acad Sci U S A **2009**; 106:2348–52.
29. Mombelli M, Lugrin J, Rubino I, et al. Histone deacetylase inhibitors impair antibacterial defenses of macrophages. J Infect Dis **2011**; 204:1367–74.
30. Roger T, Lugrin J, Le Roy D, et al. Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. Blood **2011**; 117:1205–17.
31. Roger T, Out T, Mukaida N, Matsushima K, Jansen H, Lutter R. Enhanced AP-1 and NF-kappaB activities and stability of interleukin 8 (IL-8) transcripts are implicated in IL-8 mRNA superinduction in lung epithelial H292 cells. Biochem J **1998**; 330 (Pt 1):429–35.
32. Roger T, Miconnet I, Schiesser AL, Kai H, Miyake K, Calandra T. Critical role for Ets, AP-1 and GATA-like transcription factors in regulating mouse Toll-like receptor 4 (Tlr4) gene expression. Biochem J **2005**; 387:355–65.
33. Kerschbaumer RJ, Rieger M, Volkel D, et al. Neutralization of macrophage migration inhibitory factor (MIF) by fully human antibodies correlates with their specificity for the beta-sheet structure of MIF. J Biol Chem **2012**; 287:7446–55.
34. Al-Abed Y, Dabideen D, Aljabari B, et al. ISO-1 binding to the tautomerase active site of MIF inhibits its pro-inflammatory activity and increases survival in severe sepsis. J Biol Chem **2005**; 280:36541–4.
35. Rescigno M, Urbano M, Rimoldi M, et al. Toll-like receptor 4 is not required for the full maturation of dendritic cells or for the degradation of gram-negative bacteria. Eur J Immunol **2002**; 32:2800–6.
36. Lee JS, Frevert CW, Matute-Bello G, et al. TLR-4 pathway mediates the inflammatory response but not bacterial elimination in *E. coli* pneumonia. Am J Physiol Lung Cell Mol Physiol **2005**; 289:L731–8.
37. Swant JD, Rendon BE, Symons M, Mitchell RA. Rho GTPase-dependent signaling is required for macrophage migration inhibitory factor-mediated expression of cyclin D1. J Biol Chem **2005**; 280:23066–72.
38. Wiersinga WJ, Calandra T, Kager LM, et al. Expression and function of macrophage migration inhibitory factor (MIF) in melioidosis. PLoS Negl Trop Dis **2010**; 4:e605.
39. Onodera S, Suzuki K, Matsuno T, Kaneda K, Takagi M, Nishihira J. Macrophage migration inhibitory factor induces phagocytosis of foreign particles by macrophages in autocrine and paracrine fashion. Immunology **1997**; 92:131–7.
40. Sander LE, Davis MJ, Boekschoten MV, et al. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature **2011**; 474:385–9.
41. Blander JM, Medzhitov R. Regulation of phagosome maturation by signals from toll-like receptors. Science **2004**; 304:1014–8.
42. Underhill DM, Goodridge HS. Information processing during phagocytosis. Nat Rev Immunol **2012**; 12:492–502.
43. Yates RM, Russell DG. Phagosome maturation proceeds independently of stimulation of toll-like receptors 2 and 4. Immunity **2005**; 23:409–17.
44. Flannagan RS, Jaumouille V, Grinstein S. The cell biology of phagocytosis. Annu Rev Pathol **2012**; 7:61–98.
45. Jeyaseelan S, Young SK, Yamamoto M, et al. Toll/IL-1R domain-containing adaptor protein (TIRAP) is a critical mediator of antibacterial defense in the lung against *Klebsiella pneumoniae* but not *Pseudomonas aeruginosa*. J Immunol **2006**; 177:538–47.

46. Juttner S, Bernhagen J, Metz CN, Rollinghoff M, Bucala R, Gessner A. Migration inhibitory factor induces killing of *Leishmania major* by macrophages: dependence on reactive nitrogen intermediates and endogenous TNF-alpha. J Immunol **1998**; 161:2383–90.
47. Satoskar AR, Bozza M, Rodriguez Sosa M, Lin G, David JR. Migration-inhibitory factor gene-deficient mice are susceptible to cutaneous Leishmania major infection. Infect Immun **2001**; 69:906–11.
48. Rodriguez-Sosa M, Rosas LE, David JR, Bojalil R, Satoskar AR, Terrazas LI. Macrophage migration inhibitory factor plays a critical role in mediating protection against the helminth parasite *Taenia crassiceps*. Infect Immun **2003**; 71:1247–54.
49. Diefenbach A, Schindler H, Donhauser N, et al. Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity **1998**; 8: 77–87.
50. Shiloh MU, MacMicking JD, Nicholson S, et al. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity **1999**; 10:29–38.