Targeted Disruption of Migration Inhibitory Factor Gene Reveals Its Critical Role in Sepsis

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Summary

To study the biologic role of migration inhibitory factor (MIF), a pleiotropic cytokine, we generated a mouse strain lacking MIF by gene targeting in embryonic stem cells. Analysis of the role of MIF during sepsis showed that MIF−/− mice were resistant to the lethal effects of high-dose bacterial lipopolysaccharide (LPS), or Staphylococcus aureus enterotoxin B (SEB) with d-galactosamine and had lower plasma levels of tumor necrosis factor (TNF-α) than did wild-type mice, but normal levels of interleukin (IL)-6 and IL-10. When stimulated with LPS and interferon γ, macrophages from MIF−/− mice showed diminished production of TNF-α, normal IL-6 and IL-12, and increased production of nitric oxide. MIF−/− animals cleared gram-negative bacteria Pseudomonas aeruginosa instilled into the trachea better than did wild-type mice and had diminished neutrophil accumulation in their bronchoalveolar fluid compared to the wild-type mice. Thiglycollate elicited peritoneal exudates in uninfected MIF−/− mice, but showed normal neutrophil accumulation. Finally, the findings of enhanced resistance to P. aeruginosa and resistance to endotoxin-induced lethal shock suggest that the counteraction or neutralization of MIF may serve as an adjunct therapy in sepsis.

Key words: migration inhibitory factor • gene-deficient mice • sepsis • lipopolysaccharide • Pseudomonas aeruginosa

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine released by macrophages, T cells, and the pituitary gland during inflammatory responses (1, 2). It has been shown to act as a proinflammatory cytokine, playing a major role in endotoxin shock (3) and counter-regulating the antiinflammatory effects of dexamethasone (4). Antibodies to MIF diminish the manifestations of autoimmunity in certain experimental models (5, 6). Furthermore, recent studies have shown that MIF enhances resistance to the pathogen Leishmania major (7, 8). Its ubiquitous expression (9) and developmental regulation (10, 11) suggest that MIF might have functions beyond the immune system.

To study the role of MIF, we generated a mouse strain lacking MIF by gene targeting in embryonic stem cells and analyzed mechanisms of sepsis using these MIF−/− mice. Sepsis triggered by gram-negative and gram-positive bacterial infection is a major cause of death of hospitalized patients (12). Endotoxin induces MIF release from macrophages and pituitary cells, as well as in vivo. MIF has been shown to be released in large quantities and found in the serum of mice after challenge with LPS (3, 13). A critical role of MIF in endotoxemia was suggested by the observation that recombinant MIF enhanced LPS-induced lethality, whereas anti-MIF antibodies had a protective effect (3). In this study, we describe the generation of MIF−/− mice and characterize the specific role of MIF in sepsis.

Materials and Methods

Targeting Vector Construction and Generation of MIF−/− Mice. A mouse Mif genomic fragment was isolated from a 129SV/J genomic library (14), and a 6.1-kb Xbal fragment containing the 5′ upstream region, exons 1–3, and the 3′ region was subcloned in pBluescript. The vector was digested with EcoRV (sites present in the 3′ region of the gene and in the polylinker of the plasmid), releasing a 0.7-kb fragment. The vector was religated and digested with AgeI, replacing the 0.7-kb fragment. The vector was religated and digested with AgeI, disrupting part of exon 2, the second intron, and exon 3. The neo' cassette was inserted by blunt ligation after end-filling the vector and the neo' cassette. The disrupted genomic vector was digested with Xbal/Xhol and ligated into the HSV-TK vector. The targeting vector was linearized with Xhol,
and 30 μg was transfected by electroporation into 107 J1 embryonic stem (ES) cells that were maintained on a feeder layer of neonatal embryonic fibroblasts in the presence of 500 U/ml of leukemia inhibitory factor. After 8 d of selection with G418 (200 μg/ml) and FIAU (2 μM), 30 clones were analyzed by Southern blot hybridization using the 0.7-kb EcoRI/V/ XbaI 3′ fragment as a probe. One clone displayed a novel 7-kb XbaI allele predicted to occur after homologous recombination. This heterozygous ES cell line was injected into day 3.5 C57BL/6 blastocysts, and the blastocysts were transferred into pseudopregnant females. Chimeric mice were bred with C57BL/6 mice and agouti offspring were analyzed for the MIF disrupted allele by Southern blot hybridization.

LPS-induced Shock and Cytokine Measurement. 8–12-wk-old, sex-matched MIF+/+, MIF−/−, and MIF+/− mice from heterozygous matings were injected intraperitoneally with 25 mg/kg of LPS from Escherichia coli serotype O111:B4 (Sigma Chemical Co.). This dose was based on previous experiments in C57BL/6 mice that gave a 50–60% lethal dose. The mice were monitored for signs of endotoxemia at least twice daily for 5 d. For determining the plasma cytokine levels, mice similarly treated were killed by CO2 at 90 min and bled by cardiac puncture. Plasma from three mice was pooled within each group. Two to three pools from each group were measured by ELISA.

Shock Induced with LPS, TNF-α, and Staphylococcus Enterotoxin B with d-galactosamine. Mice were injected intraperitoneally with a mixture of 20 mg/mouse of d-galactosamine (Sigma Chemical Co.) and 1 μg/mouse of LPS (E. coli type as above), 1 μg/mouse TNF-α (PharMingen), or 37 mg/kg of Staphylococcus aureus enterotoxin B (SEB; Toxin Technology). Susceptible mice all died in <24 h.

Macrophage Cultures. Macrophages elicited for 4 d with thioglycollate or resident macrophages were obtained by peritoneal lavage using 10 ml of PBS. 106 cells in 1 ml of RPMI/10% FCS supplemented with glutamine, β-ME, amino acids, penicillin, and streptomycin were cultured in 24-well plates in triplicate. After 2 h at 37°C in 5% CO2, nonadherent cells were removed by washing. Cell activation was performed by the addition of fresh media containing 100 μg of LPS and 100 U/ml of IFN-γ (PharMingen) followed by incubation for 24 h. Supernatants were collected and frozen at −20°C for subsequent analysis. For bactericidal assays, resident macrophages were prepared as described above and cultured in media with LPS and IFN-γ for 20 h; after washing with PBS, RPMI with 10% mouse serum without antibiotics was added with 107 CFU/ml Listeria monocytogenes. After 30 min, all plates were washed. 1 ml of PBS with 0.05% Triton X-100 was added to wells of the 0 time point. The other wells were incubated in RPMI with 10% FCS and 5 μg/ml of gentamycin for 2 or 6 h and the cells were lysed as above. Listeria killing was determined by counting the number of CFU per well.

Cytokine and Nitric Oxide X-100 Analysis. TNF-α, IL-6, IL-10, and IL-12 from plasma or cell culture supernatants were measured by ELISA using reagents from PharMingen following the manufacturer's directions. MIF ELISA was performed with reagents from R & D Systems, Inc. To evaluate nitric oxide (NO), NO3− and NO2− were measured from cell culture supernatants using Griess's assay (15).

Lung Infection with Pseudomonas aeruginosa. The mucoid P. aeruginosa strain A01 was used and maintenance of stocks and methods used were as previously described (16). Mice matched in age and gender (8–10 wk) were anesthetized with Ketamine (90 mg/kg) and Xylazine (10 mg/kg) (both from Sigma Chemical Co.). P. aeruginosa (3.5 × 108), freshly grown in tryptic soy broth for 18 h, were instilled into the trachea in a volume of 50 μl. The lung clearance of P. aeruginosa was measured by killing mice immediately and at 6 and 24 h after P. aeruginosa instillation. Lungs were excised and homogenized in 3 ml of ice-cold sterile distilled water. Homogenates were diluted appropriately in sterile PBS, cultured overnight in brain-heart infusion agar plates, and the number of CFU was determined. Results are expressed as CFU 6 h/CFU 0 h and CFU 24 h/CFU 0 h.

To assess microvascular injury and neutrophil accumulation, 100 μl of Evans blue (6.25 mg/ml) was administered intravenously 2 h after P. aeruginosa instillation, and bronchoalveolar lavage (BAL) × 1 ml was performed 6 h after bacterial instillation. The recovered BAL fluid was assessed for total cell counts using a standard hemocytometer, and differential cell counts were determined from Diff-Quick-stained (Dade Diagnostics) cytospin preparations. Permeability changes were determined by comparing the leakage of Evans blue into the BAL fluid to the amount remaining in the plasma (17).

Results and Discussion

Production and Characterization of MIF−/− Mice. The mouse MIF gene was disrupted by replacing part of exons 2 and 3 with a neocassette (Fig. 1 A). The targeting vector was electroporated into J1 ES cells and G418-FIAU–resistant colonies were isolated. The average frequency of homologous recombination was about 1 in 30 resistant colonies. Correctly targeted ES cells were used to generate chimeric animals by injection into C57BL/6 blastocysts. Highly chimeric animals transmitted the mutated allele through the germline, and homozygous mice were generated by intercrosses of heterozygous mice (Fig. 1 B). Northern blot analysis from liver RNA of LPS-treated animals confirmed that the gene disruption created a null mutation (Fig. 1 C). ELISA of serum from LPS-treated animals further confirmed the absence of MIF protein in the MIF−/− mice (Fig. 1 D). Of the 218 animals obtained from heterozygous matings, 16% were homozygous for the null allele. The newborn MIF−/− mice developed normally in size and behavior and were fertile. The litter size of heterozygous and homozygous matings were normal. Both gross examination and histopathological analysis of several organs (kidney, liver, spleen, adrenal, thymus, lungs, heart, brain, and intestine) of MIF−/− mice revealed no abnormalities. Flow cytometric analysis of splenocytes and thymocytes of MIF−/− mice demonstrated normal lymphocyte populations (data not shown).

Response to High-dose LPS: Survival and Cytokines. To analyze the role of MIF in endotoxemia, MIF−/−, MIF+/−, and wild-type mice were injected intraperitoneally with a high dose of LPS (25 mg/kg). MIF deficiency conferred a remarkable resistance to the lethal effects of LPS (Fig. 2 A). However, MIF−/− mice still exhibited signs of endotoxemia a few h after LPS treatment, including piloerection, shivering, and lethargy, although these signs appeared milder than in control mice. Since a cascade of inflammatory mediators triggered by LPS is important in the pathogenesis of endotoxic shock (18, 19), we measured cytokine levels in the plasma of MIF−/− mice compared with wild-type mice 90 min after LPS challenge. There was a 50% reduction in the plasma levels of TNF-α but similar levels of IL-6...
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and IL-10 (Fig. 2 B). The observed resistance to LPS could be partially due to the diminished TNF-α production secondary to lack of MIF (13); to an enhanced antiinflammatory effect of stress-induced steroids no longer counter-regulated by MIF (4); or to possible other proinflammatory properties of MIF which have yet to be determined.

Cytokines and NO of Macrophages from MIF-/- Mice. Macrophages were studied as they are critically involved in the pathogenesis of endotoxemia (20). Furthermore, MIF and TNF-α have been shown to work in an autocrine/paracrine fashion; i.e., MIF increases macrophage TNF-α, which in turn increases MIF release (13). To study the effect of MIF deficiency on macrophage activation, thioglycollate-elicited peritoneal macrophages were stimulated with LPS and IFN-γ for 6, 12, 18, and 24 h, and the supernatants were analyzed. Macrophages from MIF-/- mice showed a marked decrease in TNF-α (P < 0.001; Fig. 3 A). However, MIF-/- macrophages were able to produce IL-12 and IL-6 (Fig. 3, B and C). These results show that MIF is required for the optimal production of TNF-α during endotoxemia. The lowered plasma levels of TNF-α are probably at least partially due to diminished production by macrophages.

It has been shown that recombinant MIF enhances NO production in vitro by macrophages stimulated with IFN-γ (21). Furthermore, attenuated Salmonella transfected with the MIF gene alone, or in combination with TNF-α or IFN-γ administered orally to susceptible mice, reduced L. major infection and enhanced NO production (7). Surprisingly, there was a significant increase (P < 0.005) in NO in the MIF-/- macrophages stimulated with LPS and IFN-γ (Fig. 3 D). These results suggest that endogenous macrophage MIF either dampens NO production by these cells or increases its turnover.

Response to TNF-α, LPS, and SEB by MIF-/- Mice Receiving d-galactosamine. To further analyze the role of MIF in lethal endotoxemia, we took advantage of mouse models that have been developed to closely mimic the high sensitivity of humans to the toxic effects of bacterial products. Hepatocytes from mice sensitized with d-galactosamine have a transcriptional arrest and become highly susceptible to the cytotoxic action of TNF-α (22, 23). The reduction
of TNF-α found in MIF−/− mice suggested that MIF acted upstream from TNF-α but might still be involved in the toxic effect of TNF-α. However, injection of a lethal dose of TNF-α (1 μg/mouse) with d-galactosamine killed all five wild-type and all five MIF−/− mice (Table I), indicating that MIF is not required for the lethal effects of TNF-α.

Although MIF−/− mice were resistant to a high dose of LPS (Fig. 2 A), they were susceptible to a combination of low-dose LPS and d-galactosamine; all five mice in each group died (Table I), indicating that MIF is not required for the lethal effects of TNF-α.

Further studies showed that MIF−/− mice were resistant to the lethal effects of another bacterial product, SEB, with d-galactosamine. All five wild-type mice died from SEB injected intraperitoneally whereas all five MIF−/− mice lived. SEB acts as a superantigen for T cells, resulting in the release of inflammatory cytokines involved in toxic shock (25). Sera taken 90 min after SEB showed 65% less TNF-α in MIF−/− than in wild-type mice, 140 ± 25 and 396 ± 89 pg/ml, respectively (± SE, P < 0.05). These results indicate that MIF plays a critical role in superantigen-induced toxic shock in which T cells play a major role.

It is interesting to note that the phenotype of intracellular adhesion molecule (ICAM)-1−/− deficient mice is similar, showing resistance to high doses of LPS as well as low doses of SEB with d-galactosamine, and susceptibility to a combination of a low dose of LPS and d-galactosamine (26). ICAM-1−/− deficient mice have reduced transendothelial leukocyte migration but normal TNF-α levels in response to LPS. However, the mechanisms of action in this phenotype are different for MIF−/− mice, as the latter have reduced TNF-α production and no impairment of leukocyte migration to the peritoneum elicited by thioglycollate (mean neutrophil count after 4 h was 6.71 ± 2.23 x 10⁶ and 11.9 ± 2.15 x 10⁶ in wild-type (n = 3) and MIF−/− (n = 4) mice, respectively, P < 0.1).

Response of MIF−/− Mice to Infection. To determine the role of MIF in host defense to gram-negative bacteria, P. aeruginosa were instilled intratracheally into MIF−/− and wild-type mice. The MIF−/− mice efficiently cleared bacteria from the lungs 24 h after infection, having almost 3 log fewer bacteria than heterozygous or wild-type controls (MIF−/− 9.3 ± 4.0 x 10³, versus combined MIF+/− and MIF+/- 2.1 ± 0.9 x 10⁶, P < 0.03, Fig. 4 A). Of interest, there was a significant decrease in neutrophils in the BAL at 6 h in the MIF−/− mice compared to controls, 55.3 ± 7.6 x 10³ versus 95.9 ± 13.5 x 10³ (n = 6, P < 0.04); Fig. 4 B. This is consistent with the report that anti-MIF antibody diminishes LPS-induced neutrophil migration to the lungs and BAL fluid as well as the level of macrophage protein 2, a powerful neutrophil chemokine (27).

Inflammation has usually been thought to be a necessary part of the host's defense against microorganisms, something the body must accept to successfully defend itself. However, the findings reported here, along with the enhanced bacterial clearing and diminished inflammation in mice lacking the CD14 receptor for endotoxin (28), suggest that host defense is more efficient with certain LPS induced inflammatory cytokines. Indeed, IL-1 and TNF-α can enhance the growth and invasiveness of pathogenic gram-negative bacteria (29, 30). Moreover, MIF has been found in the alveolar airspaces of patients with adult respiratory distress syndrome (ARDS), and increases the secretion of proinflammatory cytokines from alveolar cells (31).

We have initiated studies on MIF−/− mice to determine the effect of MIF on other infectious agents. In a preliminary experiment using the gram-positive bacteria L. monocytogenes, MIF−/− mice were not more susceptible than wild-type controls. Furthermore, peritoneal macrophages

| Dose        | +/+ (dead/total) | −/− (dead/total) |
|-------------|-----------------|-----------------|
| LPS 1 μg/mouse | 5/5             | 5/5             |
| TNF-α 1 μg/mouse | 5/5           | 5/5             |
| SEB 37 mg/kg  | 5/5             | 0/5             |

Mice were injected intraperitoneally with d-galactosamine, 20 mg/mouse in conjunction with LPS, TNF-α, or SEB. Mice were observed for 3 d and the lethal effect was seen within 24 h.

- **Table I.** Effect of LPS, TNF, or SEB in conjunction with d-galactosamine

- **Figure 3.** Stimulated macrophages from MIF-deficient mice produce less TNF-α but more NO. Thioglycollate-elicited macrophages were stimulated with 100 μg/ml of LPS and 100 U/ml of IFN-γ. Supernatants were removed at 6, 12, 18, and 24 h and tested for (A) TNF-α, (B) IL-12, (C) IL-6, and (D) NO. Similar results were obtained in three other experiments at 24 h.
obtained from MIF/−/− mice and stimulated with IFN-γ and LPS were able to kill intracellular L. monocytogenes as well as wild-type macrophages (data not shown). In contrast, MIF/−/− mice were more susceptible than wild-type mice to the intracellular parasite L. major. Lymph node cells from infected MIF/−/− mice showed higher IL-4 production after antigen stimulation than did those from wild-type animals, suggesting that MIF plays a role in Th1/Th2 balance (our unpublished results).

Taken together, our results show that MIF plays a critical role in endotoxic shock and SEB toxicity without hampering the ability of mice to clear gram-negative or -positive infections. Indeed, the increased resistance to the gram-negative bacterial product LPS, as well as the enhanced ability to clear P. aeruginosa infections in the lungs in MIF/−/− mice, indicates that neutralization or counteraction of MIF might constitute an adjunct therapy for the treatment of sepsis. Further studies with this animal model should clarify the role of MIF in immunity, inflammation, and other biologic functions.

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