

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 α (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. Thioglycollate 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)¹ 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 pa-

tients (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 XbaI 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, disrupting part of exon 2, the second intron, and exon 3. The *neo^r* cassette was inserted by blunt ligation after end-filling the vector and the *neo^r* cassette. The disrupted genomic vector was digested with XbaI/XhoI and ligated into the HSV-TK vector. The targeting vector was linearized with XhoI,

¹Abbreviations used in this paper: BAL, bronchoalveolar lavage; ES, embryonic stem; MIF, migration inhibitory factor; NO, nitric oxide; SEB, *Staphylococcus aureus* enterotoxin B.

and 30 μg was transfected by electroporation into 10^7 J1 embryonic stem (ES) cells that were maintained on a feeder layer of *neo^r* embryonic fibroblasts in the presence of 500 U/ml of leukemia inhibitory factor. After 8 d of selection with G418 (200 $\mu\text{g}/\text{ml}$) and FIAU (2 μM), 30 clones were analyzed by Southern blot hybridization using the 0.7-kb EcoRV/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 $\mu\text{g}/\text{mouse}$ of LPS (*E. coli* type as above), 1 $\mu\text{g}/\text{mouse}$ 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. 10^6 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 10^7 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 $\mu\text{g}/\text{ml}$ 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 Assays. 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* (3.5×10^6), freshly grown in tryptic soy broth

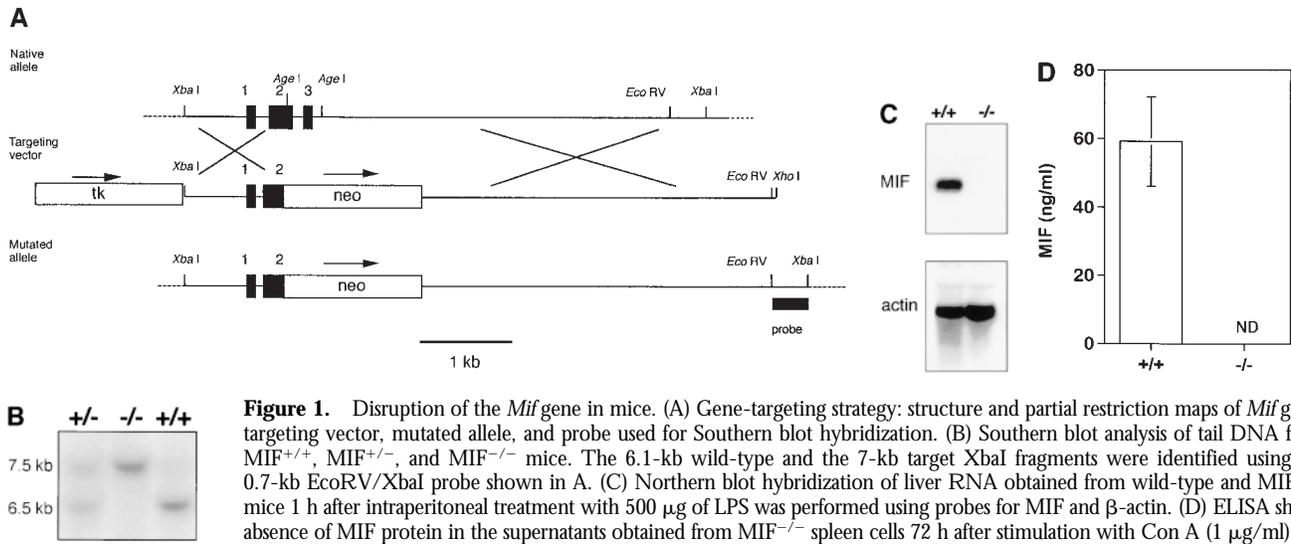
for 18 h, were instilled into the trachea in a volume of 50 μl . The lung clearance of *P. aeruginosa* was measured by killing mice immediately and at 6 and 24 h after *P. aeruginosa* instillation. Lungs were excised and homogenized in 3 ml of ice-cold sterile distilled water. Homogenates were diluted 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) \times 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 Dif-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 *neo^r* cassette (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 90 min after LPS challenge. There was a 50% reduction in the plasma levels of TNF- α but similar levels of IL-6



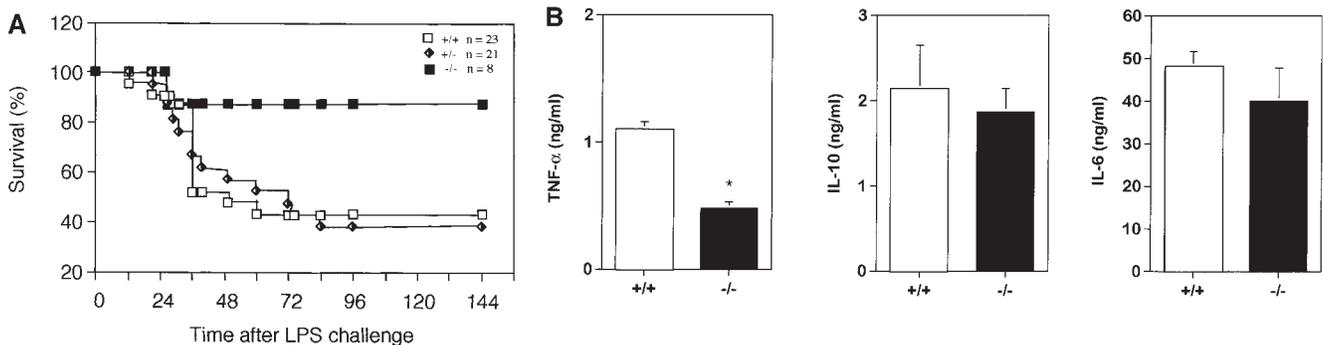
and IL-10 (Fig. 2 B). The observed resistance to LPS could be partially due to the diminished TNF- α production secondary to lack of MIF (13); to an enhanced antiinflammatory effect of stress-induced steroids no longer counter-regulated by MIF (4); or to possible other proinflammatory properties of MIF 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



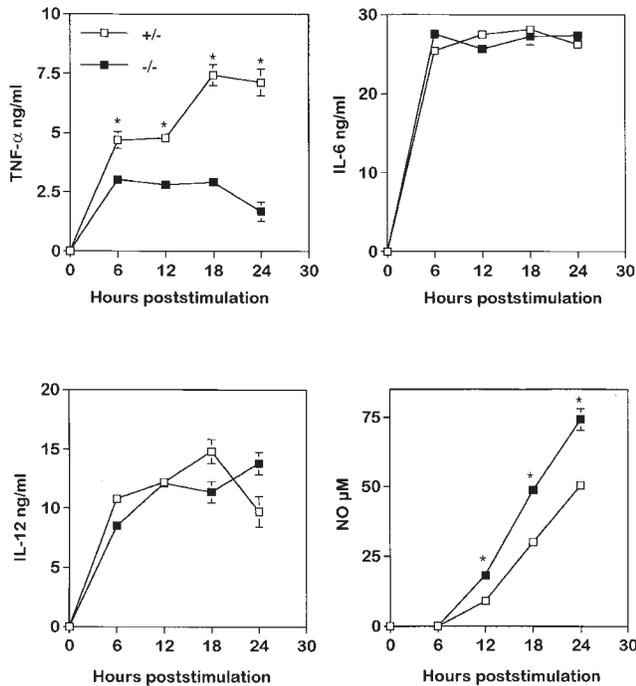


Figure 3. Stimulated macrophages from MIF-deficient mice produce less TNF- α but more NO. Thioglycollate-elicited macrophages were stimulated with 100 μ g/ml of LPS and 100 U/ml of IFN- γ . Supernatants were removed at 6, 12, 18, and 24 h and tested for (A) TNF- α , (B) IL-12, (C) IL-6, and (D) NO. Similar results were obtained in three other experiments at 24 h.

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 μ g/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). Indeed, it has been shown that the mechanisms leading to death are different in models using high or low doses of LPS (24).

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 (\pm 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

Table I. Effect of LPS, TNF, or SEB in Conjunction with D-galactosamine

	Dose	+/+ (dead/total)	-/- (dead/total)
LPS	1 μ g/mouse	5/5	5/5
TNF- α	1 μ g/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.

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 $6.71 \pm 2.23 \times 10^6$ and $11.9 \pm 2.15 \times 10^6$ 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 $^{-/-}$ $9.3 \pm 4.0 \times 10^3$, versus combined MIF $^{+/+}$ and MIF $^{+/-}$ $2.1 \pm 0.9 \times 10^6$, $P < 0.03$, 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, $55.3 \pm 7.6 \times 10^5$ versus $95.9 \pm 13.5 \times 10^5$, ($n = 6$, $P < 0.04$); 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 without 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

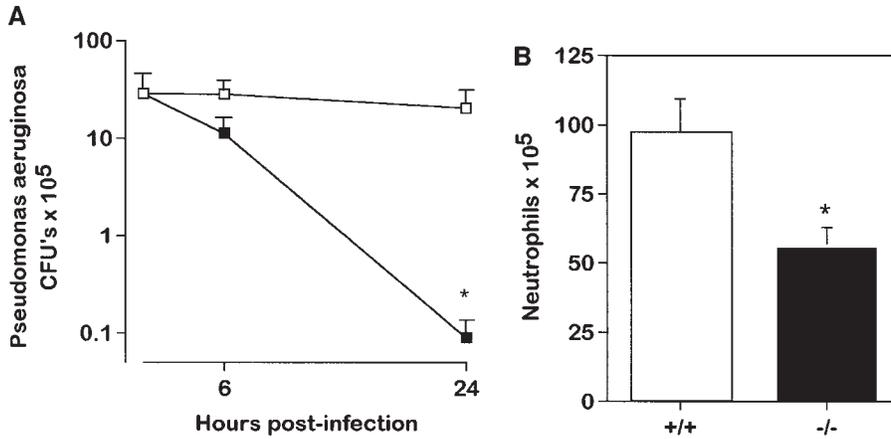


Figure 4. MIF^{-/-} mice clear *P. aeruginosa* instilled into the lungs better than do wild-type mice, and show less neutrophil accumulation. (A) Clearance of *P. aeruginosa* at 6 and 24 h. Data from wild-type (WT) and heterozygous mice were combined as they showed no difference. For 24 h: WT, *n* = 15; MIF^{-/-}, *n* = 14. For 6 h: WT, *n* = 8; MIF^{-/-}, *n* = 6. □, +/+; ■, -/-. (B) Neutrophils accumulating in the BAL fluid at 6 h after instillation of bacteria.

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.

We thank Cox Terhorst, John Samuelson, Willy Piessens, and Roberta David for their critical reading of the manuscript.

This work was supported in part by National Institutes of Health grant AI22532-13. M. Bozza was partially supported by a grant from CNPq, Brazil.

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Received for publication 22 September 1998 and in revised form 5 November 1998.

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