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Improved Resistance to Bacterial Superinfection in Mice by Treatment with Macrophage Migration Inhibitory Factor

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Nosocomial infections in immune-suppressed patients are a widespread problem in intensive care medicine. Such patients are highly susceptible to infections because their immune defenses are impaired and, therefore, unable to adequately combat invading microorganisms. To investigate the problem of sepsis-induced immune suppression, we used a model in which mice developed sublethal peritonitis induced by cecal ligation and puncture (CLP). Two days after CLP mice were in an immune-suppressed state, as measured by impaired capacity to produce tumor necrosis factor (TNF) and enhanced susceptibility to bacterial infections. Since macrophage migration inhibitory factor (MIF) is a critical mediator of septic shock by modulation of innate immune responses, the role of MIF in sepsis-induced immune suppression was analyzed. Neutralization of endogenous MIF further enhanced susceptibility to bacterial superinfection after CLP. Conversely, treatment with recombinant human MIF before the bacterial superinfection protected the animals. MIF treatment reconstituted the impaired capacity to produce proinflammatory cytokines, such as TNF and interleukin-6. This study indicates that MIF might be able to ameliorate the sepsis-induced immune suppression by reenabling the organism to react adequately to a secondary bacterial challenge.

Macrophage migration inhibitory factor (MIF) was one of the first cytokines to be identified. This compound was discovered in the 1960s as a protein released from activated T lymphocytes in a delayed-type hypersensivity reaction (4). When in 1989 human MIF was cloned, information concerning the structure and biological activities of MIF increased (26). The amino acid sequence of MIF consists of 114 amino acids with high homology in all mammals (about 90%). Over the last few years the central role of MIF in the innate immune system and inflammatory response has become known. A constitutive level of MIF can be found in sera of animals and humans, and the premade molecule is also stored in intracellular pools. MIF is released from cells of the anterior pituitary gland upon stimulation with bacterial lipopolysaccharide (LPS) (2). MIF is also released quickly from a variety of different cell types, such as macrophages and lymphocytes and also endothelial and pituitary cells, in response to stress and infection (2, 4, 6, 10). After secretion MIF enhances production of inflammatory molecules, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6), nitric oxide, and products of the arachidonic acid pathway (3, 7, 22). MIF is part of the host response to gram-negative and gram-positive bacterial products (2, 8). Protection against lethal endotoxemia and staphylococcal toxic shock, as well as lethal cecal ligation and puncture (CLP)- and *Escherichia coli*-induced infection in mice, was achieved by neutralization of endogenous MIF or deletion of the MIF gene (5, 9). On the other hand, MIF seemed to be required for killing of *Leishmania major* (15). Besides interacting with a recently described receptor (i.e., the invariant chain of major histocompatibility class II molecules detectable at a low level on the surface of cells), MIF also has enzymatic activity (16, 21, 24). MIF exhibits phenylpyruvate keto-enol isomerase and thiol-protein oxidoreductase activities in vitro. Whether these enzymatic functions play a role in vivo is not yet clear. A number of studies have provided evidence that MIF is important in inflammatory processes by documenting elevated levels of MIF in inflammatory diseases, such as glomerulonephritis, rheumatoid arthritis, and sepsis (9, 18, 19).

We intended to investigate the role of MIF in sepsis-induced immune suppression. Sepsis is a major clinical problem characterized by dysregulated systemic inflammatory responses with the presence of high levels of inflammatory and proinflammatory mediators in the blood (17). Sepsis is a life-threatening disease which patients often survive only with the help of intensive care medicine. Clinical as well as experimental studies have shown that the immune response after passage through the hyperinflammatory phase turns into a compensatory hypoinflammatory response (17). The immune system seems to be suppressed and, therefore, unable to adequately combat invading microorganisms. As a consequence, patients are highly susceptible to nosocomial infections which might lead to death because hospital germs often are multiresistant against antibiotics. To investigate the phenomenon of the sepsis-induced immune suppression, we established an animal model by using CLP as a well-characterized model of induction of polymicrobial peritonitis. Two days after sublethal CLP mice went into an immune-suppressed state, characterized by a reduced capacity to produce TNF and by high susceptibility to bacterial infections (12). Our data show that during this phase of CLP-induced immune suppression MIF can reconstitute both the immune defense to different bacterial superinfections and the capacity to produce the inflammatory cytokines TNF and IL-6.

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MATERIALS AND METHODS

Animals. Ten- to twelve-week-old (20- to 25-g) female NMRI mice were purchased from Charles River (Sulzfeld, Germany). All animals were housed in

groups of 5 or 10 mice per cage with free access to food and water and were accustomed for at least 5 days before experimentation.

Reagents. Recombinant human MIF (rhMIF), expressed in *E. coli* (plasmid pRS5a), was generously provided by Novartis, Vienna,, Austria. The material had an apparent molecular mass of 12.5 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent Western blotting. The material was active in the dopachrome tautomerization assay, as described previously (11). As a positive control for biologically active material, rhMIF was kindly provided by T. Calandra, Lausanne, Switzerland (2). An antiserum to MIF was generated by immunizing rabbits (Chinchilla Bastard; Charles River, Sulzfeld, Germany) with recombinant mouse MIF (2). Polyclonal antibodies were isolated from the rabbit antiserum by protein A affinity chromatography (Bio-Rad, Hercules, Calif.).

Bacteria. *Pseudomonas aeruginosa* strain PA 103 and *Salmonella enterica* serovar Typhimurium strain ATCC 14028s were stored in LB medium (Invitrogen, Carlsbad, Calif.) suspensions containing glycerol (30%; Merck, Darmstadt, Germany) at −80°C. *Listeria monocytogenes* strain ATCC 43251 was stored on plastic beads at −80°C. For the infection models bacteria were cultured in the following media. *P. aeruginosa* was grown in LB medium for 24 h at 37°C. *S. enterica* serovar Typhimurium was cultured for 24 h in LB medium containing ampicillin (100 μg/ml; Sigma-Aldrich, St. Louis, Mo.) because the strain is ampicillin resistant; after this 1 ml of the bacterial suspension was diluted in normal LB medium and cultured for another 24 h. *L. monocytogenes* was cultured for 24 h on Columbia agar with sheep blood (5%) and was suspended in a 0.9% NaCl solution to a McFarland index of 0.5 as measured with an ATB nephelometer (Biomerix, New York, N.Y.). Working dilutions of all bacterial strains were made, and bacterial counts were determined by plating and counting CFU. *P. aeruginosa* was diluted in LB medium and plated on agar containing Cetrimid (Oxoid Ltd., Basingstoke, United Kingdom); *S. enterica* serovar Typhimurium was diluted in LB medium and plated on XLD agar; *L. monocytogenes* was diluted in saline and plated on blood-containing agar.

CLP. For CLP mice were anesthetized intraperitoneally (i.p.) with Ketanest (75 mg/kg; Parke, Davis & Company, Munich, Germany) and Xylazin (16 mg/kg; WDT, Garbsen, Germany) in 0.3 ml phosphate-buffered saline (PBS) as described previously (13). The cecum was exposed by a 10- to 15-mm midline incision of the anterior abdomen and subjected to 30% ligation, followed by puncture with a needle (diameter of opening, 0.4 mm). The cecum was then replaced, and the abdominal wound was closed with steel clips. Two days after CLP mice were infected with *S. enterica* serovar Typhimurium (5×10^3 CFU, i.p.), *P. aeruginosa* (2×10^6 CFU, intranasally), or *L. monocytogenes* (4×10^6 CFU, i.p.). For treatment with anti-MIF mice received polyclonal anti-MIF (100 μg, i.p.) either at the same time as CLP for kinetic studies or 1 h before the bacterial challenge for survival studies. For treatment with rhMIF mice received rhMIF (0.8 mg/kg, i.p.) either 1 h before challenge with *P. aeruginosa* or 6 h before challenge with *S. enterica* serovar Typhimurium or *L. monocytogenes.*

Cytokine serum levels. After CLP blood was taken at different times, and serum was prepared. Ninety minutes before bleeding the animals were stimulated with LPS (1 μg in 200 μl PBS, intravenously [i.v.]) from *Salmonella enterica* serovar Abortus-equi (14). Control mice without CLP were stimulated with LPS, and blood was taken 90 min later. TNF and IL-6 levels in serum were determined by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minn.), and MIF levels were also determined by an ELISA (Chemicon, Hofheim, Germany).

Statistics. For comparison between treatment groups Kaplan-Meier survival curves were compared using the log rank test. The Student *t* test was used for the ELISA data (SPSS 12.0 statistical package; SPSS Inc., Chicago, Ill.). All *P* values lower than 0.05 were considered statistically significant. Each experiment was performed at least twice.

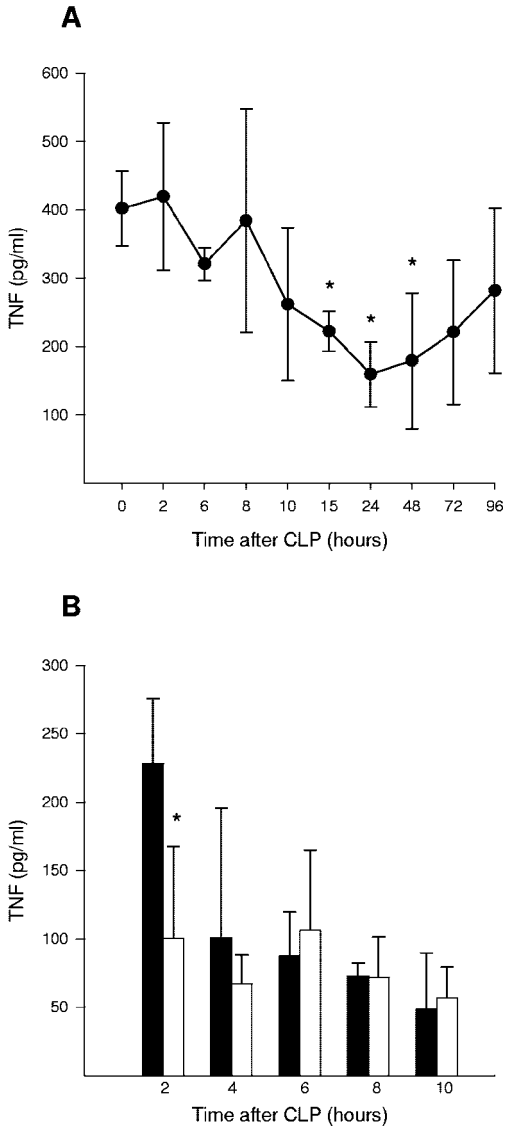

FIG. 1. TNF production capacity after CLP. At different times after CLP serum TNF levels were determined in mice after stimulation with LPS (1 μg per mouse, i.v.) 90 min previously (three mice per time). (A) Kinetics of TNF production capacity after CLP. The level at time zero represents the TNF serum level for mice without CLP before LPS stimulation. An asterisk indicates that the *P* value is <0.05. (B) TNF production capacity after CLP and anti-MIF treatment. The solid bars indicate TNF levels for control (PBS-treated) mice, and the open bars indicate serum TNF levels in mice treated with anti-MIF antibodies. An asterisk indicates that the *P* value is 0.021.

RESULTS AND DISCUSSION

Influence of MIF on TNF production capacity after CLP. To determine the innate immune status after CLP, different groups of mice were stimulated with LPS at several times after CLP, and 90 min later serum TNF levels were determined. A constant decrease in the TNF level in serum was observed over the first 24 h after CLP (Fig. 1A). Only after 2 days did LPS-induced TNF levels begin to recover. Therefore, in further experiments we considered day 2 after CLP a time when mice reproducibly exhibited CLP-induced impaired TNF production capacity.

Death after CLP could be due to overshooting inflammation, as indicated by high TNF production. Since neutralization of MIF has been shown to improve survival after CLP (9), we tested the TNF production capacity of mice treated with MIF neutralizing antibodies at the time of CLP. Neutralization of MIF led to a significant decrease in TNF production capacity when it was measured in serum 2 h after CLP (Fig. 1B). Four hours after CLP a difference in LPS-induced TNF levels could no longer be measured. The observed reduction in TNF production capacity very early after CLP by neutralization of MIF obviously reduced the extent of the hyperinflammatory reac-

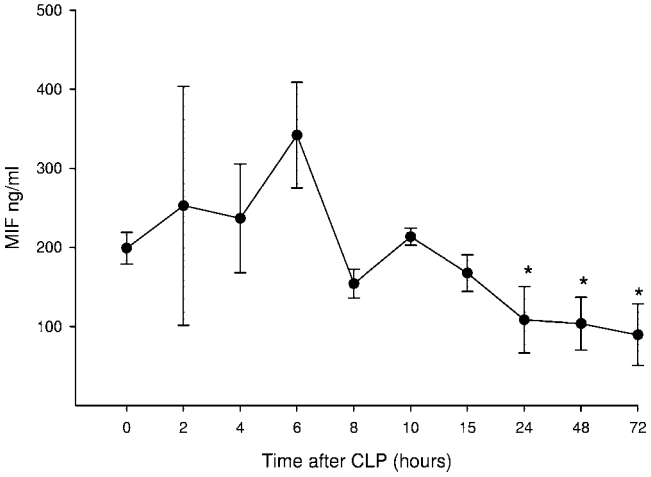

FIG. 2. MIF serum levels after CLP. At different times after CLP blood was taken from mice (three mice per time), and serum MIF levels were determined. The level at time zero represents the MIF serum level for mice without CLP. An asterisk indicates that the *P* value is <0.05.

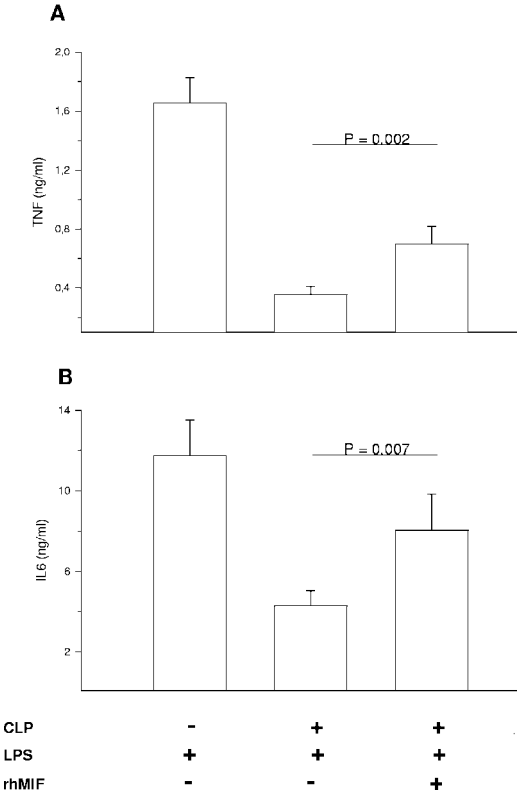

FIG. 3. Effect of treatment with rhMIF on TNF and IL-6 production capacity after CLP. Two days after sublethal CLP, groups of mice ($n = 5$) received either 100 μl PBS or 20 μg rhMIF i.p. Six hours later, all mice received 1 μg LPS i.v., and 90 min later blood was taken. Serum levels of TNF (A) and IL-6 (B) were measured by ELISA. The difference in TNF and IL-6 production capacity after treatment with rhMIF was statistically significant.

tion. Thus, since MIF has been shown to enhance inflammatory reactions (2), neutralization of MIF could contribute to the amelioration of chronic inflammation in models of arthritis (19, 20, 25) and to survival after lethal CLP (9).

Determination of the serum levels of MIF in mice after CLP clearly showed that serum MIF levels, as well as local MIF levels in the peritoneal cavity, seemed to be enhanced for only a few hours after CLP and then declined, similar to the LPS-induced serum TNF levels, and stayed below the baseline level for more than 7 days (Fig. 2; data not shown). Thus, the hypoinflammatory state of mice 2 days after CLP was reflected not only by the lower levels of the inflammatory cytokine TNF but also by the lower levels of MIF in serum of mice which had undergone CLP.

Capacity to produce proinflammatory cytokines after rhMIF treatment. Since inflammatory cytokines are essential for protection against bacterial infection and MIF has been reported to enhance the levels of proinflammatory cytokines and because TNF production capacity, as well as serum MIF levels, are reduced 2 days after CLP, we tested whether rhMIF could improve cytokine production in immune-suppressed mice. For this purpose, mice were subjected to sublethal CLP and were stimulated with LPS 2 days later. Ninety minutes after LPS injection blood was taken, and the serum levels of TNF and IL-6 were determined. As shown in Fig. 3, the reduced TNF production capacity after CLP was significantly restored by pretreatment with rhMIF (Fig. 3A), even though the levels did not reach the serum TNF levels of mice which had not undergone CLP previously. A similar result was obtained for the IL-6 production capacity (Fig. 3B). Thus, rhMIF pretreatment partly restored the TNF and IL-6 production capacity of mice immune suppressed by previous CLP.

Bacterial superinfection after neutralization of endogenous MIF. Since neutralization of MIF increased survival in *E. coli*- and LPS-induced shock models, as well as after CLP, we investigated whether MIF neutralization could also be beneficial in a superinfection model. To generate a suppressed immune status in mice characterized by low TNF production capacity, as shown in Fig. 1A, CLP was performed, and mice were infected 2 days later. One hour before challenge with *S. enterica* serovar Typhimurium mice were treated either with polyclonal antibodies directed against MIF to neutralize endogenously produced MIF or with control rabbit immunoglobulin G (IgG). Consistent with our previous results (12) the immune suppression after CLP was clearly shown by the difference in mortality between CLP-treated and *S. enterica* serovar Typhimurium-challenged mice, even though the mice received rabbit IgG 1 h prior to *S. enterica* serovar Typhimurium challenge, compared to the group which received only CLP treatment (100% survival) or the *S. enterica* serovar Typhimurium-infected group (70% survival). While all mice in the anti-MIF-treated superinfected group died within 12 days, 30% of the control-treated mice survived for 2 weeks ($P = 0.026$) (Fig. 4). Similarly, when mice were challenged with *P. aeruginosa* instead of *S. enterica* serovar Typhimurium 2 days after CLP, neutralization of MIF also clearly enhanced mortality of the superinfected animals (data not shown). These results demonstrate that while neutralization of MIF at the time of CLP or up to 8 h later protected mice from septic shock (9), neutralization of MIF during the hypoinflammatory immune-suppressed phase made animals more susceptible to a secondary bacterial infection.

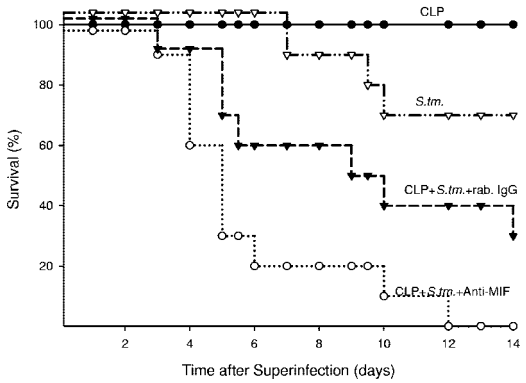

FIG. 4. Effect of MIF neutralization in *S. enterica* serovar Typhimurium (*S.tm.*) superinfection after CLP. Groups of mice were infected with 5×10^3 *S. enterica* serovar Typhimurium CFU ($n = 10$) (▽) or were subjected to CLP ($n = 7$) (■). Additional groups of mice were treated 2 days after CLP and 1 h before the bacterial challenge either with 100 μg rabbit IgG (rab. IgG) ($n = 10$) (▼) or 100 μg rabbit anti-MIF ($n = 10$) (○) i.p. Survival was monitored for14 days. The reduced survival of anti-MIF-treated mice after CLP plus *S. enterica* serovar Typhimurium infection was statistically significant ($P = 0.026$).

Bacterial superinfection after treatment with rhMIF. Since the MIF levels in serum of mice 2 days after CLP were lower than those in naïve mice and neutralization of MIF did not improve survival of these immune-suppressed mice but rather enhanced their susceptibility to superinfection, treatment with rhMIF was performed. To do this, immune-suppressed mice received a single dose of rhMIF 2 days after CLP. Different bacterial strains were used for the challenge, as follows: (i) *S. enterica* serovar Typhimurium, as a classical mouse pathogen; (ii) *P. aeruginosa*, representing bacteria typically known for nosocomial infections; and (iii) *L. monocytogenes*, as a representative of the gram-positive bacteria. Mice were challenged with *P. aeruginosa* 1 h and with *S. enterica* serovar Typhimurium or *L. monocytogenes* 6 h after rhMIF treatment. Previous experiments had shown that rhMIF treatment was equally effective when it was administered between 1 and 6 h before bacterial challenge (data not shown).

In all three superinfection models a synergistic lethal effect of CLP plus bacterial superinfection 2 days later was evident (Fig. 5A to C). In the *S. enterica* serovar Typhimurium superinfection model all PBS-treated control mice died within 8 days after bacterial challenge (Fig. 5A). Treatment with rhMIF restored the resistance of CLP-treated mice to the level of resistance of naïve mice infected with the same dose of *S. enterica* serovar Typhimurium ($P = 0.013$). Also, in the *P. aeruginosa* superinfection model rhMIF treatment significantly increased survival in immune-suppressed mice ($P = 0.026$) (Fig. 5B). In the third model of superinfection rhMIF treatment also improved survival of CLP-treated mice after a secondary bacterial challenge with *L. monocytogenes* by delaying death and improving the number of surviving animals significantly ($P = 0.027$) (Fig. 5C). A single dose of rhMIF reconstituted the ability of the CLP-treated mice to cope with the secondary bacterial infection, whether it was infection with *S. enterica* serovar Typhimurium, infection with *P. aeruginosa*, or infection with *L. monocytogenes.*

Taken together, these results demonstrate that rhMIF treatment can have a beneficial effect in immune-suppressed mice by enhancing resistance to bacterial superinfections. A similar effect was observed by us previously with recombinant human TNF (rhTNF) treatment in the same experimental system for CLP-induced immune suppression (12). Treatment with a single dose of rhTNF improved the survival of mice after *S. enterica* serovar Typhimurium or *P. aeruginosa* superinfection 2 days after CLP. However, while in the experiments reported

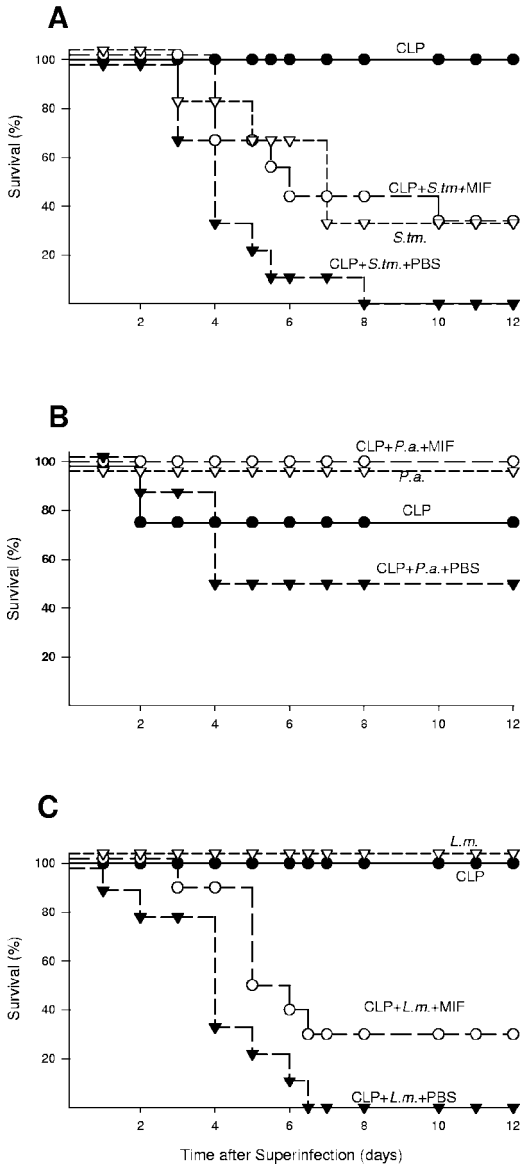

FIG. 5. Effect of treatment with rhMIF in bacterial superinfection after CLP. Two days after CLP groups of mice received either 20 μg MIF (○) or 100 μl PBS (▼) i.p. 6 h (A and C) or 1 h (B) before a secondary bacterial infection. Groups of mice were superinfected with 5×10^3 CFU *S. enterica* serovar Typhimurium (*S.tm.*) ($n = 6$) (A), 2×10^6 CFU *P. aeruginosa* (*P.a.*) ($n = 4$) (B), or 4×10^6 CFU *L. monocytogenes* (*L.m.*) ($n = 9$) (C) (▽) or underwent CLP without superinfection ($n = 4$) (●). Improved survival of rhMIF-treated mice compared with PBS-treated mice was statistically significant (panel A, $n = 9$ and $P = 0.013$; panel B, $n = 8$ and $P = 0.026$; panel C, $n = 10$ for rhMIF-treated mice and $n = 9$ for PBS-treated mice and $P = 0.027$).

here rhMIF treatment also improved the survival of mice after *L. monocytogenes* superinfection, mortality increased upon treatment with rhTNF in the previous *L monocytogenes* superinfection experiments. In addition, while larger amounts of rhTNF were harmful after CLP, indicating that there was a narrow therapeutic window for the TNF dosage (12), the dose of rhMIF used here could be 10-fold higher and still be protective (data not shown). Therefore, treatment of immune-suppressed mice with rhMIF seems to be safer than rhTNF treatment.

Knowledge of the immune status in sepsis patients is essential before immunotherapy is started. At the onset of sepsis or early in septic shock neutralization of the proinflammatory cytokine MIF seems to be beneficial for attenuating the hyperinflammatory phase (9). Two days after CLP, however, the immune status has completely changed. Experimental animals are in a hypoinflammatory phase (i.e., immune suppressed) and, therefore, unable to react adequately to invading microorganisms. Neutralization of MIF during this phase seems to worsen the situation by enhancing the mortality in the superinfection models. Conversely, MIF treatment of immune-suppressed mice could make mice more resistant to secondary infections. This could be due in part to the enhanced capacity to produce inflammatory cytokines after rhMIF treatment since higher LPS-stimulated TNF and IL-6 serum levels correlated with an improved capacity to combat the invading microorganisms in the superinfection models. Enhancement of TNF and IL-6 levels alone, however, most likely does not account for the beneficial MIF effect in immune-suppressed mice because treatment with rhTNF was deleterious in previous *L. monocytogenes* superinfection experiments (12). Additional mechanisms, such as counterregulation of immunosuppressive glucocorticoid actions, enhanced phagocytosis, or inhibition of apoptosis of macrophages and neutrophils could be considered to be involved (1, 7, 23). Further investigations of the detailed mechanisms involved in the protective effect of rhMIF in overcoming the immune-suppressed state of mice after CLP might help answer some of these questions. So far, treatment with rhMIF seems to provide an interesting new option for immunotherapy to confer protection against nosocomial infections during immune suppression.

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