Human mesenchymal stem cells reduce mortality and bacteremia in gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes

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IN THE PAST DECADE, cellular therapy with mesenchymal stem cells (MSC) has been tested in several models of organ injury and dysfunction. MSC were used first in a clinical trial of osteogenesis imperfecta (15), then in lysosomal storage disease (18), and subsequently in graft vs. host disease (20). MSC have been also tested in early clinical trials in cardiac disease, inflammatory bowel disease, stroke, and several other clinical disorders (10, 20, 28, 36). Severe sepsis is a major cause of death in critically ill patients, with 750,000 new cases every year and >200,000 fatalities (1). With increased use of invasive surgical procedures and immunosuppression, the incidence is likely to increase in the next few years. New therapeutic strategies are needed to improve the outcome of septic patients. Bone marrow-derived MSC have been protective in preclinical animal models of acute lung injury (ALI) induced by endotoxin and live Escherichia coli (5, 12, 13, 19), as well as in endotoxin-induced ALI in an ex vivo perfused human lung (21). More importantly, there is new evidence that MSC have a beneficial effect in preclinical models of polymicrobial sepsis (11, 30, 32). The protective role of MSC in these studies has been attributed primarily to their immunomodulatory properties mediated by soluble paracrine factors such as IL-10, PGE2, and TNF-α-induced protein 6.

These prior experiments suggest that MSC could be a novel therapeutic strategy for the treatment of human sepsis. All the results from published in vivo mouse models of sepsis were obtained by intravenous injection of syngeneic MSC. Little is known about the behavior of human MSC in similar conditions, although there is evidence of their beneficial effects in a mouse model of myocardial infarction (6, 24), LPS-induced ALI (5), and E. coli pneumonia (19). Similar to their murine homologs, human MSC are multipotent adult stem cells, found in the bone marrow and other anatomic niches, that have the capacity to differentiate into multiple cell types, such as osteoblasts, adipocytes, and chondroblasts, under in vitro conditions (7, 34, 36). No experiments have tested human MSC in an in vivo mouse model of sepsis. Consequently, the primary hypothesis for this study was that bone marrow-derived human MSC would exert a therapeutic effect in a mouse model of severe gram-negative peritoneal sepsis. Compared with the two controls, there was a beneficial effect of MSC on increasing survival. Therefore, we studied the mechanisms for the protective effect, including the levels of pro- and anti-inflammatory cytokines, the number of bacteria in the peritoneum, spleen, and blood, and the phagocytic capacity of neutrophils and monocytes in the septic mice.

MATERIALS AND METHODS

Animals. C57BL/6 male mice (8–12 wk old; Jackson Laboratory) were maintained in the animal facility at the University of California, San Francisco (UCSF). All experimental protocols were approved by the Institutional Animal Care and Use Committee at UCSF.
Cell culture. Allogeneic bone marrow-derived human MSC were cultured as previously described (21). Briefly, human MSC were obtained from the Texas A & M Health Science Center College of Medicine, Institute for Regenerative Medicine (Temple, TX), a National Institutes of Health repository. The cells met all the criteria for classification as MSC, as defined by the International Society of Cellular Therapy (7). In addition, the cells were found by immuno-fluorescence to be negative for CD45 and CD19. Cells were thawed and expanded in tissue culture flasks (BD Falcon) at a density of 5 × 10^6 cells/150 cm². Cells were passaged every 3–4 days by trypsinization when they reached 70–80% confluency and were used for the experiments at passages 3–10, as in our prior studies (21). Between each passage, viability was measured with Trypan blue exclusion. MSC were cultured in α-minimum essential medium without ribonucleosides or deoxyribonucleosides containing 2 mM L-glutamine and 16.5% FBS (HyClone, Thermo Scientific) and 1% penicillin-streptomycin. Cells were cultured in a humidified incubator in 5% CO₂ at 37°C under sterile conditions. Before each experiment, cells were trypsinized, counted, washed twice with PBS, and resuspended in PBS. Each experiment was done at least in triplicate with cells from different batches. The same approach and criteria were used to thaw, culture, passage, and wash the control cells, murine 3T3 fibroblasts (American Type Culture Collection, Manassas, VA). The 3T3 fibroblasts were grown in α-minimum essential medium without ribonucleosides or deoxyribonucleosides containing 10% FBS and 1% penicillin-streptomycin.

Bacterial culture and preparation. The Pseudomonas aeruginosa strain PAK was used. The methods used to passage, store, amplify, and quantify the bacteria are described elsewhere (39). The K1 (23) were placed in sterile Eppendorf tubes in RPMI medium supplemented with 5% FBS. After collecting the peripheral blood in EDTA sterile tubes, we followed an isolation technique described previously (27). Briefly, the blood was collected over Histopaque-1119 and -1077 (polysucrose/sodium diatrizoate, densities 1.119 and 1.077, respectively; Sigma-Aldrich) and centrifuged at 700 g for 30 min. After centrifugation, the buffy coat, containing monocytes and lymphocytes, and the layer beneath the buffy coat, containing neutrophils, were collected and placed in separate 15-ml polypropylene tubes. Cells were washed three times, with 10 ml of PBS added each time, and centrifuged at 200 g for 10 min, and then the supernatant was removed. Residual red blood cells were lysed by suspension of the pellet in a hypotonic solution (0.2% NaCl) for 30 s. Isotonicity of the solution was reestablished by addition of hypertonic solution (1.2% NaCl) and centrifuged at 200 g for 10 min. After an additional wash with 10 ml of PBS, the pellet was suspended in 1 ml of PBS, and the total cells were measured by a Coulter counter (Z1 series, Beckman Coulter). In the end, aliquots of 1 × 10^6 cells were placed in sterile Eppendorf tubes in RPMI medium supplemented with 5% FBS.

Measurement of total cell counts with differential in blood. After collecting the peripheral blood in EDTA sterile tubes, we followed an isolation technique described previously (27). Briefly, the blood was collected over Histopaque-1119 and -1077 (polysucrose/sodium diatrizoate, densities 1.119 and 1.077, respectively; Sigma-Aldrich) and centrifuged at 700 g for 30 min. After centrifugation, the buffy coat, containing monocytes and lymphocytes, and the layer beneath the buffy coat, containing neutrophils, were collected and placed in separate 15-ml polypropylene tubes. Cells were washed three times, with 10 ml of PBS added each time, and centrifuged at 200 g for 10 min, and then the supernatant was removed. Residual red blood cells were lysed by suspension of the pellet in a hypotonic solution (0.2% NaCl) for 30 s. Isotonicity of the solution was reestablished by addition of hypertonic solution (1.2% NaCl) and centrifuged at 200 g for 10 min. After an additional wash with 10 ml of PBS, the pellet was suspended in 1 ml of PBS, and the total cells were measured by a Coulter counter (Z1 series, Beckman Coulter). In the end, aliquots of 1 × 10^6 cells were placed in sterile Eppendorf tubes in RPMI medium supplemented with 5% FBS.

Antimicrobial activity of neutrophils and mononuclear cells. Direct inhibition of bacterial growth by mononuclear cells and/or neutrophils was assessed by counting CFU in the agar plates. Briefly, neutrophils or mononuclear cells (1 × 10^6 cells) infected with 2 × 10^5 CFU of E. coli strain K1 (23) were placed in sterile Eppendorf tubes in RPMI medium supplemented with 5% FBS and incubated for 1 h in a humidified CO₂ incubator. Aliquots of culture medium were obtained from each tube, serially diluted with sterile PBS, and plated on LB-agar plates (TEKnova, Hollister, CA). Colonies were counted after overnight incubation at 37°C. The tubes were centrifuged at 400 g for 10 min and washed twice with PBS. A cell smear was made using a Cytospin centrifuge (Thermo Shandon), and cells were visualized by Wright-Giemsa staining (Fisher Scientific). A differential of the white blood cell count was obtained by counting 100 cells.

Measurement of total cell counts with differential in blood. White blood cells, hemoglobin, hematocrit, and platelet number with differential were measured using Hemavet (Drew Scientific).

Peritoneal fluid and blood mediator measurements. Blood samples were centrifuged at 8,000 rpm for 10 min, and the supernatants were collected and stored at −80°C. Plasminogen activator inhibitor 1 (PAI-1), TNF-α, IL-10, and C5a levels were measured by ELISA or DuoSet kits (R & D Systems). PGE₂ was measured by ELISA (Cayman Chemical). Peritoneal fluid samples were centrifuged at 2,000 rpm for 10 min, and the supernatants were collected and stored at −80°C. TNF-α and macrophage inflammatory protein 2 (MIP-2), mouse neutrophil chemokines, were measured by ELISA (R & D Systems).

Neutrophil and mononuclear cell isolation from peripheral blood. After collecting the peripheral blood in EDTA sterile tubes, we followed an isolation technique described previously (27). Briefly, the blood was collected over Histopaque-1119 and -1077 (polysucrose/sodium diatrizoate, densities 1.119 and 1.077, respectively; Sigma-Aldrich) and centrifuged at 700 g for 30 min. After centrifugation, the buffy coat, containing monocytes and lymphocytes, and the layer beneath the buffy coat, containing neutrophils, were collected and placed in separate 15-ml polypropylene tubes. Cells were washed three times, with 10 ml of PBS added each time, and centrifuged at 200 g for 10 min, and then the supernatant was removed. Residual red blood cells were lysed by suspension of the pellet in a hypotonic solution (0.2% NaCl) for 30 s. Isotonicity of the solution was reestablished by addition of hypertonic solution (1.2% NaCl) and centrifuged at 200 g for 10 min. After an additional wash with 10 ml of PBS, the pellet was suspended in 1 ml of PBS, and the total cells were measured by a Coulter counter (Z1 series, Beckman Coulter). In the end, aliquots of 1 × 10^6 cells were placed in sterile Eppendorf tubes in RPMI medium supplemented with 5% FBS.

Fluorescence immunohistochemistry. For immunohistochemistry, paraffin sections of the spleen were processed through xylene and graded alcohol, rinsed in PBS, and blocked with 5% goat serum for 2 h. Sections were incubated with a primary antibody for CD163 or CD206 (goat anti-mouse, Santa Cruz Biotechnology) at a dilution of 1:50 in 5% goat serum overnight at 4°C. Then sections were washed three times with PBS for 10 min and exposed to the secondary antibody, rabbit FITC-conjugated anti-goat IgG (in 5% goat serum), for 1 h at room temperature. After they were washed, the sections were mounted with Vectashield mounting medium. Images were obtained using a Leica DM 1000 microscope.
Flow cytometry. Blood samples were obtained by cardiac puncture of the right ventricle and collected in EDTA tubes, 100 μl of blood were added to sterile polypropylene tubes, Fc receptors were blocked by preincubation with anti-mouse CD16/CD32 antibody (eBioscience, San Diego, CA) for 30 min on ice, and different combinations of antibody (Table 1) were added and additionally incubated for 30 min on ice in the dark. Red blood cells were then lysed with red blood cell lysis buffer (eBioscience), washed twice with flow cytometry staining buffer (eBioscience), and immediately subjected to flow cytometric analysis. To assess phagocytic activity, the pHrodo E. coli phagocytosis kit (Invitrogen, Life Technologies, Carlsbad, CA) was used: pHrodo E. coli particles were added after incubation with the antibody cocktail according to the manufacturer’s instructions and additionally incubated for 2 h at 37°C with slight agitation; control samples were incubated on ice. Phagocytosis was terminated by transfer of the samples on ice; samples were then processed according to the kit instructions and subjected to flow cytometric analysis. Spleens were collected, a single cell suspension was obtained by the standard procedure, and splenocytes were resuspended in RPMI medium supplemented with 10% FBS (HyClone) and further processed as described above. The cells were analyzed on a flow cytometer (model LSRII, Becton Dickinson). Monocytes were gated as SSClowCD11bhiCD115hiCD11bhiCD115hiCD11c, neutrophils were gated as SSCmed, and lymphocytes as SSClowCD45R/B220 APC-eFluor 780 eBioscience. The percentage of monocytes and neutrophils were calculated as: pHRodo E. coli + cell count/pHrodo E. coli + cell count, and immediately subjected to flow cytometric analysis. To assess phagocytic activity, the pHrodo E. coli phagocytosis kit (Invitrogen, Life Technologies, Carlsbad, CA) was used: pHrodo E. coli particles were added after incubation with the antibody cocktail according to the manufacturer’s instructions and additionally incubated for 2 h at 37°C with slight agitation; control samples were incubated on ice. Phagocytosis was terminated by transfer of the samples on ice; samples were then processed according to the kit instructions and subjected to flow cytometric analysis. Spleens were collected, a single cell suspension was obtained by the standard procedure, and splenocytes were resuspended in RPMI medium supplemented with 10% FBS (HyClone) and further processed as described above. The cells were analyzed on a flow cytometer (model LSRII, Becton Dickinson). Monocytes were gated as SSClowCD11bhiCD115hi cells; neutrophils were gated as SSCmed, CD11bhiCD115hiCD11c, and lymphocytes as SSClowCD45R/B220 APC-eFluor 780 eBioscience. The percentage of monocytes and neutrophils were calculated as: pHRodo E. coli + cell count/pHrodo E. coli + cell count.

Statistical analysis. Comparisons between two groups were done by unpaired t-test; P < 0.05 was considered to be statistically significant. Comparisons among more than two groups were made by ANOVA with Bonferroni’s correction. Survival data are presented as Kaplan-Meier curves, and the statistical significance was assessed by the Cox model. All the normally distributed data (temperature, macrophages and neutrophils, and antibacterial activity) are expressed as means ± SD. Results that were not normally distributed (cytokine and mediator levels, cell counts, and P. aeruginosa CFU) are expressed as median and 25–75 percentile. Comparisons among groups were made with Wilcoxon-Mann-Whitney analyses with Bonferroni’s corrections after Kruskal-Wallis test. All statistical analyses were performed with Stata 9.0. A two-sided P < 0.05 was considered statistically significant.

RESULTS

Treatment with MSC improves survival. Survival rate was significantly improved in the mice treated with MSC compared with the mice treated with 3T3 fibroblasts or PBS. The survival rate at 48 h was ~75% in the MSC group and ~40% in the control groups (Fig. 1).

Mice in all three groups showed a decrease in their mean body temperature, reaching the nadir at 12 h, when the mice began to die. MSC administration attenuated the decline in temperature compared with the control groups. The temperature was significantly higher at 12 h in the mice treated with MSC than in the 3T3 and PBS groups (Fig. 2).

Effect of MSC treatment on plasma level of PAI-1 and number of platelets. Because elevated levels of plasma PAI-1 have been reported to be a biomarker of prognosis for severe sepsis (2), PAI-1 levels were measured in plasma at 12 h after intraperitoneal injection of P. aeruginosa. PAI-1 levels were significantly lower in mice treated with MSC than in the 3T3 and PBS groups (Fig. 3A), suggesting that the improvement in survival in the MSC-treated mice was associated with an improvement in the severity of sepsis. Severe sepsis is also associated with thrombocytopenia (26), and we found that the number of platelets was significantly higher in MSC-treated mice than in the control groups (Fig. 3B), suggesting again an improvement in sepsis with MSC therapy. The mean white blood cell and neutrophil counts in the peripheral blood were higher in the MSC-treated mice than in the control groups, but the differences did not reach significance (Table 2). Taken together, these results reflect a protective effect of MSC following acute gram-negative peritonitis.

Effect of MSC treatment on levels of cytokines in plasma and peritoneal fluid. To test whether the beneficial effect of MSC was related to their immunomodulatory properties, plasma concentrations of pro- and anti-inflammatory cytokines were...
measured, with the focus on paracrine soluble factors that have been identified elsewhere (12, 21, 30, 32) to be potentially important in mediating their therapeutic effect. However, by ELISA, there were no statistically significant differences in the plasma levels of TNF-α, PGE₂, or IL-10 among all three groups (Fig. 4). In addition, to determine if MSC had a local immunomodulatory effect at the site of infection, we measured TNF-α and MIP-2 concentrations in the peritoneal fluid. There was no difference in the levels of these two mediators in the peritoneal fluid among the three groups, although TNF-α was statistically lower in the MSC-treated mice than in the PBS control group (Fig. 5). Thus the beneficial effects of MSC administration in this P. aeruginosa peritonitis model were not apparently associated with a change in the pro- or anti-inflammatory responses, at least among the factors that were measured.

Effect of MSC on bacterial clearance in peritoneum, lung, spleen, and blood. To further investigate the potential mechanism for the beneficial effects of MSC, the bacterial counts in the peripheral blood, peritoneal fluid, spleen, and lungs at 12 h after P. aeruginosa injection were assessed. In peritoneal fluid and lungs, bacterial counts were lower in the MSC-treated group, but these differences were not significant (Table 3). In the spleen, the MSC-treated mice showed a strong trend to lower counts, with differences that almost reached statistical significance (P = 0.05, MSC vs. 3T3; P = 0.06, MSC vs. PBS; Fig. 6A). However, the number of P. aeruginosa CFU was significantly lower in the peripheral blood of MSC-treated mice than in the two control groups (Fig. 6B).

Effect of MSC treatment on phagocytic activity of blood monocytes and neutrophils. To explain the enhanced bacterial clearance in the peripheral blood, we investigated the phagocytic activity of blood neutrophils and mononuclear cells. Separate fractions of neutrophils and mononuclear cells were isolated from the peripheral blood of mice treated with MSC, 3T3, and PBS at 12 h following infection. Isolated cells were incubated for 1 h with E. coli. Subsequently, phagocytic activity and inhibition of bacterial growth were measured.

No difference was found in phagocytic activity of neutrophil fractions isolated from the three groups of mice: the percentage of phagocytosis (mean ± SD, n = 4) was 20 ± 1%, 20 ± 4%, and 20 ± 3% for the MSC, 3T3, and PBS groups, respectively, and PI was 27 ± 2, 28 ± 3, and 31 ± 4 for the MSC, 3T3, and PBS groups, respectively. The capacity to inhibit bacterial growth also did not differ among the experimental groups: bacterial CFU in the neutrophil-conditioned medium was 2,184 ± 221, 1,669 ± 296, and 1,756 ± 259 CFU × 10³/ml for the MSC, 3T3, and PBS groups, respectively (n = 4).

Phagocytic activity was significantly higher in mononuclear cells isolated from the blood of MSC-treated septic mice than

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**Table 2. Cell counts in the circulating blood**

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<thead>
<tr>
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<th>3T3 (n = 21)</th>
<th>MSC (n = 23)</th>
<th>PBS (n = 33)</th>
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<tr>
<td>WBC</td>
<td>3,470 (2,355–4,860)</td>
<td>5,560 (1,940–9,620)</td>
<td>2,500 (1,580–5,320)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1,910 (947–3,350)</td>
<td>2,930 (1,310–6,590)</td>
<td>1,240 (780–3,035)</td>
</tr>
<tr>
<td>Platelets</td>
<td>440 (291–619)</td>
<td>897* (600–1,023)</td>
<td>655 (325–797)</td>
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</table>

Values (medians [25th–75th percentiles]) are expressed as 10⁶ cells/µl. 3T3, 3T3 fibroblasts; MSC, mesenchymal stem cells; WBC, white blood cells. *P < 0.01; †P = 0.01 (by Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney test).

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**Fig. 3. Plasma plasminogen activator inhibitor 1 (PAI-1) levels (A) and blood platelet counts (B) in mice treated with MSC (n = 19), 3T3 (n = 24), or PBS (n = 33). Values are medians and 25th–75th percentiles. *P < 0.01, MSC vs. 3T3; †P < 0.01, MSC vs. PBS (by Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney test).**

**Fig. 4. Plasma cytokine levels. A: plasma levels of TNF-α in mice treated with MSC (n = 12), 3T3 (n = 10), or PBS (n = 11). B: plasma levels of PGE₂ in mice treated with MSC (n = 11), 3T3 (n = 10), or PBS (n = 9). C: plasma levels of IL-10 in mice treated with MSC (n = 9), 3T3 (n = 11), or PBS (n = 9). Values are medians and 25th–75th percentiles. No difference was found in plasma levels of TNF-α, IL-10, or PGE₂ among the 3 groups (by Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney test).**
non-MSC-treated septic mice. Since B and T lymphocytes do not have a significant capacity for phagocytosis, the phagocytic cells in the mononuclear cell fraction were most likely monocytes. The percentage of phagocytosis was 32% in the mononuclear cells from the mice treated with MSC and <20% in the mononuclear cells from both control groups (Fig. 7A). In addition, PI was significantly higher in the MSC group than in the 3T3 and PBS groups (Fig. 7B). The total bacterial counts were markedly reduced in the conditioned medium of mononuclear cells from the MSC-treated mice compared with controls (Fig. 7C). To further confirm this finding and exclude contamination by nonmononuclear cells in the total mononuclear cell fraction, we used flow cytometry to analyze the phagocytic capacity of blood monocytes and neutrophils. Monocytes were defined as SSClowCD11bhiCD115+ cells; for assessment of phagocytosis, we used pHrodoE.coli fluorescent particles, which have the capacity to become brightly fluorescent only in an acidic environment, which occurs in the phagolysosome. This analysis also showed that the percentage of cells positive for ingested bacteria within the monocyte population was significantly higher, by 45%, in the mice treated with MSC (Fig. 7, D–F) than in the PBS group. Consistent with the previous finding, the phagocytic activity of neutrophils did not differ between the MSC- and PBS-treated mice: percentage of phagocytosis was 19.6 ± 31.4% and 16.1 ± 15.6% for the MSC and PBS groups, respectively (P = 0.75, n = 8–9 per group). Also, by flow cytometry, the percentage of monocytes or neutrophils did not differ among experimental groups (Table 4).

These results suggest that improvement in bacterial clearance in the blood of MSC-treated mice could be in part mediated through enhancement of phagocytic activity of blood monocytes.

**Table 3.** *P. aeruginosa* at 12 h in peritoneal fluid and lung

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<th>3T3</th>
<th>MSC</th>
<th>PBS</th>
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<tr>
<td>Peritoneal fluid</td>
<td>1.2 × 10^6 (1.0 × 10^5–4.7 × 10^6)</td>
<td>1.1 × 10^7 (1.1 × 10^6–2.7 × 10^7)</td>
<td>5.4 × 10^7 (3.0 × 10^6–3.0 × 10^8)</td>
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<td>Lung</td>
<td>5.0 × 10^6 (1.6 × 10^5–1.6 × 10^6)</td>
<td>1.5 × 10^6 (1.5 × 10^5–4.0 × 10^5)</td>
<td>2.3 × 10^6 (1.1 × 10^5–9.8 × 10^5)</td>
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Values (medians and 25th–75th percentiles) are expressed as colony-forming units/ml (n = 10, 9, and 10 for MSC, 3T3, and PBS in peritoneal fluid, and n = 10, 16, and 16 for MSC, 3T3, and PBS in lung).
group (Fig. 10B). No difference between experimental groups was observed in the levels of CD11b expression on the neutrophils: 17,016 ± 3,671 and 17,876 ± 2,429 mean fluorescence intensity in the MSC- and PBS-treated groups, respectively. Thus, MSC administration is associated with moderate upregulation of C5 activation, which could be in part responsible for the improved bacterial clearance in the blood by direct bactericidal activity of MAC and enhanced phagocytic activity of the blood monocytes through upregulation of CD11b.

DISCUSSION

The main findings from these experiments can be summarized as follows: 1) human bone marrow-derived MSC reduced mortality in a mouse model of gram-negative peritonitis and sepsis; 2) the protective effect of MSC was associated with a significant decrease in the number of bacteria in the blood of the Pseudomonas-infected mice; and 3) the improvement in bacterial clearance was partially mediated through enhancement of phagocytic activity of peripheral blood mononuclear cells.

Other investigators used the cecal-ligation model to study the effect of MSC on peritoneal-induced sepsis. Nemeth et al. (32) and Mei et al. (30) studied the effect of syngeneic MSC given intravenously, while Gonzales-Rey et al. (11) used human MSC injected intraperitoneally. Our study investigated the effect of human MSC given intravenously, by tail vein or...
jugular vein injection, as a treatment for severe, acute gram-negative infection induced by intraperitoneal injection of a high dose of \textit{P. aeruginosa}. The value of our approach was that injection of a constant number of \textit{P. aeruginosa} permitted us to measure bacterial growth from several compartments as an end point and to follow the effects of MSC therapy.

MSC treatment resulted in an improved survival rate in the MSC-treated mice (Fig. 1). By 12 h, the majority of mice in the control groups developed severe hypothermia. Mice treated with MSC appeared more active than those in the control groups, and the decrease of their body temperature was significantly less (Fig. 2). Most MSC-treated mice had no hypothermia and were able to recover rapidly. PAI-1 has been described as a biological marker of severe sepsis (2). The levels of PAI-1 were significantly reduced in MSC-treated mice compared with controls (Fig. 3A), consistent with other results, suggesting a protective role of MSC administration in this model of \textit{P. aeruginosa}-induced peritonitis. To further evaluate the effect of MSC treatment on the severity of sepsis, we measured total cell count with differential in peripheral blood, as well as leukocytes and neutrophils in the peritoneal fluid. There were no differences in the peritoneal fluid cell count (data not shown) or in the bacterial counts (Table 2). In the blood, the white blood cells and neutrophils were elevated compared with the control group but did not reach statistical significance (Table 2). However, the number of platelets was significantly higher in MSC- than 3T3- and PBS-treated mice (Fig. 3B, Table 2). According to recent findings (26), platelets could form complexes with neutrophils, resulting in an increase in

<table>
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<th>Characterization of cell populations in the blood by flow cytometry</th>
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<tr>
<td><strong>MSC</strong></td>
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<tr>
<td>T lymphocytes (CD3⁺)</td>
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<tr>
<td>B lymphocytes (CD45R⁺)</td>
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<tr>
<td>Monocytes (CD11b⁺CD115⁺)</td>
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<tr>
<td>Neutrophils (CD11b⁺CD115⁺)</td>
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Values (means ± SD) are expressed as percentage of total nucleated cells (\( n = 9–10 \) mice per group).

Fig. 8. MSC treatment is associated with enhanced expression of CD163 and CD206 (markers of alternatively activated macrophages). \( A–C \): splenic sections stained with CD206-specific antibody (\( A \), green), CD163-specific antibody (\( B \), green), and hematoxylin-eosin (\( C \)). Images are representative for each condition: 2 sections per mouse and 3 mice per group. Original magnification \( \times 200 \). \( D \) and \( E \): representative data for percentage of CD11b⁺CD206⁺ cells within CD11b⁺ population of splenocytes from mice treated with MSC and PBS, respectively; only CD11b⁺ splenocytes are shown. \( F \): CD206⁺ cells in spleens from mice treated with MSC and PBS. Values are means ± SD (\( n = 7–8 \) mice per group). *\( P < 0.05 \) vs. PBS.
the severity of ALI and sepsis. In the present study, the number of platelets in the peripheral blood was restored in the MSC group, and this was correlated with a higher number of neutrophils than in the 3T3- or PBS-treated mice, although the differences in neutrophils were not significant (Table 2). The higher levels of platelets in the MSC-treated mice may reflect modulation of the early phase of sepsis by MSC. Several studies (11, 30, 32), including those carried out by our group (12, 21), attributed part of the protective effect of MSC to their immunomodulatory properties. To investigate if the beneficial effect of MSC in these acute bacterial sepsis experiments was associated with a change in the cytokine profile, we measured major pro- and anti-inflammatory mediators in the blood and peritoneal fluid. We did not find a difference in the levels of TNF-α, IL-10, and PGE2 in the blood (Fig. 4) or in the levels of TNF-α and MIP-2 in the peritoneal fluid (Fig. 5). These findings could be explained by differences in the design of the studies, timing of measurement of the mediators, or the possibility that other mediators not measured could be more significantly affected by MSC administration. Nemeth et al. (32), Gonzales-Rey et al. (11), and Mei et al. (30) used a polymicrobial (cecal-ligation) model of sepsis; in the present study, we used a model of sepsis induced by a high dose of a single gram-negative bacterium. Also, the present study was an acute model, creating a lethal injury in ~12–24 h; in other studies (11, 12, 30, 32), the injury was created and observed for 48–96 h. Importantly, in the present study, MSC were used as a treatment, with an injection 1 h after *P. aeruginosa* infection, while two of the prior studies used a pretreatment approach, where MSC were given 24 h before induction of the injury (30, 32). Still, our data are in line with the findings of Mei et al., who reported no difference in the blood levels of IL-10 when MSC were given 6 h after cecal-ligation puncture. Taken together, these results indicate that the effect of MSC was probably not primarily linked to their immunomodulatory properties in this study and also not dependent on local mechanisms, since no difference was found in peritoneal fluid in terms of bacterial counts, cell counts, and the levels of cytokines.

However, we found that hypothermia, elevated PAI-1 levels, and thrombocytopenia were associated with a rapid growth of bacteria CFU in the peritoneum, spleen, lungs, and blood in the control groups (Table 3, Fig. 6). Although numerically less, the bacterial burden in the peritoneum and lungs of MSC-treated mice was similar to that of the two control groups (Table 3). Importantly, the number of bacteria in the spleen of MSC-treated mice was lower (*P* < 0.05; Fig. 6A) and the blood *P. aeruginosa* CFU were significantly reduced (Fig. 6B) compared with controls.

To explain the reduced number of bacteria with MSC treatment, we tested the phagocytic capacity of mononuclear and neutrophil cell fractions of peripheral blood after MSC admin-
istration. We isolated cells from blood 12 h after *P. aeruginosa* infection, incubated them with *E. coli*, and calculated the percentage of phagocytosis and PI. Percentage of phagocytosis, as well as PI, was higher in mononuclear cells isolated from the blood of MSC-treated mice than the control groups (Fig. 7). In addition, the number of *E. coli* CFU was significantly reduced in the conditioned medium from mononuclear cells from MSC-treated mice compared with 3T3- and PBS-treated mice (Fig. 7C). Assuming that B and T lymphocytes do not have significant phagocytic capacity, we attributed the enhanced phagocytic activity to blood monocytes. To be more accurate, we also assessed phagocytic activity of blood monocytes by flow cytometry. For assessment of phagocytosis, we used fluorescent pH-RoDo *E. coli* particles, which become fluorescent only when located inside the phagosome. Data obtained by flow cytometry confirmed our initial findings (Fig. 7F). Importantly, there was no difference in the phagocytic activity of neutrophils by both approaches. Thus these results suggest that MSC, when given intravenously, improve bacterial clearance in the blood, in part by enhancing phagocytic activity of blood monocytes. Kim and Hematti (17) reported that human MSC improved phagocytic activity of monocyte-derived macrophages when cocultured in vitro. In that study, coculture of human MSC and macrophages caused an alternative state (M2) of macrophage activation that is characterized by greater anti-inflammatory properties, more potent phagocytic activity, and increased expression of the surface markers CD163 and CD206 (mannose binding receptor). Also, Mei et al. (30) reported that administration of mouse MSC in their cecal-ligation puncture sepsis model enhanced pathways responsible for phagocytosis and provided evidence that CD11b + cells, isolated from the spleen of animals that received MSC, demonstrated an improved phagocytic activity. The spleen plays a significant role in humoral and cellular immune responses and recently was reported to be a reservoir site for monocytes, which could be recruited to the site of inflammation (25, 41). To further test the effect of MSC administration on macrophage/monocyte activation, we examined mouse spleens for the expression of CD206 and CD163, two molecular markers that are commonly considered to indicate alternative macrophage activation (8, 14, 17, 35), by immunofluorescence. The red pulp regions from the spleen in MSC-treated mice displayed more pronounced immunofluorescence for both markers, in sharp contrast to the spleen sections from the two control groups (Fig. 8, A and B). These results were partially confirmed by flow cytometry analysis, which also demonstrated a significant increase in the number of CD11b +CD206 + splenocytes in the MSC-treated group (Fig. 8F). This finding is in line with the recent publication of Dayan et al. (6), who reported that human mesenchymal stromal cells from bone marrow and umbilical cord perivascular cells mediated a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. Based on these observations, we hypothesized that MSC induced alternative activation of spleen monocytes and macrophages, which may be more efficient than classically activated macrophages in resolving inflammation and the clearance of bacteria (17, 35). The data on *P. aeruginosa* CFU in the spleen of MSC-treated mice showed a strong trend toward CFU reduction (*P* = 0.05), supporting this hypothesis. Also, splenic monocytes may have been released into the circulation and participated in the enhanced bacterial clearance in the blood. However, using flow cytometry, we did not find higher phagocytic capacity in total CD11b + or CD11b +CD206 + splenocytes from MSC-treated mice than in the same populations of splenocytes from the PBS group (Fig. 9, A and B). Nor did we detect any difference in the level of CD206 expression in the peripheral blood monocytes (Fig. 9C), suggesting that activation of phagocytic activity of blood monocytes in the MSC-treated group is not directly linked to the switch of their activation state, which possibly takes place in the spleen. The time sequence of events in the blood and in the spleen requires further study.

To investigate another possible mechanism by which phagocytic activity of blood monocytes could be increased, we measured the level of complement activation in MSC-treated and control mice. Interestingly, levels of C5a complement fragment were significantly elevated in MSC-treated mice compared with the control group. This finding was not expected, as MSC generally are considered to have a predominantly immunosuppressive phenotype, whereas increased complement activation could lead to more inflammatory responses. Moreover, Tu et al. (42) recently reported that human MSC are able to secrete factor H (complement activation inhibitor) in quantities that were functionally active in vitro. However, in this model of sepsis, MSC administration was associated with moderate (15%) upregulation of C5a plasma levels and a better outcome. C5a complement fragment induces expression of the phagocytosis receptor CD11b on monocytes and macrophages and, by that mechanism, enhances their phagocytic activity (3, 40). Interestingly, using flow cytometry, we observed a ~15% higher level of expression of CD11b receptor in peripheral blood monocytes from MSC-treated mice than the PBS group (Fig. 10). Curiously, in one study, C5a was the key factor responsible for neutrophil dysfunction in late sepsis (>24 h), compromising their ability to phagocytose and develop the oxidative burst (16). This could partially explain the fact that, in our model, we did not detect an effect on neutrophils in terms of their phagocytic activity and CD11b expression. Thus we speculate that the increased C5a release into the bloodstream, which is associated with the upregulation of CD11b receptor on the blood monocytes, could be one of the mechanisms responsible for their enhanced phagocytic activity with MSC treatment.

The mechanism by which MSC activate complement and/or blood monocytes requires further study. Whether cell contact between monocytes and MSC is necessary needs to be clarified. As determined in previous studies, >90% of human MSC injected intravenously accumulated in the lung in a short time (5–10 min after injection) and were detectable there for 24 h (6, 37). At 10 min after injection, 2–3% of initially injected cells reappeared in the circulation and <1% were detected in other organs, including the spleen. It is possible that contact between MSC and monocytes could occur in lung capillaries. It is also possible that MSC could reprogram and activate monocytes through the secretion of paracrine soluble factors (32).

Another proposed mechanism of improved bacterial clearance in the MSC group could be related to the previously reported ability of MSC to reduce neutrophil activation and delay cell death from apoptosis (38). In the present study, we observed a higher number of neutrophils in the blood of MSC-treated mice, although these data did not reach significance. Taking into account that the percentage of phagocytosis...
and PI of neutrophils were not different among the groups, we speculate that even if neutrophils are not more active in direct bacterial killing, they could have a longer life span and, thereby, contribute to the reduction in bacteremia. Further studies are needed to understand the interaction between neutrophils and MSC.

In conclusion, bone marrow-derived human MSC improved survival from acute, severe gram-negative peritonitis and sepsis in mice. The improvement in survival was associated with increased bacterial clearance in the blood, which was in part explained by enhanced phagocytic activity of blood monocytes.

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REFERENCES

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