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Intrapulmonary Delivery of Bone Marrow-Derived Mesenchymal Stem Cells Improves Survival and Attenuates Endotoxin-Induced Acute Lung Injury in Mice¹

Naveen Gupta,^{2*} Xiao Su,* Boris Popov,[†] Jae Woo Lee,* Vladimir Serikov,[†] and Michael A. Matthay*

Recent *in vivo* and *in vitro* work suggests that mesenchymal stem cells (MSC) have anti-inflammatory properties. In this study, we tested the effect of administering MSC directly into the airspaces of the lung 4 h after the intrapulmonary administration of *Escherichia coli* endotoxin (5 mg/kg). MSC increased survival compared with PBS-treated control mice at 48 h (80 vs 42%; $p < 0.01$). There was also a significant decrease in excess lung water, a measure of pulmonary edema (145 ± 50 vs 87 ± 20 μ l; $p < 0.01$), and bronchoalveolar lavage protein, a measure of endothelial and alveolar epithelial permeability (3.1 ± 0.4 vs 2.2 ± 0.8 mg/ml; $p < 0.01$), in the MSC-treated mice. These protective effects were not replicated by the use of further controls including fibroblasts and apoptotic MSC. The beneficial effect of MSC was independent of the ability of the cells to engraft in the lung and was not related to clearance of the endotoxin by the MSC. MSC administration mediated a down-regulation of proinflammatory responses to endotoxin (reducing TNF- α and MIP-2 in the bronchoalveolar lavage and plasma) while increasing the anti-inflammatory cytokine IL-10. *In vitro* coculture studies of MSC with alveolar macrophages provided evidence that the anti-inflammatory effect was paracrine and was not cell contact dependent. In conclusion, treatment with intrapulmonary MSC markedly decreases the severity of endotoxin-induced acute lung injury and improves survival in mice. *The Journal of Immunology*, 2007, 179: 1855–1863.

Acute lung injury (ALI)³ and the acute respiratory distress syndrome are major causes of acute respiratory failure in critically ill patients. Despite improvements in supportive care, recent data indicate that the mortality of acute respiratory failure from ALI is still high at ~40% (1, 2). Recent studies have demonstrated that bone marrow-derived mesenchymal stem cells (MSC) have the ability to participate in the repair of experimental models of bleomycin-induced lung injury (3, 4). Our goal was to test the effects of MSC in a mouse model of endotoxin-induced ALI, because the potential role of MSC in experimental ALI has not been well studied.

MSC are pluripotent adult stem cells found in the bone marrow that have the capability of differentiating into multiple cell types (5, 6). In the lung, MSC can engraft as type I and II epithelial cells, endothelial cells, and fibroblasts (3, 4, 7, 8). However, in non-bone marrow-suppressed mice, the levels of engraftment have been consistently <5%. Despite the low levels of MSC incorporation into the lung, the administration of these cells does attenuate the se-

verity of bleomycin-induced lung injury (3, 4), implying that the cells are acting in a manner independent of their ability to engraft. Supporting this concept is recent evidence that MSC have the ability to modulate the activity of innate and adaptive immune cells such as dendritic cells, T cells, and B cells (9–12). These effects were mediated by both contact-dependent and -independent mechanisms.

In this study, we hypothesized that the administration of MSC in an endotoxin model of ALI might have beneficial functional and survival effects related to the immunomodulatory properties of MSC. We used the intrapulmonary route of administration to maximize the efficiency of delivery of MSC to the injured lung. Local delivery of stem cells has been shown to be of benefit in both experimental and clinical studies of myocardial infarction (13–15). The results indicate that intrapulmonary treatment with MSC confers a survival and functional advantage in the mouse model of endotoxin-induced ALI, and that this benefit is mediated by a down-regulation of the acute inflammatory response to endotoxin.

Materials and Methods

Animal care

C57BL/6 male mice (8–10 wk old; The Jackson Laboratory) were used in all experiments. Animals 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.

Experimental design

Mice were first anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) *i.p.* ALI was then induced by the instillation of LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich) at 5 mg/kg intratracheally (*i.t.*) as described below. Mice were then allowed to recover in a 100% oxygen chamber for 1–2 h when they awakened from anesthesia. Then, 4 h after the induction of injury, mice were reanesthetized and given either MSC (750,000 cells in 30 μ l of PBS) or 30 μ l of PBS *i.t.* Additional experiments were done in which a fibroblast cell line, 3T3, and nonviable MSC were

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³ Abbreviations used in this paper: ALI, acute lung injury; MSC, mesenchymal stem cell(s); GM, growth medium; BAL, bronchoalveolar lavage; MPO, myeloperoxidase; *i.t.*, intratracheal(*ly*).

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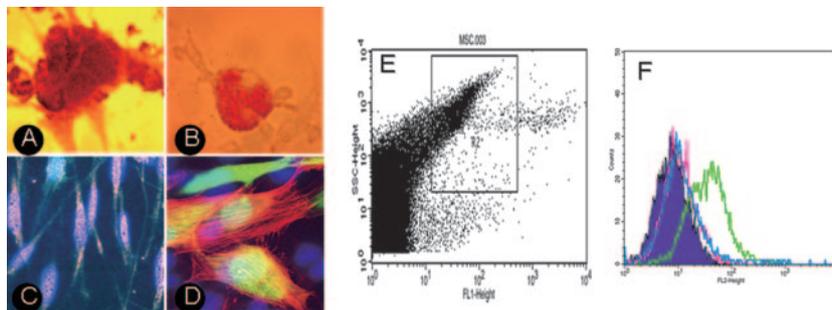


FIGURE 1. MSC retained multipotent property after 15–25 passages in culture as assessed by confocal microscopy images of MSC. A–C, Differentiation into osteoblasts (Alizarin staining, red) (A), adipocytes (Oil Red staining, red) (B), and myofibroblasts (Desmin staining, red) (C) was seen. D, MSC also expressed actin (red staining). E and F, Flow cytometry of MSC demonstrated that cells do not express the hemopoietic markers, CD45 and CD11b. Dot plot for GFP green fluorescence (E); histogram for MSC staining for CD45 and CD11b (dark blue, negative control; red, CD11b; blue, CD45; green, isotype Ab) (F).

instilled i.t. 4 h after the instillation of endotoxin as additional controls (750,000 cells in 30 μ l of PBS). MSC were made nonviable by incubating with an apoptosis induction kit containing cycloheximide, actinomycin-D, camptothecin, etoposide, and dexamethasone for 24 h in culture (Chemicon). Cells were then stained with 0.4% trypan blue to ascertain viability. Mice were then similarly recovered in a 100% oxygen chamber, and were subsequently followed for 24–72 h. Survival in each group was noted as well as body temperature and activity in the two groups. At the end of either 24 or 48 h, samples were collected from each mouse for assessment of lung injury, biochemical analysis, and histology.

Mouse MSC cultures

MSC cultures were obtained from GFP⁺-C57BL/6 mice. Bone marrow was obtained by flushing the tibias and femurs using 1 ml of growth medium (GM)-DMEM with 25 mM HEPES and 10% FBS (Invitrogen Life Technologies). The cells were washed, counted with trypan blue for evaluation of viability, and resuspended in GM to a density 5×10^6 /ml. The resuspended cells were cultured immediately at a density of 2×10^6 /ml in a humidified 5% CO₂ incubator at 37°C. After 72 h of cultivation, the nonadherent cells were removed from the culture by changing the medium. Adherent cells were then subsequently propagated in culture with IMDM containing 10% FBS, 10% horse serum, 1% L-glutamine, and 1% penicillin-streptomycin. Cells were passaged every 3–4 days by trypsinization and were used for in vivo experiments after 15–25 passages.

Differentiation assays

MSC from 20 to 25 passages were cultured at an initial concentration of 2×10^5 /ml in GM in dishes containing sterile cover glasses. Adipogenic differentiation was induced by seeding the MSC for 21 days in GM supplemented with 10^{-8} ml/L dexamethasone (Sigma-Aldrich) and 5 μ g/ml insulin (Sigma-Aldrich). The cells containing drops of fat were identified by staining the culture with 3.75% Oil Red. Osteogenic differentiation was induced by cultivation of the MSC in GM containing 10 mM β -glycerol phosphate, 50 μ g/ml ascorbic acid, and 10^{-8} ml/L dexamethasone for 21 days. The calcium containing precipitates were visualized after staining with 2% Alizarin Red S (Sigma-Aldrich) adjusted to a pH of 4.2 with ammonium hydroxide. Myogenic differentiation was induced by culturing MSC for 14 days in GM containing 1.5 μ g/ml amphotericin B, and then for 1 wk in the medium without amphotericin. The cells were then fixed for 10 min in 4% paraformaldehyde and in 70% ethanol overnight at 4°C fol-

lowed by evaluation of expression of the early (desmin) and late (myosin H chain) markers of muscle differentiation by immunohistochemistry with D3 and MF20 mAbs (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA).

Flow cytometry

Flow cytometry for expression of a panel of surface markers was done using a BD Biosciences FACS machine using standard techniques. MSC were harvested by trypsinization, washed in PBS, and stained by PE- or allophycocyanin-labeled Abs to CD11b, CD31, CD34, and CD45. This analysis was done at passages 15–25, concurrent with the time MSC were used in the in vivo model of ALI.

Intrapulmonary delivery of instillate

The details of this method have been previously published by our group (16). Briefly, mice were anesthetized with ketamine and xylazine, and then fixed at a 60° angle. A fiber optic light source was placed immediately over the neck, and the oropharynx was opened with forceps, allowing for the visualization of the trachea. The instillate (endotoxin, PBS, MSC, fibroblasts, or nonviable MSC) was then injected into the trachea using a PE-10 catheter attached to a 0.5-ml syringe.

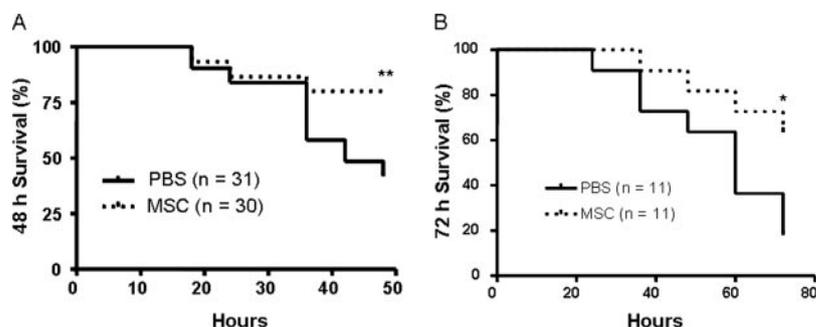
Excess lung water/lung wet-to-dry ratio

Gravimetric lung water determination was done as follows: the whole lung was excised, weighed, and then homogenized after the addition of 1 ml of double distilled H₂O. The homogenate was centrifuged at 12,000 rpm for 10 min to obtain the supernatant, which was weighed; the hemoglobin of the supernatant was also measured. A blood sample was obtained by needle puncture of the right ventricle, and the wet weight, hemoglobin, and hematocrit of the blood sample was obtained. All samples were placed in a drying oven at 55°C for 24 h, and the dry weights were subsequently determined. The final excess lung water and lung wet-to-dry ratio were calculated as described in our previous publications (17–19).

Bronchoalveolar lavage (BAL) and plasma cytokine and protein measurements

BAL and plasma samples were obtained from mice at 24 and 48 h after endotoxin-induced lung injury in the MSC, fibroblast, apoptotic MSC, and PBS-treated groups. BAL was done after euthanizing the mice and then

FIGURE 2. Administration of MSC improved 48 and 72 h survival in an endotoxin model of ALI. MSC (750,000 cells/30 μ l) or PBS (30 μ l) was administered i.t. 4 h after i.t. instillation of endotoxin (5 mg/kg). A, Forty-eight-hour survival was 80% in MSC group vs 42% in PBS group ($n = 30$ for MSC group, $n = 31$ for PBS group; **, $p < 0.01$ using a log-rank test). B, At 72 h, survival was 64% in the MSC group vs 18% in the PBS group ($n = 11$ per group; *, $p < 0.05$ using a log-rank test).



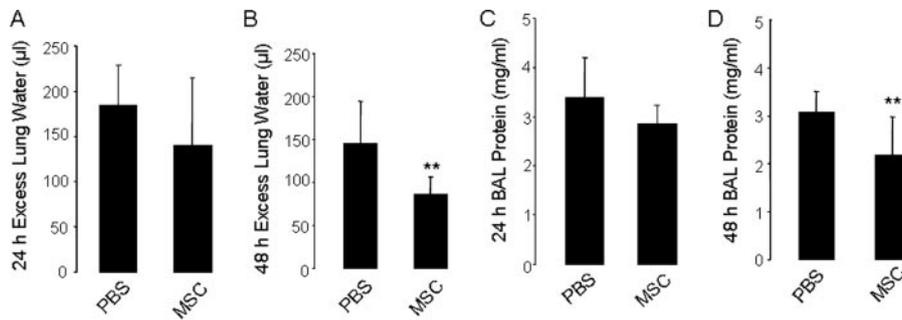


FIGURE 3. MSC significantly improved severity of lung injury (excess lung water and lung wet-to-dry ratio) and endothelial/epithelial permeability (BAL protein) at 48 h, but not at 24 h. *A*, At 24 h, the MSC-instilled group had a reduced excess lung water compared with the PBS group; however, this comparison was not significant ($n = 8-9$ per group; $p = 0.16$). *B*, At 48 h, mice that received MSC had significantly improved excess lung water compared with mice that received PBS ($n = 12$ per group; **, $p < 0.01$). *C* and *D*, Similarly, BAL protein was nonsignificantly reduced in the MSC group at 24 h ($n = 5-6$ per group, $p = 0.19$) (*C*), but was significantly reduced in the MSC group by 48 h ($n = 10-11$ per group; **, $p < 0.01$) (*D*). Data are expressed as mean \pm SD.

placing a 20-gauge catheter into the trachea through which 1 ml of cold PBS was flushed back and forth three times. TNF- α , MIP-2, IL-1ra, and IL-10 were measured in both the BAL and plasma samples with ELISA kits (R&D Systems). In addition, the level of PGE₂, a lipid mediator known to be produced by MSC, was measured in selected BAL samples with an ELISA kit (R&D Systems). BAL and plasma samples from MSC- and PBS-treated mice were also collected at 8 and 24 h for analysis by luminex assay (LINCO Diagnostic Services). Protein was measured in the BAL fluid from all experimental groups, as a marker of endothelial and epithelial permeability, by the Bio-Rad protein assay kit.

Histology and immunohistochemistry

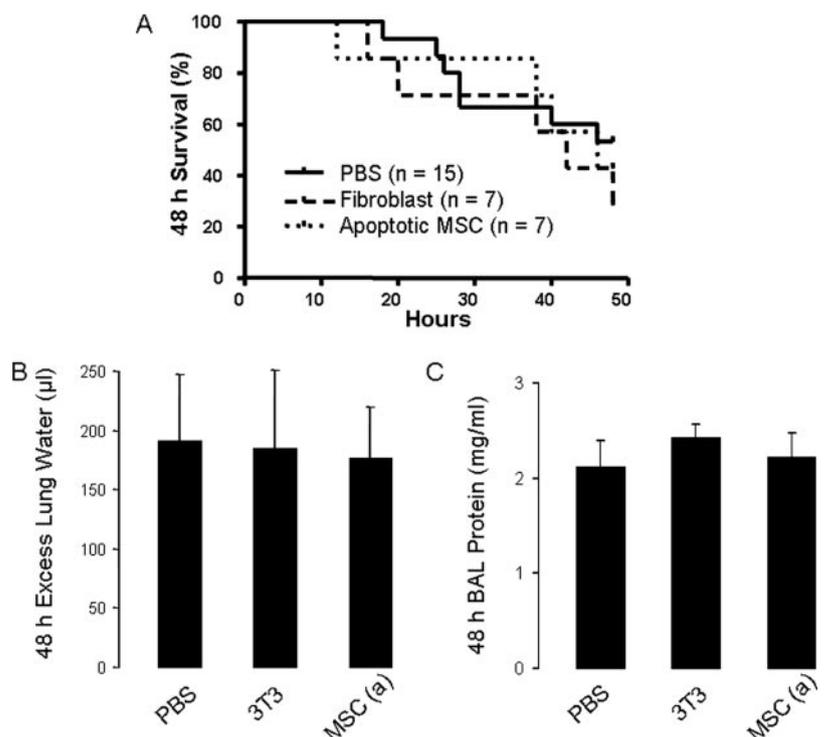
Lungs from both MSC and PBS groups were excised 24 and 48 h after endotoxin-induced lung injury and fixed with 100% ethanol i.t. under 20 cm of H₂O pressure ($n = 3$ per group per time point). After fixation, lungs were embedded in paraffin, cut into 5- μ m sections, and stained with H&E. The degree of hemorrhage and edema was scored in six sections from the lower lobes using the following criteria: no injury, 0; injury to 25% of the field, 1; injury to 50% of the field, 2; injury to 75% of the field, 3; diffuse injury, 4 (20). For immunohistochemistry, slides with lung sections were deparaffinized, washed with PBS, postfixated with ethanol, exposed to 0.2% Triton X-100 in PBS for 5 min, and then washed with PBS twice again.

Slides were further blocked with 3% goat serum, 2% horse serum, 3% BSA/0.1% Tween 20 in 4 \times SSC for 20 min. A primary Ab to GFP (Sigma-Aldrich; in 2 \times SSC/1% BSA/0.1% Tween 20) was added for 1 h at 37°C at a 1/100 dilution. Slides were washed three times with PBS and then blocked with 3% BSA/0.1% Tween 20 in 4 \times SSC for 20 min. A FITC-conjugated secondary Ab (in 2 \times SSC/1% BSA/0.1% Tween 20) was added for 1 h at 37°C. Control slides were stained with isotype Ab. Slides were washed three times with PBS, mounted on glass slides in Fluoro-Guard antifade reagent (Bio-Rad), and coverslips applied. Laser confocal fluorescence microscopy (Axiovert 100, LSM 510; Zeiss) was performed ($n = 3$ per group per time point).

Assessment of neutrophil counts and myeloperoxidase (MPO) activity in BAL

BAL was done on both MSC- and PBS-treated mice at 8 and 24 h after injury to obtain total cell count and percentage of neutrophils as well as MPO activity. Total cell count was determined with the use of a counter (Beckman Coulter). A cell smear was made using cytopsin (Thermo Shandon), and the cells were visualized using Wright-Giemsa staining (Fisher Scientific). A differential of the white blood cells was then obtained by counting 100 cells from a representative portion of the slide. The activity of MPO was assessed using previously described, standard methods (21).

FIGURE 4. Neither fibroblasts nor apoptotic MSC (MSC (a)) improve survival or decrease severity of endotoxin-induced ALI. 3T3 fibroblasts, apoptotic MSC (750,000 cells/30 μ l of PBS), or PBS (30 μ l) was administered i.t. 4 h after the i.t. instillation of endotoxin (5 mg/kg). *A*, Forty-eight-hour survival was not different among the three groups ($n = 15$ for the PBS group and $n = 7$ for the fibroblast and apoptotic MSC groups). *B*, Excess lung water was not reduced in the control cell groups ($n = 32$ for the PBS group, $n = 16$ for the 3T3 group, and $n = 12$ for the apoptotic MSC group). *C*, In addition, BAL protein was not reduced in the fibroblast and apoptotic MSC groups ($n = 4-5$ per group). Data for *B* and *C* are expressed as mean \pm SD.



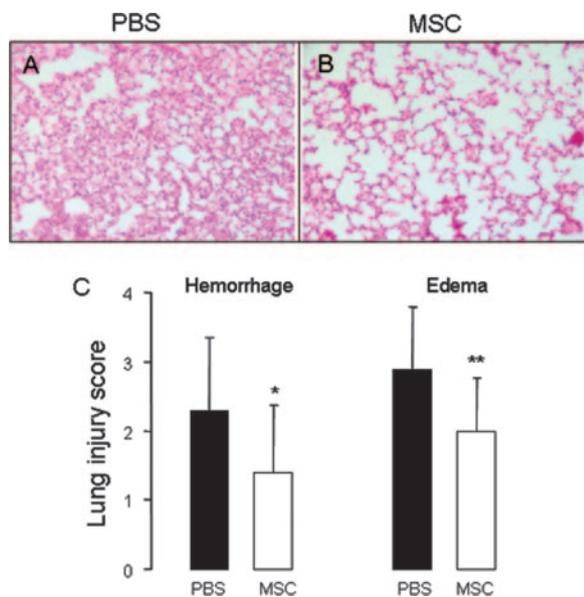


FIGURE 5. MSC improved lung injury as assessed by histological methods despite the low levels of engraftment detected. *A* and *B*, H&E staining of lung sections demonstrated attenuated lung injury in the MSC group (*B*) vs the PBS group (*A*) at 48 h after instillation of endotoxin. *C*, Quantification of lung injury showed a significant reduction in the degree of hemorrhage and edema in the mice receiving MSC (*, $p < 0.05$; **, $p < 0.01$). Data are expressed as mean \pm SD.

RNA isolation and RT-PCR for TLR4, CD14, and MD2

RNA was isolated from MSC and RAW 264.7 cells, a macrophage cell line, using the Qiagen RNeasy kit. After isolation, RNA samples were treated with DNase for 60 min at room temperature to remove contaminating DNA. The purity of the RNA was then analyzed by measuring the absorbance ratio at 260 and 280 nm using a spectrophotometer (Beckman Coulter).

Primers for TLR4, MD2, and CD14 were custom made (Sigma Genosys). The sequences were as follows: TLR4, 5'-AAACTGCGCTTCAAAACCTGGC; TLR4, 3'-ACCTGAACATCAATGGTCACATC; CD14, 5'-CCAGTCAGCTAAACTCGCTCAATC; CD14, 3'-TCCAGCCTGTTGTAACAGATCC; MD2, 5'-ATGTTGCCATTTATTCTCTTTTCGACG; and MD2, 3'-ATTGACATCACGGCGGTGAATGATG.

Primers for the housekeeping gene, *GAPDH*, were used as a control (Qiagen). RT-PCR was done using the One-Step RT-PCR protocol from Qiagen. The resulting amplified DNA product was run on a 2% agarose gel, and bands were visualized with the use of ethidium bromide.

Protein isolation and Western blot for TLR4, CD14, and MD2

Protein was isolated from MSC and RAW 264.7, a positive control, using the bicinchoninic acid assay and quantified using a Beckman Coulter spectrophotometer. A total of 40 μ g of reduced, denatured protein from each cell type was then loaded and run on a 4–12% gradient Bis-Tris gel (Invitrogen Life Technologies). The gel was run at 100 V for 2 h using a MOPS SDS running buffer (Invitrogen Life Technologies). The proteins were then transferred to a nitrocellulose membrane and incubated with blocking buffer for 1 h. The membrane was then exposed to rabbit polyclonal primary Abs (Abcam) for TLR4, CD14, and MD2 at 1/1000 dilution overnight at 4°C. The membrane was washed and then incubated with an anti-rabbit HRP-labeled Ab (GE Healthcare) at 1/2500 dilution for 30 min. The protein bands were subsequently visualized with an ECL kit (Amersham Biosciences).

Distribution of ^{51}Cr -labeled endotoxin in MSC-treated mice

Endotoxin was labeled with chromium as has been previously described (22, 23). Briefly, 2 mg of dissolved endotoxin was incubated with $\text{Na}^{51}\text{CrO}_4$ (Amersham Biosciences) for 24 h. This solution was then dialyzed for 4 days with distilled water until the free ^{51}Cr was near undetectable in the dialyzed solution. Mice were then given 5 mg/kg of endotoxin (15% labeled, 85% unlabeled) i.t. as described previously. MSC or PBS was then given 4 h after the endotoxin, and mice were then euthanized at

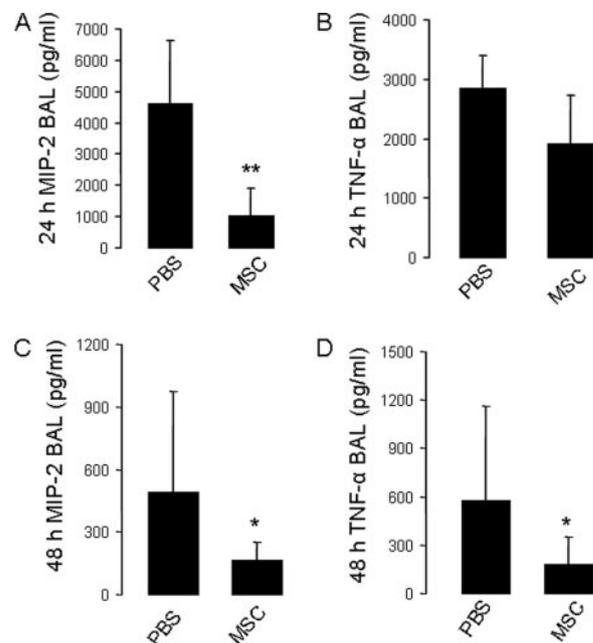


FIGURE 6. Levels of the proinflammatory cytokines, TNF- α and MIP-2, were reduced in mice receiving MSC at both 24 and 48 h after endotoxin-induced ALI. *A* and *B*, MIP-2 was significantly reduced in the BAL of mice treated with MSC at 24 h, whereas levels of TNF- α in the BAL showed a trend toward reduction in the MSC group ($n = 5$ –6 per group; **, $p < 0.01$ for MIP-2 in the BAL; $p = 0.06$ for TNF- α in BAL). *C* and *D*, At 48 h, MIP-2 and TNF- α were reduced in the BAL of MSC-treated mice ($n = 9$ –12 per group, *, $p < 0.05$). Data are expressed as mean \pm SD.

8 and 24 h after endotoxin administration. BAL and plasma samples were collected from each mouse, and the lungs, liver, and spleen were also harvested. The radioactivity of each sample was then assessed with a gamma scintillation counter (Packard Instrument) as a measure of the endotoxin concentration.

Binding and uptake of ^{51}Cr -labeled endotoxin by MSC and RAW 264.7 cells

MSC and RAW 264.7 cells were plated in six-well plates (Corning) and allowed to reach 50–70% confluency. The cells were then incubated with 4 μ g of labeled endotoxin per well for 3 h at 37°C for assessment of uptake and at 4°C for binding. Control wells included one well per plate with no endotoxin, and one well that had polymyxin-B at 50 μ g/ml to block endotoxin binding and uptake. The medium was then aspirated from each well, and cells were washed with cold PBS three times. Cells were then harvested with 0.5 ml of 0.05% trypsin, and then solubilized with 0.5 ml of 2% Triton X-100. The radioactivity in the resultant 1-ml suspension was recorded with a gamma scintillation counter (Packard Instrument).

Coculture of LPS-stimulated alveolar macrophages and MSC

To determine whether MSC are able to regulate production of proinflammatory cytokines by stimulated macrophages by a cell-cell contact-dependent or -independent mechanism, alveolar macrophages were cocultured with MSC in either a standard single well or in a Transwell (0.4- μ m pore size; Costar; Corning), and then stimulated with LPS. Alveolar macrophages were isolated from normal C57BL/6 mice by BAL using PBS. After isolation, the cells were washed once, and then resuspended in RPMI 1640 containing 5% FBS and 1% penicillin/streptomycin at a concentration of 20,000 cells/400 μ l. MSC were harvested from tissue culture after trypsinization and resuspended in RPMI 1640 medium at a concentration of 20,000 cells/100 μ l. Alveolar macrophages and MSC were then cocultured in the presence of LPS (4 μ M) for 4 h at 37°C in a standard 24-well plate (Costar; Corning). To test the effects of coculture without cell contact, additional wells were prepared in which a Transwell insert was used with the macrophages cultured in the bottom compartment and the MSC in the upper compartment (see Fig. 11A). Control wells were prepared with only macrophages stimulated with LPS, and MSC stimulated

Table I. Fibroblasts and apoptotic MSC do not decrease proinflammatory or increase anti-inflammatory cytokines^a

	3T3		Apoptotic MSC		PBS	
	24 h	48 h	24 h	48 h	24 h	48 h
TNF- α						
Plasma	0	78 \pm 109	0	105 \pm 57	0	60 \pm 32
BAL	918 \pm 257	515 \pm 286	1149 \pm 468	851 \pm 268	706 \pm 573	865 \pm 703
MIP-2						
Plasma	799 \pm 485	7314 \pm 5263	805 \pm 684	2365 \pm 1366	1030 \pm 1442	4775 \pm 2879
BAL	1810 \pm 1364	2987 \pm 2706	1303 \pm 652	1837 \pm 1290	974 \pm 1107	1598 \pm 663
IL-10						
Plasma	615 \pm 490		967 \pm 789		462 \pm 417	
BAL	143 \pm 144		249 \pm 159		88 \pm 94	

^a All comparisons of 3T3 fibroblasts and apoptotic MSC vs PBS are not significant ($p > 0.05$). Units are picograms per milliliter for all measurements. $n = 4-5$ per group for each time point. Data are expressed as mean \pm SD.

with LPS alone. The cell culture supernatants were then collected after the 4-h incubation to assay for the levels of TNF- α , MIP-2, and IL-10 by ELISA (R&D Systems).

Statistical analysis

Comparisons between two groups were made using an unpaired t test. For comparisons between multiple groups, ANOVA with Bonferroni's correction was used (see Fig. 8). The log-rank test was used for comparing survival data at 48 and 72 h. A value of $p < 0.05$ was considered statistically significant. Analyses were done using SPSS software and GraphPad Prism software. Data are shown as mean \pm SD.

Results

MSC characterization

Differentiation assays demonstrated that MSC retained their ability to form osteoblasts, adipocytes, and myofibroblasts at passages 15–25 (Fig. 1, A–C). Flow cytometry analysis demonstrated that

the cells did not express the cell surface markers CD45, CD11b, CD34, and CD31 (Fig. 1, E and F). Morphologically, the cells expressed GFP (Fig. 1D) and had a spindled, fibroblast appearance in culture that is consistent with MSC.

MSC improve survival over 72 h and indices of lung injury at 24 and 48 h

Over 48 h, mice that received i.t. MSC had a significantly higher rate of survival vs control mice that received i.t. PBS (80 vs 42%; Fig. 2A). This difference between the two groups became apparent after the initial 24 h. To determine whether the survival advantage persisted beyond the initial 48 h, additional experiments were done in which mice were followed for 72 h, and those mice that received MSC had a significantly higher rate of survival compared with control mice (Fig. 2B).

Mice given MSC showed a trend toward lower excess lung water at 24 h, and a significant difference at 48 h (Fig. 3, A and B). In addition, BAL protein, a marker of endothelial and epithelial permeability, was nonsignificantly reduced in the MSC group at 24 h, but significantly reduced in the MSC group at 48 h (Fig. 3, C and D).

In contrast, mice that received the fibroblast cell line, 3T3, or nonviable MSC had no improvement in survival or severity of lung injury (excess lung water and BAL protein) compared with PBS-treated mice at 48 h (Fig. 4).

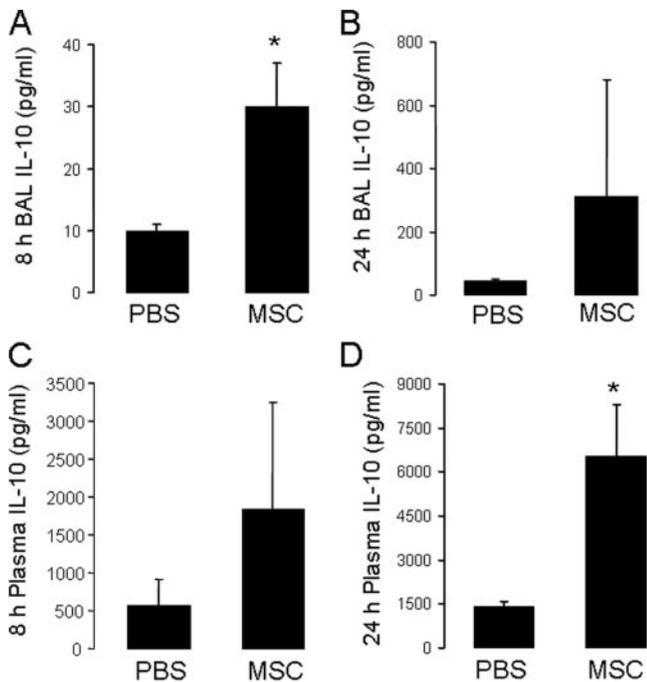


FIGURE 7. Levels of IL-10 in the BAL and plasma were increased with MSC therapy. A and B, Luminex analysis demonstrated higher levels of IL-10 in the BAL at 8 and 24 h ($n = 3$ per group per time point; *, $p < 0.05$ at 8 h, $p = 0.28$ at 24 h). C and D, There were also higher levels of IL-10 in the plasma at 8 and 24 h ($n = 3$ per group per time point; $p = 0.21$ at 8 h; *, $p < 0.05$ at 24 h). Data are expressed as mean \pm SD.

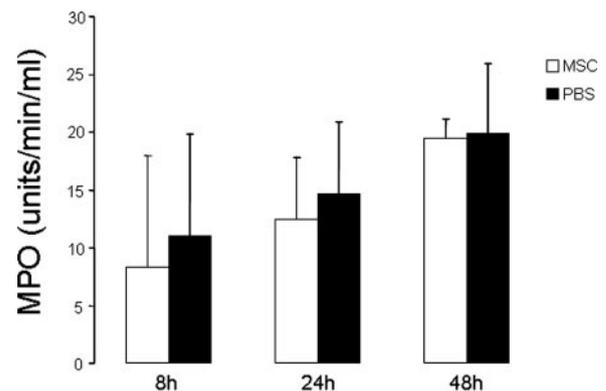


FIGURE 8. MSC did not decrease the number of neutrophils after endotoxin-induced ALI as determined by MPO activity. MPO activity was not significantly different between MSC- and PBS-treated mice at 8, 24, or 48 h after injury ($n = 3-6$ per group per time point). Data are expressed as mean \pm SD.

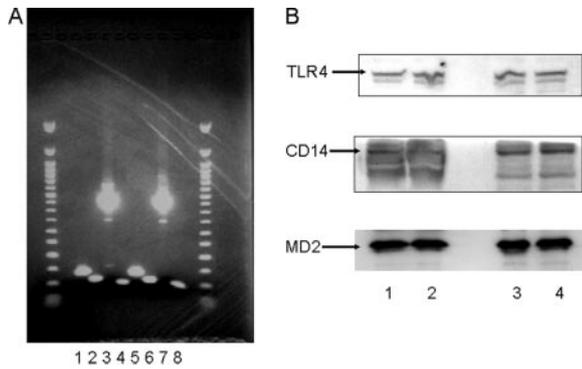


FIGURE 9. MSC express the LPS receptor complex: TLR4, CD14, and MD2. *A*, RT-PCR demonstrates that MSC express the genes TLR4, CD14 and MD2 (lanes 1–3, respectively). The positive control cell line, RAW 264.7, also expressed these three genes (lanes 5–7, respectively). *GAPDH* was used as a housekeeping gene and was expressed by both MSC and RAW 264.7 (lanes 4 and 8, respectively). *B*, Western blot confirmed that MSC and the RAW cell line express the proteins for TLR4, CD14, and MD2 (top panel, TLR4 at 90 kDa; middle panel, CD14 at 50 kDa; bottom panel, MD2 at 20 kDa; arrows indicate relevant bands).

Histology

At 48 h, H&E staining of lung sections from MSC-treated mice had significantly less injury compared with mice given PBS (Fig. 5, *A* and *B*). Mice treated with MSC also had a significant improvement in the degree of hemorrhage and edema as assessed by the lung injury score (Fig. 5*C*). Engraftment was assessed through the use of confocal microscopy as described previously. MSC expressing GFP were seen scattered through the lungs of mice treated with the cells; however, overall engraftment levels were <5% at both 24 and 48 h. In addition, there was no evidence that the MSC engrafted as clusters or formed clusters of cells in the lung parenchyma. The mice treated with PBS had no evidence for GFP-stained cells.

MSC instillation decreased levels of proinflammatory cytokines and increased anti-inflammatory cytokines following endotoxin injury

At 24 h, the levels of MIP-2 in the BAL were significantly lower in the MSC-treated mice compared with the PBS-treated mice, and TNF- α showed a trend toward a reduction in the BAL of the MSC-treated mice (Fig. 6, *A* and *B*). MIP-2 was also 6-fold lower in the plasma of MSC-treated mice at 24 h (3165 ± 449 vs 537 ± 304 pg/ml; $n = 5$ –6 per group; $p < 0.01$), whereas TNF- α in the plasma was not detectable in either group at 24 h. At 48 h, MIP-2 levels and TNF- α were also significantly lower in the BAL of the MSC-treated mice (Fig. 6, *C* and *D*). Plasma levels of MIP-2 were also 15-fold lower in the MSC-treated mice at 48 h (608 ± 819 vs

40 ± 55 pg/ml; $n = 9$ –11 per group; $p < 0.05$), whereas plasma levels of TNF- α were nonsignificantly reduced in the MSC group.

In separate experiments, mice treated with fibroblasts and apoptotic MSC did not have a significant reduction in the proinflammatory response to endotoxin compared with PBS-treated mice. At 24 and 48 h, MIP-2 and TNF- α levels in the BAL and plasma of mice treated with the control cell lines were not different compared with PBS mice (Table I).

To screen for possible anti-inflammatory cytokines up-regulated by MSC, the concentration of candidate factors was measured in the BAL and plasma of MSC and control mice at 8, 24, and 48 h. In particular, IL-10 was significantly higher in the BAL of MSC-treated mice at 8 h and remained higher in the plasma at 24 h (Fig. 7). By 48 h, IL-10 levels were not different between MSC and control mice. In contrast, compared with PBS-treated mice, those mice treated with fibroblasts and apoptotic MSC did not have a significant elevation in 24 h BAL and plasma levels of IL-10 (Table I).

In addition to IL-10, other anti-inflammatory cytokines were up-regulated by MSC administration including IL-1ra and IL-13. MSC-treated mice had higher levels of IL-1ra in the BAL at 24 h (8319 ± 2146 vs 5351 ± 1754 pg/ml; $n = 7$ –8 per group; $p = 0.01$) and IL-13 in the plasma at 8 h (118 ± 18 vs 61 ± 9 pg/ml; $n = 3$ per group; $p = 0.04$). These differences were not sustained at the other time points.

Of note, the levels of PGE₂, a lipid mediator with anti-inflammatory properties known to be secreted by MSC, was not different in the BAL between MSC- and PBS-treated mice (data not shown).

MSC administration did not prevent neutrophil chemotaxis to the alveolus

Given the significant reduction in the neutrophil chemokine, MIP-2, in the MSC-treated mice, BAL studies were done at 8, 24, and 48 h after LPS instillation to determine whether neutrophil counts were lower in the MSC-treated vs PBS-treated mice. Total cell counts and absolute neutrophil counts were not different between the two groups at any time point (data not shown). MPO activity was also similar between the MSC- and PBS-treated mice at all time points (Fig. 8).

MSC express the LPS receptor complex but do not clear endotoxin or affect its distribution

To determine whether MSC expressed the LPS receptor complex, TLR4/CD14/MD2, RT-PCR was done for these genes and the RAW 264.7 cell line was used as a positive control. The results indicate that MSC do express all three of these genes in a manner similar to the macrophage cell line (Fig. 9*A*). To confirm that the MSC expressed the proteins for TLR4, CD14, and MD2, a Western blot was done, using the RAW cell line as a positive control.

FIGURE 10. MSC do not internalize ⁵¹Cr-labeled endotoxin in vitro or affect its distribution in vivo. *A*, RAW 264.7 cells internalized significantly more labeled endotoxin compared with MSC in culture ($n = 4$ wells per cell line; **, $p < 0.01$). *B* and *C*, MSC did not affect the distribution of labeled endotoxin in the lung or BAL compared with PBS at either 8 or 24 h after endotoxin instillation ($n = 3$ mice per group per time point). Data are expressed as mean \pm SD.

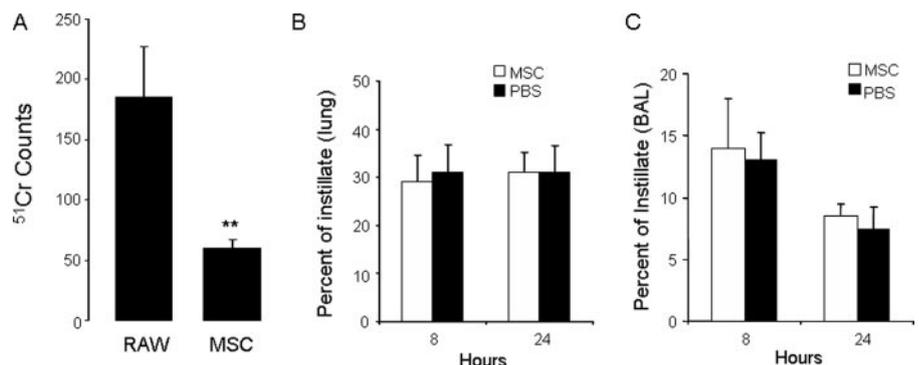
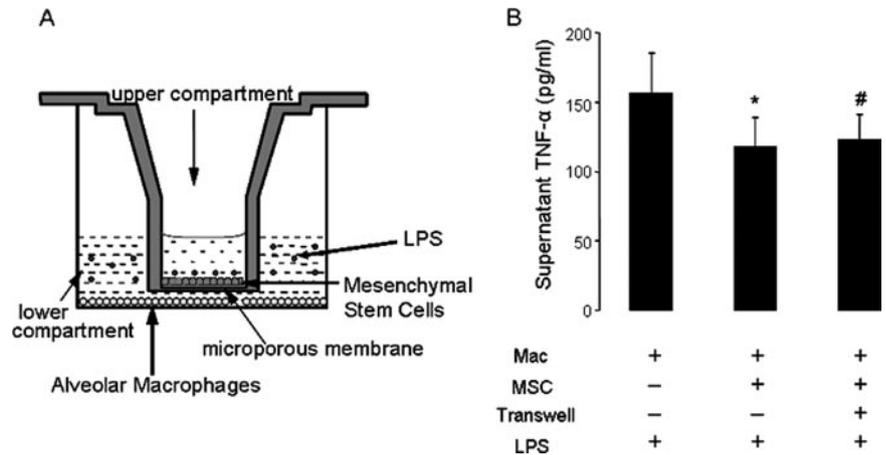


FIGURE 11. MSC down-regulate the production of TNF- α by LPS-stimulated alveolar macrophages in a cell-to-cell contact-independent manner. *A*, MSC and alveolar macrophages were co-cultured in the presence of LPS, and Transwell inserts were used in some wells to prevent cell-cell contact. *B*, TNF- α was measured in the supernatant of each well. The presence of MSC reduced the production of TNF- α by LPS-stimulated alveolar macrophages, and this reduction was similar when a Transwell insert was placed to prevent direct cell-cell contact ($n = 8-9$ wells per condition; *, $p < 0.01$; #, $p = 0.01$ for comparison with TNF- α production by LPS-stimulated alveolar macrophages alone). Data are expressed as mean \pm SD.



This demonstrated that MSC and the RAW cell line express all three proteins (Fig. 9B). These results are consistent with recent reports demonstrating that MSC and hemopoietic stem cells do express TLRs (24–26).

Next, it was determined whether MSC could internalize endotoxin in an in vitro system or affect its distribution in vivo. Binding and uptake studies with chromium-labeled endotoxin demonstrated that MSC internalized a significantly smaller amount of endotoxin compared with the control cell line, RAW 264.7 (Fig. 10A). Using the chromium-labeled endotoxin in the in vivo model of ALI, MSC did not alter the kinetics of endotoxin distribution in the mouse (Fig. 10, B and C). These results suggest that clearance of endotoxin is not part of the mechanism by which MSC exert their beneficial effects.

Coculture of alveolar macrophages and MSC reduced level of macrophage stimulation by LPS

The addition of MSC to LPS-stimulated macrophages reduced the level of TNF- α in the cell supernatant compared with only LPS-stimulated macrophages. The degree of reduction was similar in the presence of a Transwell that prevented cell contact between the macrophages and MSC (Fig. 11B). MSC alone did not produce TNF- α in response to LPS stimulation. In contrast to the in vivo data, levels of MIP-2 were not reduced with the addition of MSC to LPS-stimulated macrophages, and IL-10 was not detectable in any of the conditions.

Discussion

There are three major findings of this study: 1) the i.t. administration of MSC demonstrated both a functional and survival benefit in the mouse model of endotoxin-induced ALI when given 4 h after the administration of LPS; 2) there was a significant histological improvement in the severity of lung injury following MSC administration despite a level of MSC engraftment of <5% at 48 h after injury; 3) the beneficial effects with MSC appear to be mediated by a shift from a proinflammatory to an anti-inflammatory response to endotoxin.

Previous studies using MSC in lung injury have used only the bleomycin model of lung injury (3, 4). In these studies, the i.v. administration of cells ameliorated the severity of bleomycin-induced injury despite the low engraftment levels seen. This beneficial effect was primarily demonstrated by an improvement in histology, although Rojas et al. (4) did report a significant survival advantage when the MSC were given to bone marrow-suppressed mice. Despite these interesting results, there is little known of the effects of MSC in experimental models of ALI and of the possible

benefit of administering cells by the i.t. route. Our objective was to study the effects of the i.t. delivery of MSC in the mouse model of endotoxin-induced ALI when given as a treatment, and our primary endpoints were functional and survival effects. The data obtained in our study demonstrate, for the first time, a significant beneficial effect of the local delivery of MSC in experimental ALI. MSC therapy reduced lung wet-to-dry ratio and excess lung water, overall markers of severity of lung injury, and BAL protein, a marker of endothelial and epithelial permeability, at 48 h. However, the most striking finding of this study was the significant survival advantage conferred with MSC treatment. In addition, the survival data demonstrate that cell therapy exerted its beneficial effects after the initial 24 h of endotoxin-induced injury, because this is when the survival curves diverge. Furthermore, the benefits with MSC administration were not replicated with the i.t. delivery of fibroblasts or nonviable MSC. This result suggests that undifferentiated, viable MSC are required to produce a functional and survival benefit in endotoxin-induced ALI.

The second major finding of this study was that MSC administration led to a significant histologic improvement in the extent of lung injury despite the low level of engraftment. The improvement in histology was assessed quantitatively by the use of a lung injury score described previously (20). The extent of hemorrhage and edema was significantly reduced in the mice treated with MSC, which supports the findings of less excess lung water in these mice. MSC engraftment was estimated by the use of confocal microscopy. The low level of engraftment seen (<5%) is consistent with prior studies in the field (3, 7), which have reported engraftment levels <1% even in the presence of lung injury. These data support the concept that MSC might be protective against lung injury through mechanisms independent of their ability to engraft in the lung.

Given the rare incorporation of administered MSC into the lung, as well as recent literature reporting the immunomodulatory properties of MSC (9–12), we hypothesized that the benefit with MSC treatment was mediated through a down-regulation of the proinflammatory responses to endotoxin. Analysis of BAL and plasma samples from MSC-treated and control mice confirmed this hypothesis demonstrating lower levels of TNF- α and MIP-2 in the MSC-treated mice. Despite this reduction in the level of the chemokine MIP-2, neutrophil influx into the alveolus was not ameliorated in the MSC-treated mice because the levels of MPO and absolute neutrophil counts were not reduced. This suggests that the protection provided by MSC is not dependent on a reduction in neutrophil recruitment to the lung. This finding may be explained by the fact that MSC are administered 4 h after i.t. endotoxin by

which time there has been activation of alveolar macrophages and recruitment of neutrophils to the lung. In addition, MSC may also have effects on neutrophil function and activity that have yet to be described.

To determine whether the anti-inflammatory effect of MSC was related to an ability to internalize and act as a scavenger of free endotoxin, *in vitro* and *in vivo* studies with ^{51}Cr -labeled endotoxin were done. These studies showed that, despite the expression of TLR4 by MSC, the cells did not take up endotoxin as well as a macrophage cell line and did not alter the distribution of endotoxin in the mouse. Therefore, the beneficial effects of MSC are not related to clearance of endotoxin and reduction of its free concentration.

Given these results, other potential mechanisms of MSC regulation of the response to endotoxin were investigated. Of these, the possibility that MSC were either producing or up-regulating the production of anti-inflammatory cytokines seemed most likely given previous reports that MSC can modulate the activity of immune cells (9–11). To test for this, the levels of a variety of candidate factors were assessed. These analyses demonstrated that the levels of IL-10 were significantly higher in the BAL at 8 h and in the plasma at 24 h in MSC-treated mice. This result is consistent with recent reports demonstrating that MSC do increase production of IL-10 by APCs (9, 27). Furthermore, this finding may indicate an important mechanism by which MSC produce their beneficial effect because IL-10 has well-described protective effects in lung inflammation (28, 29). In addition to increasing IL-10 levels, MSC administration led to higher levels of IL-13 and IL-1ra. These findings indicate that MSC shift the balance from a proinflammatory to an anti-inflammatory cytokines in response to endotoxin in part by leading to the production of soluble factors such as IL-1ra and by inducing a Th2 type response. This result is also consistent with recent reports that MSC can down-regulate T cell responses by shifting from a Th1 to Th2 type response (9, 30, 31). However, it is still not known whether the MSC are the source of the regulatory cytokines or whether they are inducing immune cells to secrete them, or both. Separate experiments done with the fibroblast and apoptotic MSC control cells did not demonstrate this shift in the inflammatory response to endotoxin. It is worth noting that the absolute levels of the specific cytokines measured (TNF- α , MIP-2, and IL-10) are different in Table I compared with the levels in Fig. 6, perhaps because the experiments for Table I were done at a later time to provide evidence that the control cells (fibroblasts and apoptotic MSC) did not change the levels of BAL and plasma cytokines. These differences can most likely be ascribed to the inherent variability in the cytokine response to endotoxin, which may be explained by changes in temperature, age, and environmental conditions (32, 33). Despite these differences, the finding that the two control cell populations did not decrease the proinflammatory or increase the anti-inflammatory response to endotoxin, in comparison with PBS, is important and suggests that the immunomodulatory effect observed with MSC is specific.

In vitro co-culture experiments with endotoxin-stimulated alveolar macrophages and MSC were done to determine whether direct cell-to-cell contact is required to down-regulate the acute inflammatory response to endotoxin. Alveolar macrophages were used in these studies because they are the initial innate immune cell in the lung that is exposed to endotoxin in this model and orchestrate the inflammatory response and recruitment of other cells, such as neutrophils (34). Using a Transwell to physically separate the macrophages and MSC and thereby inhibit cell-cell contact, we found that the stem cells can inhibit macrophage production of TNF- α by endotoxin in a contact-independent manner. These experiments suggest that endotoxin-stimulated MSC produce soluble factors

that can regulate the macrophage response to endotoxin and may account for the beneficial effects with MSC treatment in the endotoxin model of ALI *in vivo*.

One of the limitations of using MSC is the lack of specific cell surface markers to isolate and characterize them. There has been significant variability in the methods used to isolate and culture bone marrow-derived MSC in the prior literature (3, 8, 35). Initial studies by Kotton et al. (8) used the plastic adherent fraction of whole bone marrow to select for MSC, whereas Ortiz et al. (3) used bone marrow cells that were immunodepleted for cells expressing CD45, CD11b, and CD34 to isolate a purified population of MSC. More recently, Miyahara et al. (35) isolated MSC from adipose tissue of rats, and similar to Kotton et al. (8), retained only the plastic adherent fraction for propagation. The MSC used in this study were originally isolated by selecting for the adherent fraction of whole mouse bone marrow and then subsequently propagating this subset of cells in culture. To verify that these cells represented a homogenous population of MSC, flow cytometry was done and demonstrated that the cells did not express CD11b, CD31, CD34, or CD45. In addition, differentiation assays were done after 20–25 passages and confirmed that the cells retained the ability to differentiate into osteoblasts, adipocytes, and myofibroblasts. Thus, the cells used in this study do represent a population of MSC that have retained their multipotent capability. However, until there are more specific cell surface markers for MSC, there will be continued difficulty in standardizing the MSC used by investigators in this field.

In summary, this study demonstrates that intrapulmonary treatment with MSC in experimental endotoxin-mediated ALI provides a significant survival advantage that is explained by a reduction in lung vascular permeability and the quantity of lung edema. This protection is mediated by a shift from a proinflammatory to an anti-inflammatory response to endotoxin that includes enhanced production of IL-10. These findings are the first evidence that intrapulmonary MSC have beneficial effects in an animal model of ALI and raise the possibility of using cell-based therapy for ALI in patients.

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Disclosures

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