Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice

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Submitted 1 November 2006; accepted in final form 30 March 2007

Xu J, Woods CR, Mora AL, Joodi R, Brigham KL, Iyer S, Rojas M. Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. Am J Physiol Lung Cell Mol Physiol 293: L131–L141, 2007. First published April 6, 2007; doi:10.1152/ajplung.00431.2006.—Bone marrow-derived mesenchymal stem cells (BMDMSCs) appear to be important in repair of the chronic lung injury caused by bleomycin in mice. To determine effects of these BMDMSCs on an acute inflammatory response, we injected C57BL/6 mice intraperitoneally with 1 mg/kg endotoxin followed either by intravenous infusion of 5 × 10^5 BMDMSCs, the same number of lung fibroblasts, or an equal volume of normal saline solution. Lungs harvested 6, 24, and 48 h and 14 days after endotoxin showed that BMDMSC administration prevented endotoxin-induced lung inflammation, injury, and edema. Although we were able to detect donor cells in the lungs at 1 day after endotoxin, by 14 days no donor cells were detected. BMDMSC administration suppressed the endotoxin-induced increase in circulating proinflammatory cytokines without decreasing circulating levels of anti-inflammatory mediators. Ex vivo cocultures of BMDMSC and lung cells from endotoxemic animals demonstrated a bilateral conversation in which lung cells stimulated proliferation and migration of stem cells and suppressed proinflammatory cytokine production by lung cells. We conclude that BMDMSCs decrease both the systemic and local inflammatory responses induced by endotoxin. These effects do not require either lung engraftment or differentiation of the stem cells and are due at least in part to the production of stem cell chemoattractants by the lungs and to humoral and physical interactions between stem cells and lung cells. We speculate that mobilization of this population of BMDMSCs may be a general mechanism for modulating an acute inflammatory response.

ACUTE LUNG INJURY (ALI) is a clinical syndrome associated with respiratory dysfunction, usually a consequence of sepsis and a systemic inflammatory response. The severest form of ALI (acute respiratory distress syndrome, ARDS) carries an ~30–50% mortality, and, except for low volume ventilation, no interventions have been shown to be effective (10, 21). Because the cause of common cause of ALI in humans is sepsis, infusion of gram-negative bacterial endotoxin has been used as a model of sepsis-related lung injury. Endotoxemia in several animal species (including mice) causes an acute systemic inflammatory response associated with lung edema, altered pulmonary function, deposition of extracellular matrix, and accumulation of inflammatory cells in the lungs (25). Low doses of endotoxin activate macrophages (19) and mobilize inflammatory cells from bone marrow, which localize to the lung and release cytokines and other chemoattractant factors. Higher doses of endotoxin cause tissue injury that includes apoptosis and necrosis of lung parenchymal cells, a consequence of a persistent and severe inflammatory response.

Results from several studies suggest that mobilization of progenitor cells into the injured lung occurs as a consequence of lung injury, and these cells have been considered critical to repair of injured lung. We (3) have shown that humans with high numbers of circulating endothelial progenitor cells (EPCs) are more likely to recover from ARDS than patients with similar degrees of lung injury but low numbers of circulating EPCs. Yamada and associates (29) reported that cells from whole bone marrow transformed into lung epithelial and endothelial cells in radiated mice treated with systemic endotoxin and implicated these cells as important in limiting endotoxin-induced lung injury.

We (26) reported that bone marrow-derived mesenchymal stem cells (BMDMSCs), expanded in culture and delivered intravenously to C57BL/6 mice, protected animals from lung injury and fibrosis induced by a single intratracheal dose of bleomycin. However, this protective effect was observed only when the cells were given early on after injury and the numbers of cells persisting in the lungs did not appear sufficient to account for the magnitude of the protective effect. Administered BMDMSCs appeared to engraft the lung and assume multiple lung cell phenotypes, but the markedly increased survival and decreased lung injury was also accompanied by reduced lung inflammation, inhibition of the systemic inflammatory response, and increased production of soluble factors involved in endogenous stem cell mobilization and localization.

We hypothesized that BMDMSCs can inhibit an acute inflammatory response and thus would protect the lungs from endotoxin-induced acute injury. To test that hypothesis, we delivered BMDMSC intravenously to mice after systemic administration of endotoxin and made in vivo and ex vivo observations. Endotoxin caused a systemic inflammatory response and alterations in lung structure and function; BMDMSC prevented the lung injury and suppressed systemic inflammation. The stem cells localized acutely to the lung but did not assume lung cell phenotypes and did not engraft the lung. Ex vivo experiments demonstrated that endotoxin-injured lungs produced chemoattractants for BMDMSC and stimulated their proliferation. In addition, in coculture experiments, we observed that reduction in inflammatory cytokines and increase

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in anti-inflammatory mediators produced by lung cells obtained from endotoxemic animals did not require contact between BMDMSC and lung cells, but the effect was greater when such cell-cell contact was present.

METHODS

Mice. Eight- to 10-wk-old female wild-type C57BL/6 and transgenic C57BL/6 mice that express green fluorescent protein (eGFP) driven by a β-actin promoter described previously (4) were used. Blood samples were collected before they were randomly assigned to one of four experimental groups that received 1) saline solution plus BMDMSC, 2) endotoxin plus BMDMSC, 3) endotoxin plus mouse lung fibroblasts, or 4) endotoxin alone. All of the animals were maintained in the Division of Animal Resources at Emory University, an Assessment and Accreditation of Laboratory Animal Care-approved facility. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

LPS-induced lung injury. Mice were inoculated intraperitoneally with 1 mg/kg endotoxin (LPS) prepared from Escherichia coli O111:B4 (Sigma, St. Louis, MO). Briefly, recipient animals were anesthetized by isoflurane inhalation. While anesthetized, endotoxin dissolved in PBS or an equal volume of PBS was injected intraperitoneally. Subgroups of animals were killed at 6, 24, and 48 h and 14 days after endotoxin. Lungs were harvested for histological analysis and determination of wet-dry ratio. Blood samples were collected from each animal before death for measurements of cytokine and growth factor concentrations.

Generation and administration of BMDMSC or lung fibroblasts. Transgenic mice, in which eGFP was driven by a β-actin promoter, were used to obtain BMDMSCs and lung fibroblasts. We used the mouse strain C57BL/6-Tg (Act-eGFP)C14-Y01-FM131 OS, a line that highly expresses GFP in all cells. For BMDMSCs, fresh bone marrow cells were isolated by flushing DMEM containing 1% penicillin-streptomycin through both femurs. The harvested cells were washed once with DMEM containing penicillin-streptomycin and plated at 10^6 cells per 100-mm plastic cell culture dish in complete media: RPMI 1640 containing 9% FCS, 9% horse serum, supplemented with HEPES, 1% penicillin-streptomycin, and pyruvate and cultured in 5% CO2. After 48 h, nonadherent cells were removed, and fresh media were added to the culture. Adherent cells were cultured in complete expansion media: Iscove’s modified Dulbecco’s medium (IMDM) containing 9% FCS, 9% horse serum, supplemented with HEPES, 1% penicillin-streptomycin, and pyruvate. At day 10, cells were harvested by treating the culture with 0.25% trypsin for 5 min followed by gentle scraping to remove cells. Macrophages were depleted by negative selection using anti-CD11b antibody conjugated with phycocyanin (PE), and hematopoietic cells were removed using anti-CD45 conjugated with PE. Cells were sorted with the help of magnetic beads coated with anti-PE (Miltenyl). After culture and negative depletion, purity of the cell preparation was evaluated for expression of CD45 and CD11b by fluorescence-activated cell sorting (FACS) analysis. Lung fibroblasts were isolated from sections of fresh lungs cultured for 4 wk in DMEM containing 10% FCS, supplemented with 1% penicillin-streptomycin and pyruvate, and cultured in 5% CO2. Before infusion, cells were washed twice with warm PBS and resuspended in a concentration of 5 × 10^7 cells per 0.1 ml of isotonic saline solution. Mice were anesthetized by inhalation of isoflurane, and cells were infused 1 h after LPS administration through a vein puncture using a 27-gauge needle.

Wet-dry analysis. Lungs were removed from mice, placed into previously weighed microcentrifuge tubes, and weighed. Lungs were then desiccated under a vacuum (Vacufuge; Eppendorf, Westbury, NY) overnight at 45°C and weighed again. The wet lung mass was divided by the dry lung mass to give the wet-dry ratio.

Histopathology. To harvest the lungs, the trachea was cannulated, and the lungs were fixed by inflation with 4% paraformaldehyde. Following overnight fixation, tissue was embedded in paraffin, sectioned, and stained. Hematoxylin and eosin (H&E) stained sections were made to determine morphology and inflammatory infiltrate. To determine BMDMSC engraftment and transformation, we used frozen sections. Briefly, after harvest, lungs were fixed with an intratracheal infusion of Tissue Teck (Sakura Finetek, Torrance, CA), rapidly placed in liquid nitrogen, and stored at −70°C. To analyze the samples, frozen lung sections were fixed with 4% paraformaldehyde for 30 min and treated with 1% bovine serum albumin plus 0.1% Triton X-100 (Sigma) for another 30 min. Sections were then blocked with normal donkey serum (Sigma) for 30 min at room temperature. For double labeling, sections were incubated with monoclonal anti-GFP antibody (Molecular Probes, Eugene, OR) and polyclonal goat anti-surfactant protein-C (SPC) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or polyclonal rabbit anti-aquaporin-5 antibody (Chemicon, Temecula, CA), polyclonal rabbit CD31 antibody (BD Biosciences), or polyclonal goat anti-vimentin antibody (Santa Cruz Biotechnology). FITC-conjugated donkey anti-mouse and rhodamine-conjugated donkey anti-rabbit or goat IgG (Jackson Immunoresearch Laboratory, West Grove, PA) were used as secondary antibodies. Each one of the experiments included a control for the secondary antibody to demonstrate the specificity of the reaction; these controls were negative. Endothelial cells were detected using anti-CD31 Texas red conjugated antibody (Sigma); sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Photographs were taken in an Olympus EX41 fluorescence microscope (Olympus America, Melville, NY) using ×100 and ×40 lenses with an Olympus MagnaFire camera.

Measurement of mobilization of neutrophils. For measurement of the number of neutrophils in the lungs, we modified the technique published by Everhart et al. (8). Briefly, numbers of neutrophils were counted in 10 randomly selected fields in three histological sections per mouse from a total of four mice. Histological sections were stained with H&E and analyzed at high-power magnification (×100).

In vitro chondrogenesis. We have reported previously that BMDMSC, as we prepare them, can assume phenotypes of multiple lung cell types. To further document the plasticity of these cells, we demonstrated that they could be made to differentiate into chondrocytes in vitro. Two hundred thousand cells were placed in each well of a six-well plate. Cell pellets were cultured at 37°C with 5% CO2 in chondrogenic basal media that consisted of DMEM supplemented with 50 mg/ml ascorbate-2-phosphate (Sigma), 40 mg/ml proline (Sigma), 100 mg/ml pyruvate (Sigma), and 50 mg/ml ITS premix (6.25 mg/ml insulin, 6.25 mg/ml transferrin, 6.25 mg/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 mg/ml linoleic acid; Becton Dickinson, San Jose, CA). The cells were cultured for 10 days and then stained with Alcian blue and counterstained with nuclear fast red.

Detection of cytokines and growth factors. These measurements were made using a Luminex platform. An anti-mouse multiplex kit was obtained from Linco (St. Charles, MO). Well filters were pre-washed, and 1:1 diluted samples were applied to each well. Specific antibody-coated beads were added to the wells and incubated for 18 h at 4°C. After incubation, the plate was washed twice. Biotinylated antibodies against the growth factors were added, and the mixture was incubated for 1 h. Afterward, the cytokine-antibody complexes were detected by adding streptavidin coupled to PE. The number of positive complexes was determined by reading each sample in a Luminex XYP platform. Data were analyzed using MasterPlex 1.2 software from MiraiBio, and data related to concentration were expressed in picograms per milligram.

Detection of Toll-like receptor 4 by RT-PCR. RT-PCR was performed following one-step SuperScript III protocol (Invitrogen). The reaction contained 1 μl of 10 μM mouse Toll-like receptor 4 (TLR4) PCR primer pair (RDP-269, R&D Systems), 5 μl of template RNA (0.1 μg), 25 μl of 2× reaction mix, 2 μl of SuperScript III RT/platinum Taq mix, and 17 μl of water. The RT step involved incubation at 55°C for 30 min. The PCR cycling conditions included
an initial denaturation of 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. The samples were run on a 1% agarose gel and visualized via a MultiImage Light Cabinet (Alpha Innotech).

**FACS analysis.** Cell suspensions of cultured BMDMSCs were prepared. Cells were stained with specific antibodies using allophycocyanin (APC)-anti-CD11c, PE-anti-TLR4, and FITC-anti-CD45 (Pharmingen). Analysis was performed on a FACScan cytometer using CellQuest software (Becton Dickinson), and the data were further analyzed using FlowJo software (Tree Star, San Carlos, CA). Forward angle and side light scatter were used to exclude dead cells.

**Coculture experiments.** We used a technique that we have published previously (26). Briefly, the entire lung was placed in ice-cold PBS in a 100-mm cell culture plate, and the tissue was mechanically macerated to create a suspension. The cells were pelleted, washed twice with DMEM (American Type Culture Collection, Manassas, MD), resuspended in a final volume of 2 ml, and counted. Lung cells (5 x 10^5) from mice either treated with LPS or not were placed on a 0.4- or 3-μm pore size membrane (Millipore, Billerica, MA) and inserted into a well of a six-well plate (Millipore) containing 50% confluent BMDMSC obtained from eGFP mice. The distance between the plate and the filter was 2–3 mm. After 5 days in coculture, we visualized cells in the lower well under an immunofluorescence microscope as an assessment of cell proliferation. In addition, we examined the filters and counted numbers of fluorescent cells as a measure of stem cell migration toward the lungs.

**Statistical methods.** For comparisons between groups, paired Mann-Whitney tests or unpaired t-tests with or without Welch correction and one-way ANOVA were used (P < 0.05 was considered significant). We used GraphPad Prism and GraphPad InStat to calculate the statistics.

**RESULTS**

**Characterization of the BMDMSCs.** To minimize contamination with non-BMDMSC including fibrocytes, cells expressing the surface markers CD45 and CD11b were depleted from BMDMSC cultures using magnetic beads before injection. Expression of surface markers was determined by FACS to ascertain the purity of the population. After purification, we were unable to detect CD45^+ or CD11c^+ cells. To determine possible responsiveness of BMDMSC to LPS, expression of TLR4 receptors was determined. Expression of TLR4 was determined by FACS and RT-PCR analysis. Low levels of TLR4 mRNA were detected by RT-PCR (data not shown). In contrast, expression in the cell surface of TLR4 was negative in BMDMSCs when compared with TLR4 expression in CD45^+ cells obtained from fresh bone marrow (Fig. 1A). Similar results had been reported recently by other groups (23). In cells obtained from bronchoalveolar lavage (BAL), expression of TLR4 was detected only in CD45^+ cells (Fig. 1B).

To determine the ability of the purified and cultured BMDMSCs to differentiate into different cell types, we cultured the cells with basic chondrocyte media. This media has been described to induce the transformation of mesenchymal cells into chondrocytes (5). After 10 days of culture, 50% of the cells stained positive for polysaccharides, one of the markers of chondrocytes (Fig. 1, C and D).

**Infusion of BMDMSC prevents LPS-induced ALI.** As we reported previously, injection of 1 mg/kg LPS intraperitoneally caused no mortality in any of the groups. The effect of systemic LPS on the lung was characterized by increased numbers of inflammatory cells associated with an increase in lung water content (edema). To see whether administration of BMDMSC altered endotoxemia-induced lung injury, we infused 5 x 10^5 BMDMSCs or lung fibroblasts as a control. We and others had showed previously that this number of cells decreased the severity of the lung injury induced by intratracheal instillation of bleomycin (22, 26). Lungs were harvested at baseline and 6, 24, and 48 h and 14 days after administration of endotoxin, BMDMSC, endotoxin plus BMDMSC, or endotoxin plus lung fibroblasts; histological H&E-stained sections are shown in Fig. 2A. Administration of BMDMSC alone caused no detectable alterations in lung structure. Lungs from animals receiving endotoxin but not BMDMSC showed vascular congestion and a general increase in cellularity predominantly due to the presence of neutrophils. These changes were present by 6 h and were more pronounced at 24 h after endotoxin when thickening of the alveolar septae was especially apparent. By 48 h, there were residual inflammatory cells and some alveolar wall thickening, but these changes were resolving. Lungs from animals receiving endotoxin and BMDMSC were devoid of these changes and were histologically similar to lungs from control animals that did not receive endotoxin. Infusion of lung fibroblast did not inhibit the effect of endotoxin in the lung. To quantify the effect of BMDMSC on lung inflammation, we counted the number of neutrophils in histological sections of lungs from mice from the different experimental groups at different time points (Fig. 2B). We found a significant decrease in the number of neutrophils in animals treated with BMDMSC and LPS compared with the animals treated with LPS alone; differences were larger 24 h after endotoxemia.

Pulmonary edema is a hallmark of ALI. Previously, we compared the extravascular lung water/blood-free dry lung ratios with blood-inclusive lung wet-dry ratios measured on the same lung samples either in normal lungs or in lungs with pulmonary edema induced by myocardial ischemia, oxygen toxicity, or endotoxemia with wet-dry ratios ranging from 4 to 8. Those studies showed a near perfect correlation of the two ratios regardless of how the edema was produced and that including residual blood in making the lung water measurement did not increase either precision or sensitivity of the measurement (4, 7, 27). Figure 3 summarizes the time course of wet-dry weight ratios of lungs from control, endotoxin-, endotoxin plus lung fibroblasts-, and endotoxin plus BMDMSC-treated animals. In mice treated with endotoxin alone, edema reached a peak at 24 h and was largely resolved by 48 h, similar to the animals that received lung fibroblasts. No edema developed in the lungs of animals receiving endotoxin and BMDMSC. Although the kinetics of the wet-dry ratio differed from our previous report (25), the finding of lung edema in endotoxin-treated animals is consistent.

**Infusion of BMDMSC moderates the endotoxemia-induced systemic inflammatory response.** We measured cytokine and chemokine concentrations in the serum at different time points in mice from all groups. Figure 4 summarizes the serum levels of six different cytokines and chemokines. Endotoxemia caused a consistent acute systemic inflammatory response reflected in increased serum concentrations of the proinflammatory mediators, IFN-γ, interleukin (IL)-1β, macrophage inflammatory protein (MIP)-1α, and KC (murine analog of IL-8). This response peaked at 6 h after endotoxin administration and largely subsided by 24 h. Administration of BMDMSC moderated the increase in each of these proinflamm-
Endotoxemia also caused an increase in serum concentrations of IL-12 p70 and the anti-inflammatory cytokine IL-10, effects that were not altered by BMDMSC administration. There was a persistent increase in serum concentrations of granulocyte colony-stimulating factor (G-CSF, a growth factor that is implicated in mobilizing endogenous stem cells from bone marrow) after endotoxin, and at 6 h, the increase was greater in the animals receiving BMDMSC.

Because we did not observe any biological effect by infusing lung fibroblasts, systemic and local cytokines were not measured in this group.

**Trafficicking and fate of BMDMSC in the lungs of endotoxemic mice.** We identified BMDMSC donor cells by staining the sections with an anti-GFP-specific antibody (Fig. 5A). Lungs from either control animals (data not shown) or animals given endotoxin that did not receive BMDMSC showed minimal background staining. At the 24 h time point, lungs from animals that received BMDMSC but not endotoxin contained few GFP<sup>+</sup> cells, but lungs from animals receiving both endotoxin and BMDMSC contained large numbers of these cells. By day 14 after endotoxin, very low numbers of GFP<sup>+</sup> cells could be detected in lungs from mice that received endotoxin and BMDMSC.
To determine whether donor BMDMSC engrafted the lungs and assumed lung cell phenotypes, we examined tissue sections of lungs by immunofluorescent staining for cell type-specific markers so that colocalization of GFP staining (indicating donor-derived cells) with staining for cell-specific markers would indicate the phenotype of the donor cells. Figure 5B shows fluorescent photomicrographs of histological sections from lungs obtained either 24 h or 14 days after endotoxin administration and stained for type II alveolar epithelial (pro-SPC; Ref. 2), endothelial (CD31; Ref. 9), type I epithelial (aquaporin-5), or fibroblast (vimentin) phenotypes. Although at 24 h there were many GFP+ cells, there was no colocalization with the lung cell-specific markers. At 14 days, there were few GFP+ cells and again no evidence of any lung cells that were donor stem cell derived. We found no colocalization of GFP+ with any of the cell-specific phenotypic markers analyzed. These data suggest that BMDMSC did not engraft the lung or assume phenotypic characteristics of the major cell types that compose lung parenchyma.

Fig. 2. Systemic administration of BMDMSC decreases endotoxin (LPS)-induced lung injury. Mice were inoculated intraperitoneally with 1 mg/kg LPS. One hour later, half of the animals were infused intravenously with $5 \times 10^5$ green fluorescent protein-positive (GFP+) BMDMSC or lung fibroblasts. In a group of animals, $5 \times 10^5$ GFP+ BMDMSC were infused, but no LPS was given. Animals were killed at 6, 24, and 48 h and 14 days. A: lungs were fixed in paraformaldehyde, and sections were stained with hematoxylin and eosin (H&E) and visualized at $\times 40$ magnification. B: quantification of neutrophils per high-power field on histological sections stained with H&E. The total number of neutrophils was counted in 10 different high-power fields per slide. The image represents the average from 3 to 5 histological lung sections ($^*P < 0.05$). Scale bar = 50 μm.
Concentrations of MIP-1α, IL-1β, IL-12, RANTES, and IL-6 were measured from the different groups. In the presence of BMDMSC, Figure 7 summarizes the data for cytokines in the supernatants obtained from mice 24 h after endotoxin administration on the proliferation and migration of eGFP fibroblasts into the lung cell compartment or by a membrane separated either by a membrane that permitted BMDMSC to reach the lung by migration into the lung cell compartment or by a membrane with pores too small to permit cell migration (see METHODS).

**Interactions of BMDMSC and lung cells ex vivo.** To determine whether humoral factors that affect stem cell behavior originate in the injured lung, we measured the effects of cell suspensions prepared from lung harvested 24 h after LPS administration on the proliferation and migration of eGFP BMDMSC. In coculture experiments (Fig. 6A), lung cell suspensions from either control or endotoxin-treated animals were separated from GFP+ BMDMSC by a membrane with pores large enough to permit migration of BMDMSC into the chamber containing lung cells. Fluorescent photomicrographs were made of the upper surface of the filter separating the lung and stem cells (to assess stem cell migration) after 3 days in culture. When the upper chamber contained cells from uninjured lung, there was no evidence of migration of the GFP+ stem cells from the lower chamber toward the lung cells. However, when cells from endotoxin-injured lungs were in the upper chamber, numerous GFP+ stem cells migrated to the upper chamber (Fig. 6B). The migrating cells did not appear to change their morphology. It appears that cells from endotoxin-injured lungs produce factors that stimulate BMDMSC to migrate into the lungs.

To determine whether BMDMSC produce factors that alter cytokine production by lung cells and whether such an effect requires that BMDMSC and lung cells come into direct contact, we analyzed supernatants from the lung cell chamber in coculture experiments in which BMDMSC and lung cells obtained from mice 24 h after endotoxin administration were separated either by a membrane that permitted BMDMSC migration into the lung cell compartment or by a membrane with pores too small to permit cell migration (see METHODS). Figure 7 summarizes the data for cytokines in the supernatants from the different groups. In the presence of BMDMSC, concentrations of MIP-1α, IL-1β, IL-12, RANTES, and IL-6 were consistently lower than in the absence of BMDMSC, whereas IL-10 concentrations were unaffected by stem cells. In addition, the BMDMSC effect on MIP-1α and RANTES was much greater in experiments where BMDMSC could reach the lung cell compartment than in experiments in which contact between lung cells and stem cells was not possible. It appears that BMDMSC moderate production of cytokines by endotoxin-injured lungs both by production of humoral factors and by processes that require direct contact between stem cells and lung cells.

**DISCUSSION**

In a well-defined mouse model of endotoxemia (3, 10–12), we have shown that BMDMSCs suppress the systemic inflammatory response and prevent ALI. These stem cells localized transiently to the lungs but did not engraft there or assume lung cell phenotypes. In cocultures, we demonstrated a bilateral conversation between lung and stem cells: stem cells influencing lung cell function and lung cells affecting stem cell behavior. Lung cells from endotoxemic animals produced stem cell chemoattractants and stimulated stem cell proliferation. Mesenchymal stem cells suppressed production of inflammatory cytokines by lung cells from endotoxemic animals by both cell contact-dependent and cell contact-independent mechanisms.

A transient inflammatory response is a critical body defense that controls infectious and other toxic insults. Injury of the lungs and other organs consequent to sepsis in humans and several other animal species appears to result from a dysregulated inflammatory response resulting in intense and persistent inflammation (8, 20, 25). Several factors appear to contribute to the termination of acute inflammation, including generation of anti-inflammatory cytokines (e.g., IL-10; Refs. 14, 28). Lung injury induced by systemic administration of endotoxin is a complex process. For example, we have shown that endotoxin has some direct effects on the lung but that severe lung injury only occurs when the liver is present in the circulation (27). The fact that stem cells have the capacity to suppress endotoxin-induced systemic and local acute inflammation raises the possibility that mobilization of endogenous stem cells could contribute to regulation of this response. Our earlier reported observation (3) that recovery of humans with ALI correlates with numbers of circulating progenitor cells is consistent with that notion.

In the lung, several reports in the literature demonstrate that either whole bone marrow or BMDMSCs can limit experimental bleomycin-induced lung injury and fibrosis (22, 26), but the idea that stem cells simply supply a reservoir of new lung parenchymal cells and therefore hasten repair is not adequate to explain the effect. If that were true, stem cells given after the acute inflammatory response subsides should protect against fibrosis, and that is not the case (22); stem cells must be given very early after bleomycin to be effective. In addition, although it is possible to demonstrate persistence of donor stem cells in the lungs and evident differentiation into lung cell phenotypes, the numbers of engrafted cells is small, insufficient to explain the protective effect (26). Similarly, whole bone marrow infusions protect irradiated mice made endotoxemic out of proportion to the numbers of donor cells that can be found in the lungs (29). The anti-inflammatory effect of mesenchymal stem cells demonstrated here may be an important contributor to the “therapeutic” effect of stem cell administration in these models.

Consistent with this report, local injection of BMDMSC has been shown to restore cardiac function after injury during the first 72 h after injection. This early effect cannot be attributed...
Fig. 4. Infusion of BMDMSCs alters the systemic inflammatory response to endotoxin. C57BL/6 mice were inoculated with LPS and 60 min later infused with BMDMSCs or saline solution. A 3rd group of animals received BMDMSC without LPS. Blood samples and BAL were collected at 6, 24, and 48 h. Cytokine concentrations were determined in a Luminex system. A: 6 h after infusion of BMDMSC, there was a significant decrease in systemic concentrations in the levels of LPS-induced proinflammatory cytokines IFN-γ, IL-1β, IL-6, macrophage inflammatory protein-1α (MIP-1α), and KC (murine analog of IL-8). However, we did not observe a decrease in the levels of the anti-inflammatory cytokine IL-10 or IL-12 p70, but there was a significant enhancement in the levels of granulocyte colony-stimulating factor (G-CSF), a soluble factor important for stem cell mobilization. B: in contrast, concentration of cytokines in BAL was not significantly affected by the infusion of BMDMSC (values are means ± SE; n = 9 for each time point; *P < 0.05).
to cell transformation or tissue regeneration by the BMDMSC, but rather it is described as a paracrine effect as demonstrated by the upregulation of several genes coding for soluble factors (9).

Earlier reports indicate that BMDMSCs can have an immunomodulatory effect in in vitro preparations. Reports by several groups have demonstrated that BMDMSC can inhibit T cell proliferation and prevent the reversible differentiation of human monocytes into dendritic cells both by production of soluble factors and by processes requiring direct cell-cell contact (1, 11, 30). Since maturation of effector T cells depends on the cytokine milieu when T cells are activated by the dendritic cells, the stem cell-dendritic cell interaction could be an important regulator of the balance between T helper type 1 (Th1) and Th2 cells.

BMDMSCs can also suppress the activity of T cells, and suppression of T cell activity can increase survival of transplant recipients with host-vs.-graft disease (13, 17, 18). The mechanisms involved in the suppression of T cell activity are not well defined. BMDMSC express costimulatory molecules (B7-1, B7-2, CD40), but overexpression of these molecules did not increase the frequency of T cell suppression.

Fig. 5. BMDMSCs localize transiently to endotoxemic lung without engraftment or differentiation. C57BL/6 female mice were inoculated with 1 mg/kg LPS intraperitoneally. One hour later, $5 \times 10^5$ GFP$^+$ BMDMSC were infused intravenously. An additional group of animals received BMDMSC but not LPS. Lungs were harvested, and frozen sections were used for immunofluorescence to detect expression of GFP (arrows) and several cell markers with specific antibodies. A: photomicrographs of GFP immunostaining of sections from lung harvested 24 h after BMSMSC infusion in animals treated or not with LPS. We observed a large number of cells positive for GFP in LPS-treated animals. At day 14, we observed less than 0.1% of GFP$^+$ cells in the lung (data not shown). B: immunostaining sections of lungs from animals receiving BMDMSC plus LPS at 1 and 14 days after LPS treatment and BMDMSC infusion were stained with anti-GFP (green) and pro-surfactant protein-C (SPC; red) for type II epithelial cells, anti-CD31 to detect endothelial cells (red), anti-aquaporin for type I cells (red), and vimentin for fibroblasts (red). No colocalization of cell-specific markers and GFP was observed. Essentially, no GFP$^+$ cells were present on day 14. As a negative control, lungs from C57BL/6 mice were stained with anti-GFP, and as positive control, lungs obtained from C57BL/6-GFP mice were stained with anti-GFP.

Fig. 6. Lung cells from endotoxemic mice cause BMDMSC migration in cocultures. A: diagram of the preparation. BMDMSC obtained from GFP$^+$ mice were equally distributed in a 6-well plate. Suspensions of lung cells from control mice and from animals 24 h after administering endotoxin were placed in 1.5-inch filters with 3-$\mu$m radius pores (large enough to permit cell migration) and cocultured with BMDMSC. B: fluorescent photomicrographs of the upper and lower wells after 3 days in culture showing an experiment with control lung and 1 with lung cells from an endotoxemic animal. The migration of BMDMSC GFP$^+$ was quantified by counting the number of GFP$^+$ cells in the lung cell chamber. Significantly higher numbers of cells are mobilized by cells from endotoxin-injured lungs compared with lung obtained from animals treated with PBS (values are means $\pm$ SE; $n = 6$; $*p < 0.05$).
not rescue their ability to induce T cell proliferation after allogenic stimulation, implicating soluble factors (6, 15). On the other hand, Krampera and associates (16) reported that stem cell-T cell interaction is necessary to suppress T cell activity. It seems likely that both humoral factors and physical cell-cell interactions play roles in T cell suppression by BMDMSCs. Infusion of large numbers of allogenic mesenchymal stem cells into baboons did not induce systemic T cell alloreactivity for at least 4 wk (12), suggesting that these stem cells can escape attack by cytotoxic T lymphocytes and natural killer (NK) cells (24).

Delineating the precise mechanism of the anti-inflammatory effect of BMDMSCs will require further study. Possible mechanisms include generation and secretion of soluble receptors for proinflammatory cytokines, production of prostacyclin (PGI2), which has been implicated in decreasing dendritic cell production of proinflammatory cytokines (IL-12, TNF-α, IL-6) and chemokines (MIP-1α, monocyte chemoattractant protein-1), or generation of anti-inflammatory cytokines (e.g., IL-10) directly by BMDMSCs (31).

Endotoxemia in mice results in increased circulating concentrations of several cytokines and growth factors (3). Although BMDMSC administration did not entirely prevent these responses, stem cell administration changed the pattern of cytokine responses, significantly decreasing generation of Th1 proinflammatory cytokines without increasing the anti-inflammatory Th2 cytokine IL-10. BMDMSC prevented in a significant manner the endotoxin-induced influx of inflammatory cells (predominately neutrophils) into the lungs and lung edema, responses that were temporally coincident with decreased circulating levels of proinflammatory cytokines (MIP-1α, KC) that are known to affect inflammatory cell trafficking (24, 25). BMDMSC administration suppressed production of proinflammatory mediators but did not suppress production of IL-10 and G-CSF, which would alter the milieu from pro- to anti-inflammatory. Measurement of proinflammatory cytokines in BAL may indicate that proinflammatory cytokine concentrations in BAL may not always reflect a proinflammatory interstitial milieu (since neutrophilic inflammation was demonstrated histologically). The systemic inflammatory response is a major feature of this model, and the associated lung inflammation and injury may depend largely on responses of other organs.

In summary, these studies demonstrate that BMDMSCs have the ability to suppress the endotoxin-induced systemic inflammatory response. Administration of lung fibroblasts did not alter the endotoxin response so that the BMDMSC effect is specific. BMDMSC prevented endotoxemia-induced ALI without engrafting the lung and without assuming lung cell phenotypes. BMDMSC convert the systemic endotoxin response from a proinflammatory to an anti-inflammatory milieu by suppressing generation of proinflammatory mediators without suppressing generation of anti-inflammatory mediators. Studies of interactions between lung cells and mesenchymal stem cells in coculture define a bilateral conversation in which lung cells stimulate mesenchymal stem cells to migrate, and mesenchymal stem cells promote an anti-inflammatory cytokine milieu by producing soluble factors and by mechanisms requiring physical contact of stem and lung cells.

These observations expand the concept of stem cells as immunomodulatory cells by demonstrating modulation of systemic and local acute inflammation in vivo and by identifying
interactions between mesenchymal stem cells and cells from injured lung that may comprise a homeostatic mechanism for controlling responses to inflammatory stimuli. We speculate that acute mobilization of mesenchymal stem cells from the bone marrow contributes to termination of acute inflammation in the lungs as a result of lung cell-directed homing to and proliferation of stem cells in areas of injury and of stem cell modulation of cytokine production. In addition, these findings indicate that mesenchymal stem cells could be therapeutic in acute inflammatory states that eventuate in organ injury as a result of their ability to control inflammation as well as to contribute to repair of organ injury.

ACKNOWLEDGMENTS

We thank Valerie Mac and John LaVoy for their technical assistance.

GRANTS

This research was supported by National Heart, Lung, and Blood Institute Grant 5-P01-HL-066949-02, Emory University Research Committee Grant 2003100, and the McKelvey Center for Lung Transplantation at Emory University.

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