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Study of Bone Marrow and Embryonic Stem Cell-Derived Human Mesenchymal Stem Cells for Treatment of *Escherichia coli* Endotoxin-Induced Acute Lung Injury in Mice

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Key Words. Acute lung injury • Embryonic stem cells • Matrix metallopeptidase • Mesenchymal stem cell

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ABSTRACT

Mesenchymal stem cells (MSCs) can be derived from multiple tissue sources. However, the optimal source of MSCs for cell-based therapy for acute lung injury (ALI) is unclear. In the present experiments, we studied bone marrow (BM)-derived and embryonic stem cell-derived human MSC (ES-MSCs) as a therapeutic agent in *Escherichia coli* endotoxin-induced ALI in mice. We hypothesized that ES-MSCs would be more potent than BM-MSCs owing to its more primitive source of origin. ALI was induced by the intratracheal instillation of endotoxin at 4 mg/kg into 10–12-week-old C57BL/6 mice with or without BM-MSCs, ES-MSCs, or normal human lung fibroblasts as a cellular control. Compared with the endotoxin-injured mice at 48 hours, the administration of ES-MSCs provided results similar to those of BM-MSCs, significantly reducing the influx of white blood cells and neutrophils and decreasing the secretion of the inflammatory cytokines, macrophage inflammatory protein-2 and tumor necrosis factor-α, in the injured alveolus. BM-MSCs also reduced extravascular lung water, a measure of pulmonary edema, by 60% and the total protein levels, a measure of lung permeability, by 66%. However, surprisingly, ES-MSCs did not have these protective effects, which was partially explained by the increased secretion of matrix metallopeptidase 9 by ES-MSCs, an enzyme known to increase lung protein permeability. In conclusion, both BM-MSCs and ES-MSCs markedly decreased endotoxin-induced inflammation. However, ES-MSCs did not show any beneficial effect on reducing pulmonary edema and lung protein permeability compared with BM-MSCs, suggesting that not all MSCs behave in a similar fashion. Our results highlight the need perhaps for a disease-specific potency assay for MSCs. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:832–840*

SIGNIFICANCE

To determine the optimal source of mesenchymal stem cells (MSCs) for cell-based therapy for acute lung injury, bone marrow (BM)- and embryonic stem cell-derived human MSC (ES-MSCs) were compared as therapeutic agents for *Escherichia coli* endotoxin-induced lung injury in mice. ES-MSCs behaved similarly to BM-MSCs by markedly decreasing the inflammatory response induced by endotoxin. However, unlike BM-MSCs, ES-MSCs provided no protective effects against increasing lung water and protein permeability, in part because of an increase in expression of matrix metallopeptidase 9 by ES-MSCs. In patients with acute respiratory distress syndrome, impaired alveolar fluid clearance (i.e., no resolution of pulmonary edema fluid) has been associated with higher mortality rates. Although ES-MSCs might ultimately be found to have properties superior to those of BM-MSCs, such as for immunomodulation, these results highlight the need for a disease-specific potency assay for stem cell-based therapy.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is among the most common causes of respiratory failure in critically ill patients. Despite extensive research, ARDS remains a major cause of morbidity and mortality, with no effective pharmacological treatment options [1–3]. Recently, stem cell-based therapies with mesenchymal stem cells (MSCs) have been shown to be effective in preclinical models of acute lung injury (ALI) owing to their ability to secrete several paracrine factors that can regulate lung endothelial and epithelial permeability, including growth factors, anti-inflammatory cytokines, and antimicrobial peptides [4–13]. These soluble factors can treat the major abnormalities that underlie ALI, including impaired alveolar fluid clearance, altered lung permeability, dysregulated inflammation, and infection [12, 14–24]. Because of

these promising results, 2 phase I and II clinical trials (NCT01775774, NCT01902082) are currently underway to test the effect of human bone marrow (BM)-derived MSCs in patients with moderate to severe ARDS.

However, the optimal source of MSCs for cell-based therapy remains unknown. MSCs are adult nonhematopoietic precursor cells that can be derived from a variety of tissues, such as bone marrow, adipose tissue, and placenta. Recently, we found that MSCs obtained from the chorion of human term placenta expressed embryonic stem cell markers such as OCT-4, NANOG, SSEA-3, and TRA-1-60. Compared with bone marrow-derived MSCs (BM-MSCs), chorion-derived MSC secreted significantly higher levels of hepatocyte growth factor, fibroblast growth factor-β, keratinocyte growth factor, and angiopoietin-1, soluble factors known to have reparative properties in ALI. In addition, these chorion-derived MSCs restored transepithelial resistance across Calu3, a human epithelial cell line, more effectively than BM-MSCs after oxidant injury [25]. Owing to the source of origin, we speculated that chorion-derived MSCs would potentially be an abundant autologous source of stem cells with more potency than BM-MSCs in ARDS.

Recently, MSCs have been derived from human embryonic stem cells (ES-MSCs) by a number of research groups [26–34]. These cells display standard MSC surface markers (CD105, CD90, CD73, and CD146) with the capability of osteogenic, adipogenic, and chondrogenic lineage differentiation. More importantly, ES-MSCs do not form teratomas in vivo in mice. Owing to the source of origin, ES-MSCs could be an unlimited and, potentially, a more potent source of MSCs for cell-based therapy and tissue engineering. However, little is known about their functional activity in lung disease. To determine whether MSCs derived from more primitive cells might be more efficacious, we compared ES-MSCs (H9-MSCs) [33] and BM-MSCs in *Escherichia coli* endotoxin-induced ALI in mice. We hypothesized that ES-MSCs would have more reparative properties, in part through the increased secretion of soluble factors.

Materials and Methods

Human Mesenchymal Stem Cells

Human bone marrow-derived MSCs were obtained from an NIH repository from Texas A&M Health Science Center (Temple, TX). MSCs from 3 different human donors were used in the experiments with the total passage number ≤ 8. Human MSCs derived from embryonic stem cells (H9-MSCs, ES-MSCs) were a kind gift from Dr. Jan Nolta (University of California, Davis, Davis, CA). Both adult stem cells met all the criteria for MSCs as defined by the International Society of Cellular Therapy [35]. The culture conditions and characterization performed for the two types of MSCs are summarized in supplemental online Tables 1 and 2. Normal adult human lung fibroblasts (NHLFs) were used as cellular controls (Lonza Group, Basel, Switzerland, http://www.lonza.com).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from MSCs using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, http://www.qiagen.com). The primers used for reverse transcription-polymerase chain reaction (RT-PCR) were human angiopoietin-1 (Ang-1), human keratinocyte growth factor (KGF), human matrix metallopeptidase (MMP)-9, human MMP-2, human tissue inhibitor of metallopeptidase (TIMP)-1, human TIMP-2, and human glyceraldehyde 3-phosphate dehydrogenase (SABiosciences, Qiagen, Valencia, CA, http://www.sabiosciences.com). The RT-PCR assays were conducted using the One-Step RT-PCR protocol as described by the manufacturer (SABiosciences, Qiagen).

E. coli Endotoxin-Induced ALI in Mice

C57BL/6 male mice (10–12 weeks old, ~25 g; Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) were used in all experiments. The Institutional Animal Care and Use Committee at the University of California, San Francisco, approved all experimental protocols. The mice were first anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) i.p. Acute lung injury was then induced by intratracheal instillation of a nonlethal dose of endotoxin from *E. coli* O111: B4 (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) at 4 mg/kg. The treatment groups were as follows: phosphate-buffered saline (PBS) as the carrier control; NHLFs as a cellular control (750,000 cells per mouse); BM-MSCs (750,000 cells per mouse); and ES-MSCs (750,000 cells per mouse). The different treatments were given to all groups simultaneously.

Measurement of Neutrophil Counts in Bronchoalveolar Fluid and Plasma

Both bronchoalveolar lavage (BAL) fluid and plasma samples were obtained from the mice 48 hours after endotoxin-induced lung injury. The total white blood cell (WBC) count and differential blood count were obtained using the Hemavet HV950FS (Drew Scientific, Miami Lakes, FL, http://www.drew-scientific.com).

Measurement of Cytokine and Protein Levels in Bronchoalveolar Fluid

Mouse macrophage inflammatory protein-2 (MIP-2) and tumor necrosis factor-α (TNF-α) were measured in the BAL fluid using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, http://www.rndsystems.com). Mouse MMP-9, human MMP-9, and mouse TIMP-1 were also measured in the BAL fluid using ELISA kits (R&D Systems). The total protein concentration was measured in the BAL fluid (Pierce BCA Protein Assay Kit; Thermo Scientific, Wilmington, DE, https://www.lifetechnologies.com).

Extravascular Lung Water

Gravimetric lung water determination was performed as follows. The whole lung was excised, weighed, and homogenized after the addition of 1 ml of double distilled water. The homogenate was centrifuged at 12,000 rpm for 10 minutes 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 were obtained. All samples were placed in a drying oven at 55°C for 48 hours. The dry weights were subsequently determined. The final excess lung water was calculated as described in our previous publication [6].

Histologic Findings and Lung Injury Score

The lungs from all 4 groups of mice were excised at 48 hours. The lungs were gently inflated with 0.3 ml of air, and the trachea was ligated. The lungs were then fixed in 4% paraformaldehyde. After fixation, the lungs were embedded in paraffin, cut into 5-μm sections, and stained with H&E. An investigator, who was unaware of

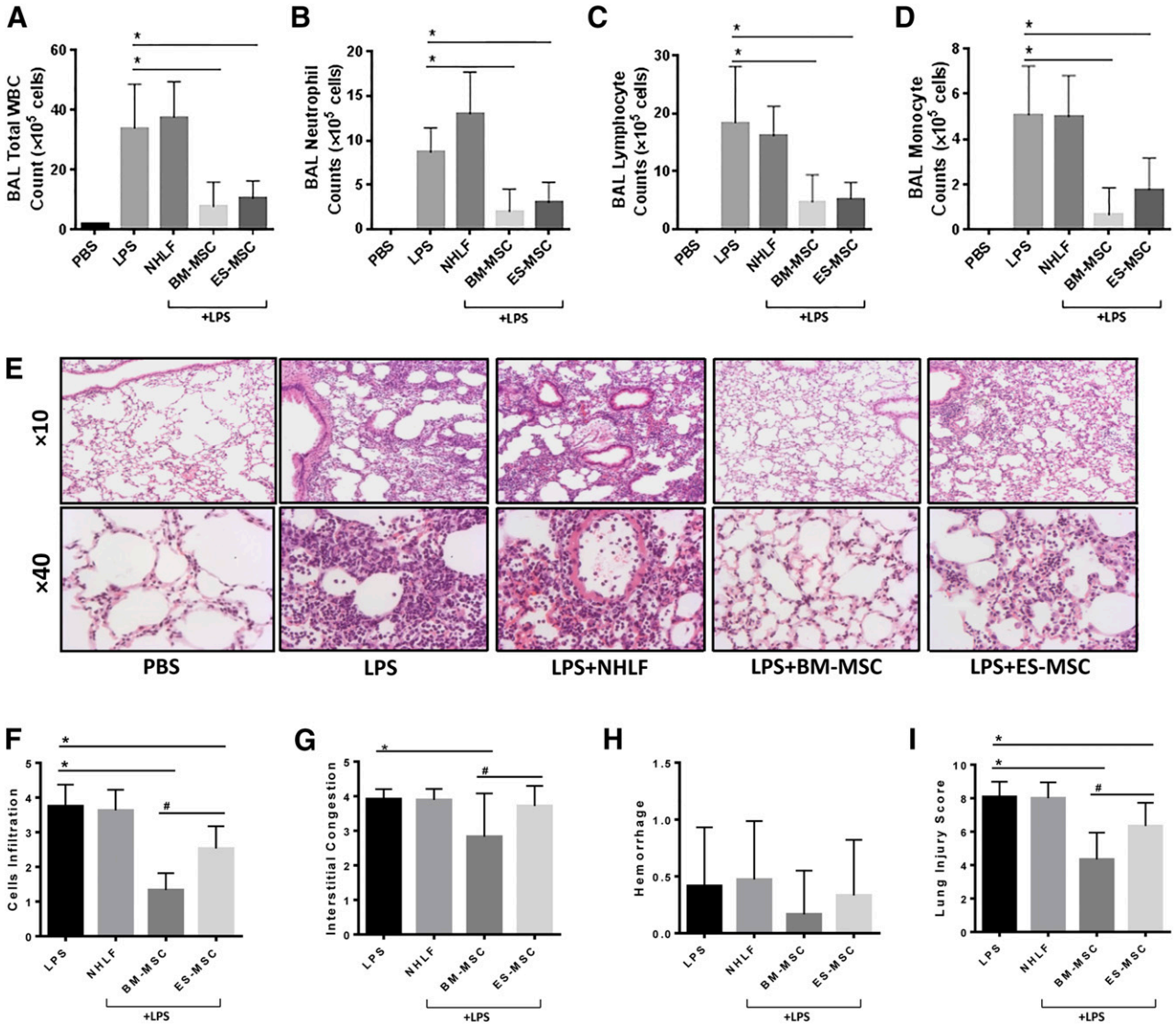

Figure 1. Effect of human BM-MSCs or ES-MSCs on influx of inflammatory cells in endotoxin-induced acute lung injury in mice. Both BM-MSCs and ES-MSCs have significant immunomodulatory properties. **(A–D):** Total counts of WBCs, neutrophils, lymphocytes, and monocytes were significantly elevated after intratracheal instillation of endotoxin compared with PBS control at 48 hours. Administration of BM-MSCs or ES-MSCs significantly reduced the influx of inflammatory cells in endotoxin-induced ALI in mice. Data are shown as mean ± SD; n = 6–7 for PBS, LPS + BM-MSCs or ES-MSCs or NHLF; n = 12 for endotoxin; ∗, p value is significant versus endotoxin-injured mice by analysis of variance (ANOVA; Bonferroni). **(E):** Intratracheal BM-MSCs also improved lung injury, as assessed by histologic examination. H&E staining of lung sections at 48 hours demonstrated a reduction in inflammatory cell influx, edema, and blood and reduced thickening of the interstitium in endotoxin-injured lungs treated with BM-MSCs. ES-MSCs had a partial effect on cellularity and no effect on interstitial congestion, leading to a higher lung injury score compared with BM-MSCs. NHLFs showed no therapeutic benefit. Images are ×10 and ×40 magnification. **(F–I):** Lung injury assessed by semiquantitative scoring. ∗, p value is significant versus endotoxin-injured mice by ANOVA (Bonferroni); #, p value is significant versus endotoxin-injured mice treated with ES-MSCs by ANOVA (Bonferroni). Abbreviations: BAL, bronchoalveolar lavage; BM-MSC, bone marrow-derived mesenchymal stem cell; ES-MSC, embryonic stem cell-derived mesenchymal stem cell; LPS, lipopolysaccharide; NHLF, normal adult human lung fibroblast; PBS, phosphate-buffered saline; WBC, white blood cell.

the group assignments, graded the level of lung injury using semiquantitative scoring as follows: infiltration or aggregation of inflammatory cells in air space or vessel wall: 1 = only wall, 2 = few cells (1–5 cells) in air space, 3 = intermediate, 4 = severe (air space congested); interstitial congestion and hyaline membrane formation: 1 = normal lung, 2 = moderate (<25% of lung section), 3 = intermediate (25%–50% of lung section), 4 = severe (>50% of lung section); and hemorrhage: 0 = absent, 1 = present. For each mouse, 20 fields of both lungs at ×20 view were examined [36].

Coculture of RAW264.7 Cells and MSC With Endotoxin

Mouse RAW264.7 cells (Sigma-Aldrich) were cocultured with either BM-MSCs or ES-MSCs in Transwell plates (100,000 cells in the upper chamber, 0.4-μm pore size; Costar, Corning, Acton, MA, http://www.corning.com/lifesciences) in the presence of endotoxin (1,000 ng/ml) at a concentration of 5×10^5 cells per well in Dulbecco's modified Eagle's medium without fetal bovine serum for 24 hours. The cell culture supernatants were then collected after coincubation to assay the levels of mouse MMP-9 with ELISA kits (R&D Systems).

Protein Permeability Across Human Lung Microvascular Endothelial Cells

The protein permeability assay was performed in a 24-well receiver plate with individual hanging cell culture inserts (Costar, Corning). The inserts contained 1-μm-pore size transparent polyethylene terephthalate membrane. Human lung microvascular endothelial cells (1×10^5 HMVEC-L; Lonza) were seeded onto the collagen-coated inserts with 1×10^5 BM-MSCs or 1×10^5 ES-MSCs in the bottom chamber. Once the endothelial monolayer had formed, the cell monolayer was treated with endotoxin (1,000 ng/ml). After treatment, high-molecular-weight fluorescein isothiocyanate-dextran (EMD Millipore, Billerica, MA, http://www.emdmillipore.com) was added on top of the cells, allowing the fluorescent molecules to pass through the endothelial cell monolayer at a rate proportional to the monolayer's permeability. The extent of permeability was determined by measuring the fluorescence of the receiver plate well solution. We also tested the effect of MMP-9 inhibitor I (EMD Millipore), a selective inhibitor of MMP-9, and TIMP-1 (R&D Systems), an endogenous MMP-9 inhibitor, on protein permeability.

Statistical Analysis

Comparisons between two groups were made using an unpaired *t* test. For comparisons between multiple groups, analysis of variance (ANOVA) with Bonferroni's correction was used. Analyses were done using SPSS software (IBM Corp., Armonk, NY, http://www-01.ibm.com/software/analytics/spss/) and GraphPad Prism software (GraphPad, Inc., San Diego, CA, http://www.graphpad.com). Data are presented as the mean $\pm$ SD.

RESULTS

Dose-Response Effect of Embryonic Stem Cell-Derived Mesenchymal Stem Cells on Endotoxin-Induced Acute Lung Injury

The ES-MSCs were morphologically similar to the BM-MSCs, appearing as spindle-shaped fibroblast-like cells (supplemental online Fig. 1A). In addition, the culture conditions and characterizations used to identify the cells as MSCs (supplemental online Tables 1 and 2) were similar. To determine the optimal therapeutic dose of ES-MSCs and compare with the effects to BM-MSCs, 3 different doses of ES-MSCs (7.5×10^4, 3.7×10^5, and 7.5×10^5 cells) were administered to endotoxin-injured mice. Administration of 7.5×10^5 ES-MSCs had the most significant effect in reducing the influx of total WBCs and neutrophils (supplemental online Fig. 1B) and inflammatory cytokines, TNF-α and MIP-2 (data not shown), in the BAL fluid at 48 hours and were similar to the effects with 7.5×10^5 BM-MSCs. In all subsequent studies, 7.5×10^5 was used for both ES-MSCs and BM-MSCs.

Therapeutic Effect of Bone Marrow- and Embryonic Stem Cell-Derived Mesenchymal Stem Cells on Endotoxin-Induced Acute Lung Injury in Mice

Intratracheal instillation of endotoxin induced a robust, but nonlethal, inflammatory response in the alveolus of mice at 48 hours. Administration of BM-MSCs or ES-MSCs simultaneously with endotoxin reduced the influx of inflammatory cells (Fig. 1A–1D) and decreased the secretion of the inflammatory cytokines, MIP-2 and TNF-α (Fig. 2), in the injured alveolus at 48 hours. Histologic examination revealed that administration of BM-MSCs reduced cellularity and interstitial thickening after endotoxin-induced ALI. In contrast, ES-MSCs had a partial therapeutic effect on cellularity and no effect on interstitial congestion, leading to a higher lung injury score compared with BM-MSCs (Fig. 1E–1I). No beneficial effect was found with administration of NHLFs (Figs. 1, 2).

Therapeutic Effect of Bone Marrow- and Embryonic Stem Cell-Derived Mesenchymal Stem Cells on Extravascular Lung Water and Protein Permeability in Endotoxin-Injured Mice

Intratracheal instillation of endotoxin increased the total extravascular lung water (EVLW), a measure of pulmonary edema, by approximately 100 μl, compared with the PBS control. Administration of BM-MSCs significantly reduced EVLW by 60%. However, surprisingly, administration of ES-MSCs had a minimal effect (Fig. 3B). The total protein levels in the BAL fluid, a marker of lung protein endothelial and epithelial permeability, were increased in the endotoxin-injured mice. Administration of BM-MSCs significantly reduced the BAL protein levels by 66%. However, similar to the results with EVLW, administration of ES-MSCs had no effect on restoring lung protein permeability (Fig. 3A). Administration of BM-MSCs reduced body weight loss by 43% compared with the endotoxin-injured mice (n = 7, 3.7 ± 1.0 for endotoxin-injured vs. 2.1 ± 1.4 for endotoxin plus BM-MSCs at 48 hours; p value was significant by ANOVA). ES-MSCs had no effect on restoring body weight (Fig. 3C).

Embryonic Stem Cell-Derived Mesenchymal Stem Cells Express High Levels of Matrix Metallopeptidase 9

To identify the differences between BM-MSCs and ES-MSCs, we measured the mRNA expression of key proteins secreted by MSCs involved in the therapeutic effect in ALI. Although ES-MSCs expressed higher levels of human Ang-1 and KGF than BM-MSCs, perhaps owing to its more primitive source of origin, the expression for both soluble factors was roughly similar between BM-MSCs and ES-MSCs by RT-PCR. We previously demonstrated that MSC Ang-1 secretion was involved in restoring lung protein permeability, and MSC KGF secretion was involved in enhancing alveolar fluid clearance (i.e., resolution of pulmonary edema fluid) after lung injury (supplemental online Fig. 2A) [37, 38]. We then screened for genes involved in barrier permeability. Compared with BM-MSCs, ES-MSCs expressed higher levels of human MMP-9 (supplemental online Fig. 2A); however, no differences was found in the mRNA expression of human TIMP-1, the endogenous inhibitor of MMP-9 (supplemental online Fig. 2B). Small significant differences were found in mRNA expression of other genes potentially involved in lung permeability during lung injury (e.g., vascular endothelial growth factor [VEGF], MMP-2, TIMP-2, TIMP-3), although not to the extent seen with MMP-9 (supplemental online Fig. 2A–C).

Matrix Metallopeptidase 9 and Tissue Inhibitor of Metallopeptidase 1 Levels in Endotoxin-Injured Mice Treated With Bone Marrow- and Embryonic Stem Cell-Derived Mesenchymal Stem Cells

The mouse MMP-9 level was undetectable in the uninjured mice. Intratracheal instillation of endotoxin significantly increased the level of mouse total MMP-9 in the BAL fluid (Fig. 4A). Compared with endotoxin-injured mice, treatment with BM-MSCs or ES-MSCs both decreased the mouse total MMP-9 BAL fluid level by 95% and 62%, respectively (Fig. 4A). However, the BM-MSCs further

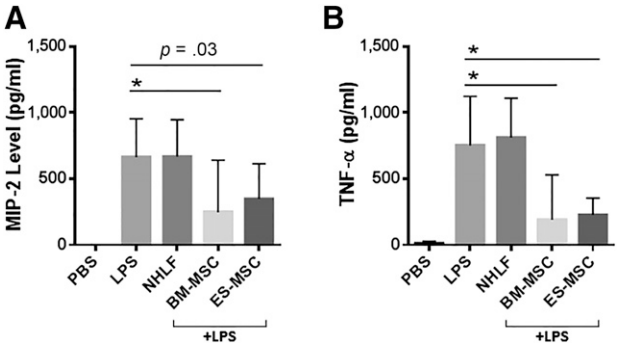

Figure 2. Effect of human BM-MSCs or ESC-MSCs on pro-inflammatory response to endotoxin-induced acute lung injury (ALI) in mice. **(A):** Both BM-MSCs and ES-MSCs significantly reduced MIP-2 levels within the BAL fluid in mice with endotoxin-induced ALI. Data are expressed as mean ± SD; $n = 6–7$ per group; ∗, p value is significant versus endotoxin-injured mice by analysis of variance (ANOVA; Bonferroni). **(B):** TNF-α levels in the BAL fluid were also significantly reduced in BM-MSC- or ES-MSC-treated mice. Data are expressed as mean ± SD; $n = 6–9$; ∗, p value is significant versus endotoxin-injured mice by ANOVA (Bonferroni). Abbreviations: BAL, bronchoalveolar lavage; BM-MSC, bone marrow-derived mesenchymal stem cell; ES-MSC, embryonic stem cell-derived mesenchymal stem cell; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein-2; NHLF, normal adult human lung fibroblast; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor-α; WBC, white blood cell.

decreased the mouse total MMP-9 levels compared with the levels in the injured mice treated with ES-MSCs. These beneficial effects were not observed in the injured mice treated with NHLFs. The levels of pro-MMP-9 and active MMP-9 in the BAL fluid showed the same pattern as total MMP-9 in terms of the effects with BM-MSCs or ES-MSCs (Fig. 4B, 4C). The level of mouse TIMP-1, an endogenous MMP-9 inhibitor, was significantly increased in endotoxin-injured mice compared with the PBS control (Fig. 4D). Administration of BM-MSCs resulted in a decrease in TIMP-1 levels at 48 hours by 66%. However, administration of ES-MSCs or NHLFs had a minimal effect on TIMP-1 release (Fig. 4D). In endotoxin-injured mice, the MMP-9/TIMP-1 ratio was significantly increased by eightfold, a large imbalance favoring protease activity at 48 hours. Although both BM-MSCs and ES-MSCs decreased the MMP-9/TIMP-1 ratio 1.4-fold and 3.5-fold, respectively (Fig. 4E), the effect was more pronounced with BM-MSCs.

To determine whether MSC secretion of MMP-9 (donor MMP-9) was involved in the increase in permeability, the human MMP-9 levels were measured in the endotoxin-injured mice treated with BM-MSCs or ES-MSCs. The human MMP-9 level was significantly higher with ES-MSC administration than with BM-MSC administration (Fig. 4F). However, the level of human MMP-9 was >1,000 lower than that of mouse MMP-9 (recipient MMP-9). The low level of human MMP-9 might not be as functionally relevant in increasing permeability but in triggering the secretion of MMP-9 in recipient mice.

Effect of Bone Marrow- and Embryonic Stem Cell-Derived Mesenchymal Stem Cells on Expression of Mouse Matrix Metallopeptidase 9 in Coculture Experiments With RAW264.7 Cells

To determine whether donor human MSCs were able to regulate the production of recipient mouse MMP-9 by stimulated macrophages, we cocultured RAW264.7 cells, a mouse macrophage cell line, with BM-MSCs or ES-MSCs in the presence of endotoxin for 24 hours. Similar to the in vivo ALI model, coculture with ES-MSCs led to significantly higher levels of mouse MMP-9 compared with those with coculture with BM-MSCs (Fig. 4G).

Effect of Bone Marrow- and Embryonic Stem Cell-Derived Mesenchymal Stem Cells on Protein Permeability Across Primary Culture of Human Lung Microvascular Endothelial Cells Injured by Endotoxin

To investigate the role of MSCs on protein permeability, we exposed primary cultures of HMVEC-L to endotoxin with or without BM-MSCs or ES-MSCs in the lower compartment of the Transwell plate (Costar, Corning). Exposure of HMVEC-L to endotoxin increased protein permeability to dextran by 70% over 12 hours (Fig. 5A). The addition of BM-MSCs grown on the bottom chamber of the Transwell plate (Costar, Corning) significantly reduced the increase in protein permeability caused by endotoxin. However, coculture with ES-MSCs on the bottom chamber had no beneficial effect on protein permeability (Fig. 5A). To understand the importance of MMP-9 secretion on protein permeability in HMVEC-L injured with endotoxin, we simultaneously added MMP-9 inhibitor I, a selective inhibitor of MMP-9, or TIMP-1, an endogenous MMP-9 inhibitor, with or without ES-MSCs. In both conditions, the addition of an MMP-9 inhibitor or TIMP-1 significantly reduced protein permeability across HMVEC-L injured by endotoxin (Fig. 5A, 5B).

Discussion

The major findings of the present study are as follows. First, treatment with BM-MSCs or ES-MSCs markedly decreased the influx of inflammatory cells (total white blood cells, neutrophils, lymphocytes, and monocytes) and cytokines/chemokines (MIP-2 and TNF-α) after endotoxin-induced ALI in mice (Figs. 1, 2). Second, BM-MSC treatment decreased EVLW, a measure of pulmonary edema, and reduced the total protein levels in the BAL fluid, a measure of lung protein permeability, after endotoxin-induced ALI. However, ES-MSC treatment did not result in these protective effects (Fig. 3). Third, compared with BM-MSCs, ES-MSCs expressed higher mRNA levels of human MMP-9, an extracellular matrix enzyme previously found to increase lung protein permeability in preclinical models of ARDS (supplemental online Fig. 2). Fourth, compared with BM-MSCs, ES-MSC-treated mice had higher levels of total and active mouse MMP-9 and a higher MMP-9/TIMP-1 ratio in the injured alveolus after endotoxin-induced ALI (Fig. 4). Fifth, in cultures of RAW264.7 cells, a mouse macrophage cell line, BM-MSCs downregulated the secretion of mouse MMP-9 induced by endotoxin. In contrast, ES-MSC administration had no beneficial effect (Fig. 4). Finally, in primary cultures of human lung microvascular endothelial cells injured with endotoxin, BM-MSC treatment restored protein permeability but ES-MSC administration had no effect. The addition of MMP-9 neutralizing antibody or TIMP-1 with or without ES-MSCs restored protein permeability, demonstrating the importance of MMP-9 on lung endothelial permeability (Fig. 5).

MSCs generated from human embryonic stem cells (ESCs) or other primitive cells with stem cell markers (i.e., chorionic stem cell) have generated interest regarding the potential for a more potent cell-based therapeutic agent compared with BM-MSCs owing to the importance of secreted growth factors for its effect. A recent study demonstrated that "younger" MSCs had more anti-inflammatory properties than did "older" MSCs [39]. To test this hypothesis, we studied the therapeutic effect of bone marrow-derived MSCs and MSC generated from human ESCs (H9-MSCs) in an endotoxin-induced ALI model [33].

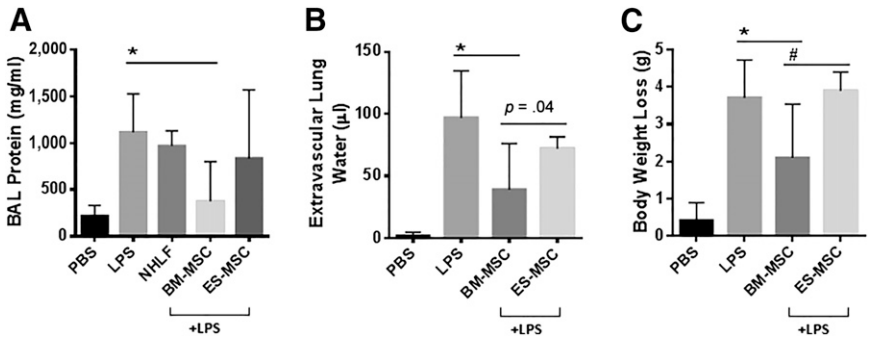

Figure 3. Effect of human BM-MSCs or ES-MSCs on protein permeability and extravascular lung water in endotoxin-induced ALI in mice. **(A):** Intratracheal BM-MSCs decreased total protein levels in the BAL fluid of endotoxin-injured mice, but ES-MSCs did not show the same beneficial effects. Data are shown as mean ± SD; $n = 6–7$; ∗, p value is significant versus endotoxin-injured mice by analysis of variance (ANOVA; Bonferroni). **(B):** Intratracheal BM-MSCs significantly reduced extravascular lung water in mice with endotoxin-induced ALI, but instillation of ES-MSCs had minimal effects. Data are shown as mean ± SD; $n = 6–7$; ∗, p value is significant versus endotoxin-injured mice by ANOVA (Bonferroni). **(C):** Intratracheal BM-MSCs decreased the body weight loss of endotoxin-injured mice at 48 hours; ES-MSC administration had no effect. Data shown as mean ± SD; $n = 6–7$; ∗, p value is significant versus endotoxin-injured mice by ANOVA (Bonferroni); #, p value is significant versus endotoxin-injured mice treated with ES-MSCs by ANOVA (Bonferroni). Abbreviations: BAL, bronchoalveolar lavage; BM-MSC, bone marrow-derived mesenchymal stem cell; ES-MSC, embryonic stem cell-derived mesenchymal stem cell; LPS, lipopolysaccharide; NHLF, normal adult human lung fibroblast; PBS, phosphate-buffered saline; WBC, white blood cell.

Both BM-MSCs and ES-MSCs had a profound anti-inflammatory effect in endotoxin-induced ALI in mice by preventing the influx of inflammatory cells and decreasing the level of chemokines/cytokines in the injured alveolus. However, unlike BM-MSCs, ES-MSCs did not restore lung protein permeability or prevent pulmonary edema formation after injury (Figs. 1–3). To understand the effect, we searched for differences in mRNA expression for key soluble factors between BM-MSCs and ES-MSCs, such as KGF, an epithelial-specific growth factor involved in alveolar epithelial type II cell repair [40], and Ang-1, a ligand for the endothelial Tie2 receptor and a known vascular stabilization factor that reduces endothelial permeability [37]. We previously demonstrated that KGF secretion by MSCs restored alveolar fluid clearance (i.e., pulmonary edema resolution) by trafficking the key epithelial sodium channel involved in fluid transport to the apical membrane of alveolar epithelial type II cells [38]. We, and other investigators, also demonstrated that Ang-1 secretion by MSCs prevented "actin stress fiber" formation and protein permeability across both lung epithelial and endothelial cells after injury [37]. Both BM-MSCs and ES-MSCs expressed roughly similar mRNA levels for both Ang-1 and KGF (supplemental online Fig. 2C).

We then screened for the expression of genes expressed by ESCs involved with growth, potentially involved in permeability. ES-MSCs expressed higher mRNA levels of both MMP-9 and VEGF than did BM-MSCs, although the effect was more pronounced with MMP-9 expression. No difference was found in mRNA expression of TIMP-1, the endogenous inhibitor of MMP-9 (supplemental online Fig. 2C). Proteases of the matrix metalloproteinase family are involved in the breakdown of extracellular matrix (ECM) in normal physiological processes, such as embryonic development and growth, reproduction, angiogenesis, and so forth. A high expression of MMPs is needed to maintain the self-renewal property of ESCs [41]; both the stiffness of the ECM and the surrounding niche are important in ESC proliferation and differentiation [42, 43]. MMP-9 is an important enzyme involved in the induction of stiffness of the ECM and is highly expressed in undifferentiated ESCs [43]. The high expression of MMP-9 might be a phenotype inherited when ESCs are differentiated to MSCs.

Under pathological conditions, MMPs are overexpressed or oversecreted to facilitate clearance of foreign and noxious agents; however, excess MMP secretion can lead to the breakdown of the ECM, disrupt resident cells, and stimulate further inflammation.

Of the MMPs secreted during lung injury such as ARDS, MMP-9 is a key enzyme, produced by inflammatory cells, including neutrophils, monocytes, macrophages, lymphocytes, and eosinophils, and alveolar epithelial and endothelial cells involved in the degradation of the extracellular matrix [44, 45]. To determine the significance of human MMP-9 expression by ES-MSCs, we measured mouse MMP-9 and TIMP-1 levels in the BAL fluid after endotoxin-induced ALI at 48 hours. Intratracheal endotoxin dramatically increased mouse MMP-9 secretion in the injured alveolus. Administration of both BM-MSCs and ES-MSCs reduced both total and active mouse MMP-9 levels. However, with ES-MSC administration, the effect was significantly less than that with BM-MSC administration. Because TIMP-1 is an endogenous inhibitor of MMP-9, the ratio of MMP-9/TIMP-1 is a key factor in the control of MMP activity. The MMP-9/TIMP-1 ratio in normal mice was less than 1. When exposed to endotoxin, the ratio increased by eightfold, favoring increased protease activity. Administration of both BM-MSCs and ES-MSCs reduced the ratio of MMP-9/TIMP-1. However, with ES-MSC administration, the effect was again significantly less than that with BM-MSCs (Fig. 4). Upregulated MMP-9 activity during lung injury results in the breakdown of the extracellular matrix and the release of matrix-bound growth factors (i.e., VEGF), which in turn facilitate increased barrier permeability.

To validate the in vivo study, we tested the effect of both BM-MSCs and ES-MSCs in the production of mouse MMP-9 by RAW264.7 cells exposed to endotoxin. During ARDS, macrophages and epithelial and endothelial cells are major sources of MMP-9 secretion. Endotoxin exposure increased the mouse MMP-9 levels dramatically, and coculture with either MSC type blunted the effect. However, compared with BM-MSCs, coculture of ES-MSCs with endotoxin-exposed RAW264.7 cells resulted in significantly higher levels of mouse MMP-9 than did cocultures with BM-MSCs (Fig. 4G).

The barrier integrity of lung microvascular endothelium is important for the prevention of the influx of protein-rich fluid and

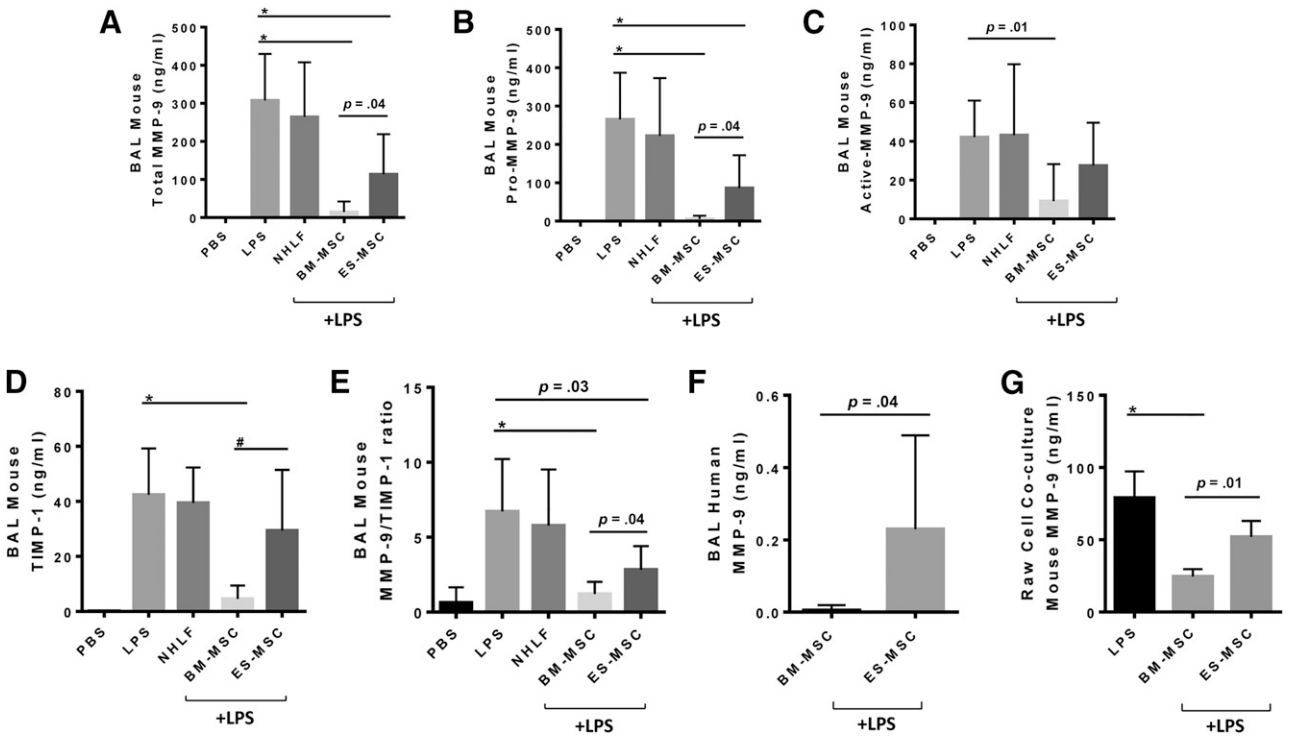

Figure 4. Effect of human BM-MSCs and ES-MSCs on the expression of human and mouse MMP-9 and TIMP-1 levels. **(A–C):** Endotoxin instillation increased BAL fluid levels of mouse total MMP-9 (both pro-MMP-9 and active-MMP-9). Mouse MMP-9 level was significantly reduced in mice treated with BM-MSC or ES-MSC. However, endotoxin-injured mice treated with ES-MSC had significantly higher levels of MMP-9 compared with mice treated with BM-MSCs. **(D–E):** The overall ratio of mouse MMP-9/mouse TIMP-1 was significantly higher for ES-MSC-treated mice than for the BM-MSC-treated group. **(F):** Human MMP-9 level in the BAL fluid of ES-MSC-treated mice was also significantly higher than that of the BM-MSC-treated mice. **(G):** When cocultured with RAW264.7 cells injured with endotoxin, ES-MSCs reduced mouse MMP-9 levels compared with endotoxin injury but to a lesser extent than in RAW264.7 cells treated with BM-MSCs. Data are expressed as mean ± SD; $n = 6–7$; ∗, p value is significant versus endotoxin-injured mice; #, p value is significant versus endotoxin-injured mice treated with ES-MSCs by analysis of variance (ANOVA; Bonferroni). Abbreviations: BAL, bronchoalveolar lavage; BM-MSC, bone marrow-derived mesenchymal stem cell; ES-MSC, embryonic stem cell-derived mesenchymal stem cell; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein-2; MMP-9, matrix metallopeptidase-9; NHLF, normal adult human lung fibroblast; PBS, phosphate-buffered saline; TIMP, tissue inhibitor of metallopeptidase; WBC, white blood cell.

inflammatory cells from the circulation, which can aggravate the ability of the lung epithelium to remove pulmonary edema fluid. In primary cultures of normal human lung microvascular endothelial cells injured with endotoxin, the addition of BM-MSCs grown on the bottom chamber of the Transwell plate (Costar, Corning) reduced the permeability to dextran. However, coculture of ES-MSCs had no effect on restoring dextran permeability. To further understand the role of MMP-9, we tested the effect of MMP-9 inhibitor I, a selective inhibitor of MMP-9, or TIMP-1 on permeability with or without ES-MSCs. Inhibiting or blocking MMP-9 activity had a protective effect on the increased protein permeability induced by endotoxin, suggesting that MMP-9 plays a critical role in mediating endothelial permeability (Fig. 5).

The present study had some limitations. First, although BM-MSCs and ES-MSCs demonstrated different biological activities in an endotoxin-induced ALI model, both adult stem cells fulfilled the criteria for MSCs as defined by the International Society for Cellular Therapy. Whether additional surface markers exist that can differentiate BM-MSCs from ES-MSCs or MSCs derived from other tissue sources remains unknown. This study highlights, perhaps, a need for a disease-specific functional assay for MSC. Second, compared with BM-MSCs, ES-MSCs expressed higher levels of human MMP-9 and induced higher levels of mouse MMP-9 after endotoxin-induced ALI. Whether the human MMP-9 secreted by ES-MSCs was directly responsible for the increase in mouse MMP-9 and the subsequent increase in lung water and protein permeability remains unknown. Additional studies are needed. However, from the in vivo results, it appears unlikely that the increase in permeability resulted from inflammatory cytokines or chemokines, given the effect of both BM-MSCs and ES-MSCs on MIP-2 and TNF-α secretion, and is unlikely to have resulted from a decrease in the secretion of antipermeability soluble factors such as Ang-1 by either BM- or ES-MSCs. Finally, although ES-MSCs had a detrimental effect on lung permeability, a major functional parameter involved in the pathogenesis of ARDS, it is unknown whether ES-MSCs might be superior to BM-MSCs in other parameters such as inflammation. Recently, Bustos et al. found that aging MSCs had an impaired migration capability and anti-inflammatory response [39]. Gruenloh et al. also recently demonstrated that ES-MSCs (H9-MSCs) retained the same cell migration capability in hypoxic conditions as adult MSCs, which might be diminished with older adult MSCs [33]. ES-MSCs have been generated by a number of research groups using different methods and different human ESC lines, including H1-hES, H7-hES, H9-hES, hES-MP002.5, and HSF-6 hES [26–34]. It remains unknown whether MSCs derived from different ES cell lines and/or differentiation methods function similarly; this is also an important issue for adult

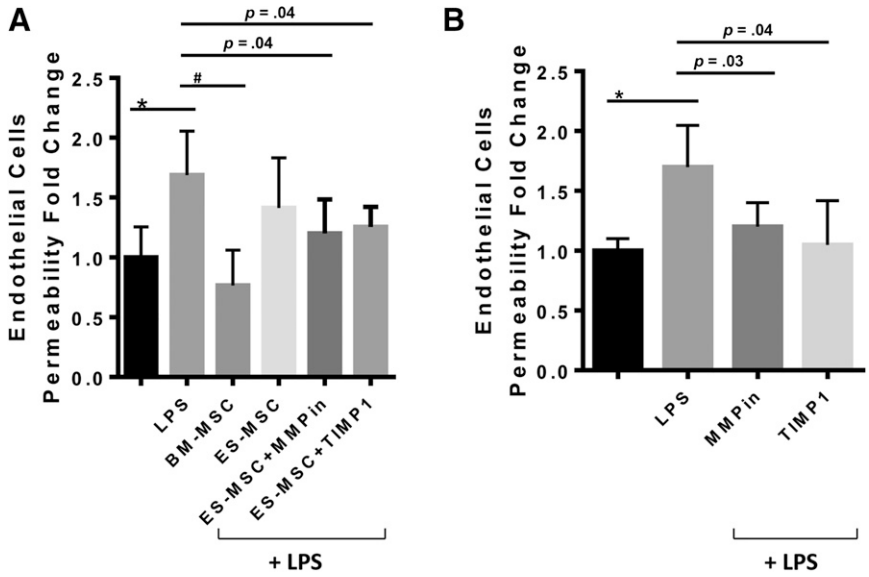

Figure 5. Effect of human BM-MSCs or ES-MSCs on lung endothelial protein permeability in primary cultures of human lung microvascular endothelial cells. **(A):** Endotoxin exposure increased protein permeability across primary cultures of human lung microvascular endothelial cells. Coculture of BM-MSCs partially restored endothelial permeability to protein. In contrast, coculture of ES-MSCs had no effect. Data are normalized to untreated endothelial cells and expressed as mean ± SD; $n = 5–6$ wells per condition. **(A, B):** Treatment with MMPin or recombinant TIMP-1 with or without ES-MSC significantly restored the increase in protein permeability induced by endotoxin. Data are normalized to untreated endothelial cells and expressed as mean ± SD $n = 3–5$ wells per condition; ∗, p value is significant versus PBS-treated human lung microvascular endothelial cells (HMVEC-L) by analysis of variance (ANOVA; Bonferroni); #, p value is significant versus endotoxin-injured HMVEC-L by ANOVA (Bonferroni). Abbreviations: BM-MSC, bone marrow-derived mesenchymal stem cell; ES-MSC, embryonic stem cell-derived mesenchymal stem cell; LPS, lipopolysaccharide; MMPin, matrix metallopeptidase-9 inhibitor; NHLF, normal adult human lung fibroblast; PBS, phosphate-buffered saline; TIMP-1, tissue inhibitor of metallopeptidase-1; WBC, white blood cell.

MSCs [46]. Additional studies are clearly necessary to determine whether all ES-MSCs behave similarly to H9-MSCs in models of ALI.

Conclusion

We studied both BM-MSCs and ES-MSCs as a therapeutic agent in an endotoxin-induced ALI model in mice. The administration of ES-MSCs behaved similarly to BM-MSCs by markedly decreasing the inflammatory response induced by endotoxin. However, unlike BM-MSCs, ES-MSCs had no protective effect on lung water and protein permeability, in part owing to an increase in the expression of MMP-9 by ES-MSCs. In patients with ARDS, increased lung water or impaired alveolar fluid clearance (i.e., no resolution of pulmonary edema fluid) has been associated with higher mortality rates [47]. Although ES-MSCs might ultimately be found to have superior properties compared with BM-MSCs, such as better anti-inflammatory properties, the results highlight the need for a disease-specific potency assay for stem cell-based therapy.

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Author Contributions

Q.H.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; Y.-g.Z., A.M., S.G., T.L., and F.X.: collection and/or assembly of data; J.-W.L.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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