Journal of Cellular and Molecular Medicine
A JOURNAL OF TRANSLATIONAL MEDICINE

J. Cell. Mol. Med. Vol 16, No 2, 2012 pp. 376-385

A therapeutic role for mesenchymal stem cells in acute lung injury independent of hypoxia-induced mitogenic factor

Lin Song [a, #], Jinfu Xu [b, #], Jieming Qu [c, *], Yin Sai [d], Chunmei Chen [d], Long Yu [d], Dechun Li [e], Xuejun Guo [f]

[a] *Department of Pulmonary Medicine, Zhongshan Hospital, Shanghai Medical College, Fudan University, Shanghai, China*
[b] *Department of Pulmonary Medicine, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China*
[c] *Department of Pulmonary Medicine, Huadong Hospital, Shanghai Medical College, Fudan University, Shanghai, China*
[d] *State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai, China*
[e] *Department of Internal Medicine, Division of Pulmonary, Critical Care, and Sleep Medicine, Saint Louis University School of Medicine, St. Louis, MO, USA*
[f] *Department of Pulmonary Medicine, Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China*

Received: November 17, 2010; Accepted: March 28, 2011

Abstract

Bone marrow mesenchymal stem cells (BM-MSCs) have therapeutic potential in acute lung injury (ALI). Hypoxia-induced mitogenic factor (HIMF) is a lung-specific growth factor that participates in a variety of lung diseases. In this study, we evaluated the therapeutic role of BM-MSC transplantation in lipopolysaccharide (LPS)- induced ALI and assessed the importance of HIMF in MSC transplantation. MSCs were isolated and identified, and untransduced MSCs, MSCs transduced with null vector or MSCs transduced with a vector encoding HIMF were transplanted into mice with LPS-induced ALI. Histopathological changes, cytokine expression and indices of lung inflammation and lung injury were assessed in the various experimental groups. Lentiviral transduction did not influence the biological features of MSCs. In addition, transplantation of BM-MSCs alone had significant therapeutic effects on LPS-induced ALI, although BM-MSCs expressing HIMF failed to improve the histopathological changes observed with lung injury. Unexpectedly, tumour necrosis factor α levels in lung tissues, lung oedema and leucocyte infiltration into lungs were even higher after the transplantation of MSCs expressing HIMF, followed by a significant increase in lung hydroxyproline content and α-smooth muscle actin expression on day 14, as compared to treatment with untransduced MSCs. BM-MSC transplantation improved LPS-induced lung injury independent of HIMF.

Keywords: mesenchymal stem cells • acute lung injury • transplantation • hypoxia-induced mitogenic factor • fibrosis

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening conditions characterized by high mortality, rapid onset of respiratory failure following extensive inflammation, lung oedema and alveolar haemorrhage [1]. In ALI and ARDS, inflammatory cells migrate into the lung parenchyma and release mediators that destroy bacteria but also cause tissue disruption and lung injury [2]. Although supportive therapies for these conditions exist, no efficient therapies or targeting strategies have been developed. Mesenchymal stem cell (MSC) transplantation has recently been proposed as a potential new alternative therapy for ALI. Reportedly, systemic transfer of MSCs effectively reduced bleomycin-induced ALI and fibrosis, likely through

[#]Both authors contributed equally to this article.
*Correspondence to: Prof. Jieming QU,
Department of Pulmonary Medicine, Huadong Hospital, Shanghai Medical College, Fudan University, Shanghai 200040, China.
Tel.: 86-21-62483180-99
Fax: 86-21-62484981
E-mail: jmqu64@yahoo.com.cn

doi: 10.1111/j.1582-4934.2011.01326.x

down-regulation of nitric oxide metabolites and pro-inflammatory and angiogenic cytokines [3]. In addition, recent work has also demonstrated the derivation of pulmonary progenitor cells such as type II pneumocytes from embryonic stem cells (hESCs) [4], as well as integrin-dependent differentiation and adhesion of stem cells in lung tissue [5].

Hypoxia-induced mitogenic factor (HIMF), an autocrine and paracrine cytokine primarily expressed in airway and alveolar epithelial cells, plays a number of roles in lung disease, including lung cell proliferation and development, in addition to mitogenic, angiogenic and vasoconstrictive effects [6–8]. Furthermore, HIMF has anti-apoptotic functions that can protect against the development of lipopolysaccharide (LPS)-induced ALI through the reservation of surfactant protein C production [9], although previous studies have shown that HIMF can promote cell proliferation, migration and production of vascular endothelial growth factor and monocyte chemotactic protein-1 in pulmonary endothelial cells, as well as the production of reactive oxygen species in murine monocyte/macrophage cells. In addition, HIMF up-regulates pro-angiogenic and pro-inflammatory factors, recruits inflammatory cells into the lungs and promotes inflammation in mice with pulmonary hypertension [10].

In the present study we sought to investigate the therapeutic role of bone marrow MSC (BM-MSC) transplantation in LPS-induced ALI and fibrosis and to explore the potential additive and/or synergistic effects of HIMF on BM-MSC transplantation. First, we transplanted BM-MSCs without any manipulation to prevent LPS-induced lung oedema and inflammation from developing. Second, in order to maintain the consistent production of HIMF, BM-MSCs were transfected with virus encoding HIMF or other vectors, then transplanted to treat LPS-induced lung injury and fibrosis.

Materials and methods

Isolation, culture and identification of BM-MSCs

Adult BALB/c mice were obtained from Slac Laboratories, Shanghai, China. The experimental protocol was approved by the Committee of Animal Care and Use of Fudan University, according to National Institutes of Health guidelines. BM cells were collected as previously described [11]. Briefly, BM-MSCs were obtained from the femurs and tibias of 6-week-old BALB/c mice and resuspended at 10^6 cells/ml in DMEM/F12 media containing 10% foetal bovine serum (Gibco, Grand Island, NY, USA). BM-MSCs at passages 4–6 were harvested for flow cytometry analysis, and stained with fluorescein isothiocyanate-, phycoerythrin- or phycoerythrin-cy5-labelled antibodies against CD14, CD44, CD105, CD19, CD73, CD29, CD34, CD45 and major histocompatibility complex (MHC)-II (eBioscience, Inc., San Diego, CA, USA). BM-MSCs from 4 to 6 passages were plated at 10^5 cells/cm^2 for adipogenic, osteogenic and chondrogenic differentiation experiments, as reported previously [11].

Lentiviral transduction and identification of MSCs

The HIMF gene was PCR subcloned from the pcDNA3.1-HIMF plasmid, a kind gift from Professor Dechun Li. High titre lentivirus vectors encoding enhanced green fluorescence protein (eGFP) or the HIMF gene were provided by Genechem Co. Ltd. (Shanghai, China). Passage 4–6 BM-MSCs were plated at 10^5/ml and transduced with lentivirus vector at different multiplicity of infection (MOI) levels.

HIMF gene and protein expression in BM-MSCs

Transduced BM-MSCs were harvested at designated time-points and total RNA was isolated, extracted and reverse transcribed (RT; Takara, Tokyo, Japan). Primers used for amplification HIMF were: sense 5′TATCCCTC-CACTGTAACGAA3′; and antisense 5′GTGGTCCAGTCAACGAGTAA3′. RT-PCR was carried out using the PrimeScript™ RT-PCR Kit from Takara. Amplified DNA products were run on a 2% agarose gel and bands were visualized using ethidium bromide. Lentivirally transduced BM-MSCs were lysed in buffer and the lysates were separated by precast polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). HIMF was detected with a 1:1000 dilution of anti-HIMF antiserum (a gift from Professor Dechun Li) and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An enhanced chemiluminescence substrate kit was used for the chemiluminescent detection of signals with autoradiography film.

Experimental design

The experimental design is outlined in Figure 1. The first part of the experiment was to determine the therapeutic effects of MSC transplantation in LPS-induced lung injury. Animals were injected intranasally with 10 μg/g body weight of LPS (*Escherichia coli*, Serotype 055:B5; Sigma-Aldrich, St. Louis, MO, USA), followed by an intravenous injection of MSCs at a concentration of 5×10^5/ml or the same volume of phosphate-buffered saline (PBS) as controls. Mice were terminated by anaesthesia overdose on days 0, 3, 7 and 14 after transplantation ($n = 6$–8 mice/time-point/group) to collect tissue for analysis. Lung wet:dry (W/D) ratio, myeloperoxidase (MPO) activity, cytokine expression and pathology were measured. In the second part of the experiment, we investigated whether HIMF treatment had an additive effect on MSC therapy. Animals were challenged with LPS and transplanted with MSCs transduced with empty control vector (MSCs-null vector) or vector encoding HIMF (MSCs-HIMF) at a concentration of 5×10^5/ml. Samples were also collected on days 0, 3, 7 and 14 after transplantation ($n = 6$–8 mice/time-point/group) for analysis.

Total protein in bronchoalveolar lavage (BAL) fluid, lung wet/dry ratio and MPO activity

Lungs of ALI mice were lavaged three times with 1 ml of cold saline. BAL fluid was collected and centrifuged at 4°C and 1000 × *g*, and the supernatant was collected for analysis of total protein and cytokine levels. The concentration of total protein in BAL fluid was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The lung W/D ratio was measured by dividing the wet weight by the dry weight. MPO activity was measured according to the manufacturers' instructions (Nanjing Jiancheng Biological Engineering Institute, Jiangsu, China).

Measurement of tumour necrosis factor α (TNF-α) and interleukin 10 (IL-10) in lung homogenates

Lung homogenate levels of the cytokines TNF-α and IL-10 were measured with murine cytokine-specific Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) as previously described [12]. Lungs were homogenized and all results were normalized (25 mg of total protein per sample) to individual protein concentrations measured from lung homogenate samples by bicinchoninic acid protein assay (Pierce). TNF-α and IL-10 mRNA levels in lung homogenates were measured by quantitative real-time PCR using SYBR Green PCR Mix (Takara). Primers used were: TNF-α, sense 5′-GACCCAGTGTGGGAAG-3′; and antisense 5′-GGTTCAGTGATGTAGCGA-3′; IL-10, sense 5′GGACAACATACTGCTAACCGACTC3′; and antisense 5′CCGCATCCTGAGGGTCTTC3′; β-actin, sense 5′-GTGGGCCGCTCTAG-GCACCAA-3′; and antisense 5′-CTCTTTGATGTCACGCACGATTTC-3′. The PCR conditions for amplification of TNF-α, IL-10 and β-actin were initially denaturated at 95°C for 10 sec., then subjected to 40 cycles at 94°C for 5 sec., 60°C for 30 sec.

Histological examination and immunohistochemical staining for α-smooth muscle actin (SMA) and hydroxyproline quantification

Tissue sections were embedded in paraffin and prepared for haematoxylin–eosin staining. Lung sections were microscopically examined by a board-certified pathologist blind to the treatment conditions according to a standard, previously described method [13]. A total lung injury score was calculated as the sum of the four criteria at an average of 10 slices. Intra-alveolar septal thickness was measured in different groups. Paraffin-embedded lung sections were used for immunocytochemistry *via* anti-α-SMA antibody (Sigma-Aldrich) at day 14. The total hydroxyproline content of the lung tissue was measured as an assessment of lung collagen content after homogenization of lung tissue in cold PBS according to the manufacturers' instructions (Nanjing Jiancheng Biological Engineering Institute).

Statistical analysis

Data are represented as means ± S.E.M. Differences between groups were assessed using analysis of variance. A value of $P < 0.05$ was considered statistically significant. Analyses were done using SPSS 11.5 software.

Results

MSC isolation, identification and transduction

After passaging, MSCs took on a spindle-shaped fibroblastic morphology. Flow cytometric analysis revealed that over 95% of passages 4–6 MSCs expressed CD105, CD73, CD29 and CD44, and were negative for CD45, CD34, CD14, CD19 and MHC-II (Fig. 2), consistent with the current criteria [14]. Passage 4–6 MSCs were tested for pluripotency. When subjected to osteogenic, adipogenic and chondrogenic media, MSCs succeeded in differentiating into osteoblasts, adipocytes and chondrocytes. Oil red O staining was used for adipocyte staining, toluidine blue for chondrocyte staining and alkaline phosphatase and Alizarin staining was used to identify osteoblasts in the corresponding media (Fig. 2). When MSCs were transduced with lentiviral vectors at various MOIs, the majority of adherent cells were GFP^+ after 96 hrs. Most transduced MSCs continuously adhered to the tissue culture dish and appeared to be viable (Fig. 3). Five days after transduction (MOI = 5) MSCs-null vector and MSCs-HIMF cells were prepared for transplantation, as HIMF was successfully expressed in transduced MSCs and detected by RT-PCR and Western blot (Fig. 3).

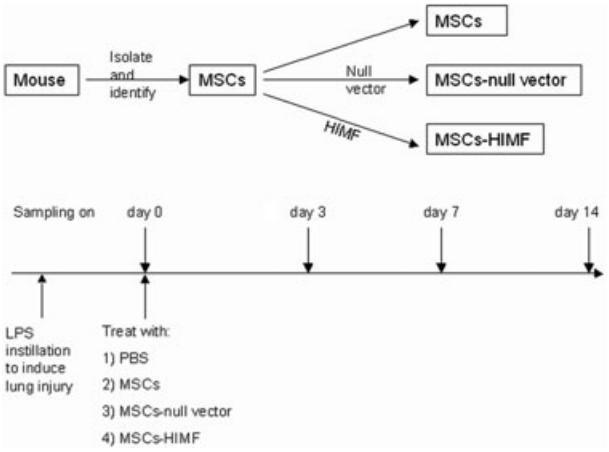

Fig. 1 Mice were treated with LPS to induce lung injury, followed by intravenous injection simultaneously with PBS, cultured MSCs, MSCs-null vector or MSCs-HIMF. Mice were then sacrificed on days 0, 3, 7 and 14 to evaluate the therapeutic efficacy.

MSC transplant improved LPS-induced lung injury independent of HIMF

The severity of LPS-induced lung injury was evaluated using a semi-quantitative histopathology scoring system. Lung injury occurred after LPS challenge, with the peak of injury observed on 3 and 7 days after treatment with PBS (Fig. 4A). The severity of LPS-induced lung injury was slightly improved 3 days after MSC transplantation and significantly improved after 7 days ($^*P < 0.05$). Transplantation of MSCs-null vector resulted in significant effects after 7 days, similar to treatment with MSCs alone (Fig. 4B), whereas treatment with MSCs-HIMF did not result in additional therapeutic effects. Unexpectedly, the level of injury in animals treated with MSCs-HIMF was significantly higher than those treated with PBS, MSCs alone or MSCs-null vector ($^*P < 0.05$, Fig. 4B).

Histological assessment of lung sections after administration of LPS revealed evidence of marked inflammatory infiltration, interalveolar septal thickening and alveolar congestion and haemorrhage in animals challenged with LPS and treated with PBS.

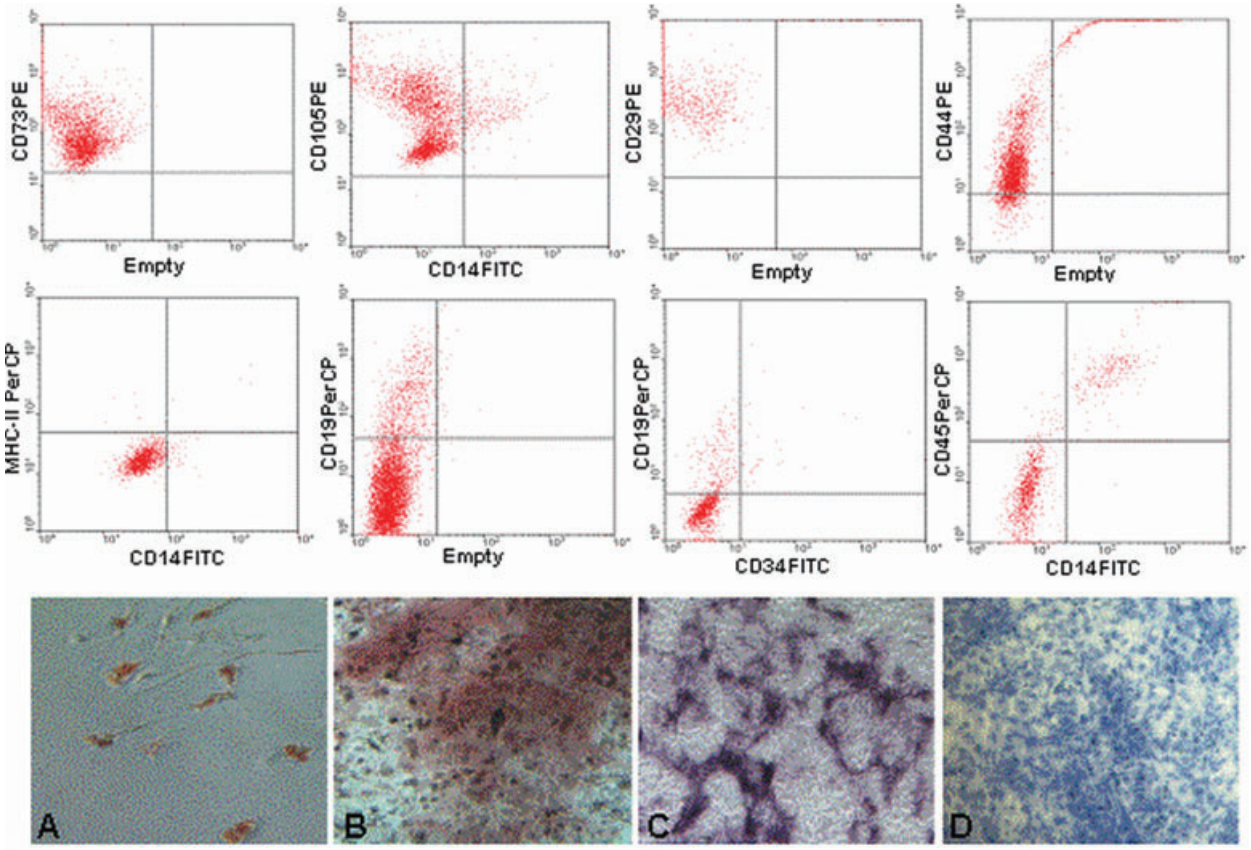

Fig. 2 *In vitro* identification of MSCs. FACS analysis of MSCs expressing CD105, CD73, CD29 and CD44, but not the typical haematopoietic or endothelial markers CD45, CD34, CD14, CD19 and MHC-II. The multipotent potential of MSCs was assessed *via* (**A**) oil red staining for adipocytes, (**B**, **C**) alizarin staining and alkaline phosphatase staining for osteoblasts and (**D**) toluidine blue staining for chondrocytes.

LPS-induced infiltration of inflammatory cells, interstitial oedema and alveolar collapse were significantly alleviated 7 days after transplantation of MSCs alone or MSCs-null vector, but not MSCs-HIMF (Fig. 5). Increased interalveolar septal thickening and fibroblast proliferation were observed 14 days after transplantation of MSCs-HIMF (Fig. 5). Intra-alveolar septal thickness increased 4-fold 3 days after LPS exposure (Fig. 4C). Transplantation of MSCs or MSCs-null vector prevented LPS-induced increases in intra-alveolar septal thickness on day 7 compared to treatment with PBS ($P < 0.05$, Fig. 4C and D). No such effect was observed after treatment with MSCs-HIMF. In fact, intra-alveolar septal thickness increased by 74% 14 days after transplantation of MSCs-HIMF, significantly higher than that observed in PBS-treated animals (Fig. 4D, $P < 0.05$).

Lung W/D ratios and MPO activity were significantly higher in animals treated with PBS 3 days after LPS challenge. This effect was significantly inhibited by transplantation of MSCs ($P < 0.05$ or 0.01, Fig. 6A and C). Treatment with MSCs alone or MSCs-null vector consistently inhibited LPS-induced lung oedema after 7 days. This did not occur after treatment with MSCs-HIMF. Animals treated with MSCs-HIMF showed significantly increased MPO values 7 and 14 days after LPS treatment compared to animals treated with PBS, MSCs alone or MSCs-null vector ($P < 0.05$, Fig. 6C and D). The total protein concentration in BAL fluid was significantly higher 3 days after LPS challenge ($P < 0.05$ *versus* day 0, Fig. 6E). However, these levels were reduced significantly after treatment with MSCs alone or MSCs-null vector when compared to PBS treatment. Treatment with MSCs-HIMF resulted in a significant increase in total BAL protein concentration 14 days after LPS challenge when compared to other treatments ($P < 0.05$, Fig. 6F).

MSCs treatment increased IL-10 and decreased TNF-α expression levels

Levels of TNF-α mRNA expression were significantly higher in lung tissue 3 days after LPS challenge in animals treated with PBS when compared to day 0 ($P < 0.05$, Fig. 7A). Levels of TNF-α mRNA in the lungs of animals treated with MSCs or MSCs-null vector were significantly lower than those treated with PBS on days 3 and 7 after LPS administration, but remained higher than observed on day 0 ($P < 0.05$). There was a significant increase

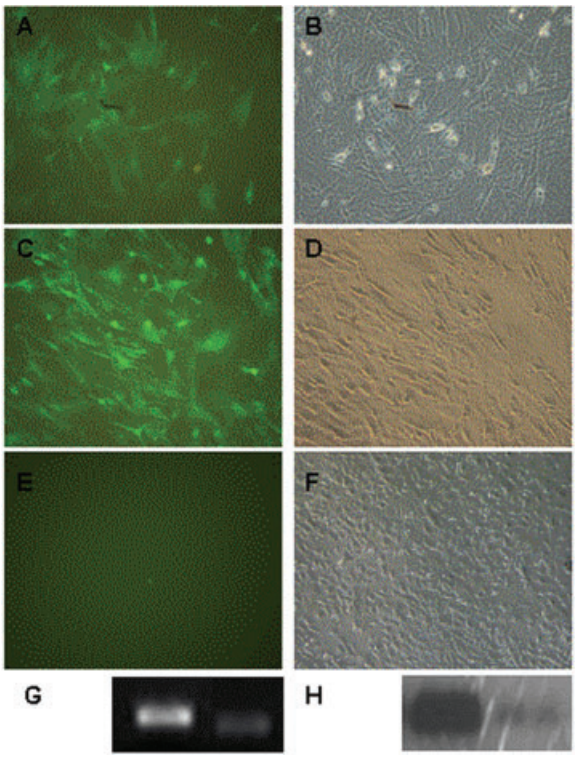

Fig. 3 Transduction of MSCs with HIMF-eGFP or eGFP and identification five days after transduction. Fluorescence (left) and phase contrast microscopy (right) showing the expression of eGFP in MSCs-HIMF (**A** and **B**) and MSCs-null vector (**C** and **D**) five days after lentiviral transduction at a MOI = 5 × 200. MSCs without eGFP transduction (**E** and **F**) are shown as a negative control for autofluorescence. RT-PCR (**G**) and Western blot (**H**) indicating HIMF expression in MSCs-HIMF.

in TNF-α mRNA expression in lung tissue from animals treated with MSCs-HIMF 7 and 14 days after LPS, compared with those treated with MSCs alone or MSCs-null vector ($P < 0.05$, Fig. 7A and B). Lung levels of TNF-α protein were consistent with the changes observed in TNF-α mRNA expression (Fig. 7C and D). Levels of IL-10 mRNA (Fig. 7E and F) and protein (Fig. 7G and H) were significantly higher 7 days after LPS challenge in lung tissue harvested from animals treated with MSCs or MSCs-null vector than those treated with PBS or MSCs-HIMF ($P < 0.05$).

MSCs decreased lung fibrosis

We observed an increased number of myofibroblasts in the MSCs-HIMF-treated group *via* staining with α-SMA antibodies. Animals treated with MSCs alone or MSCs-null vector showed less

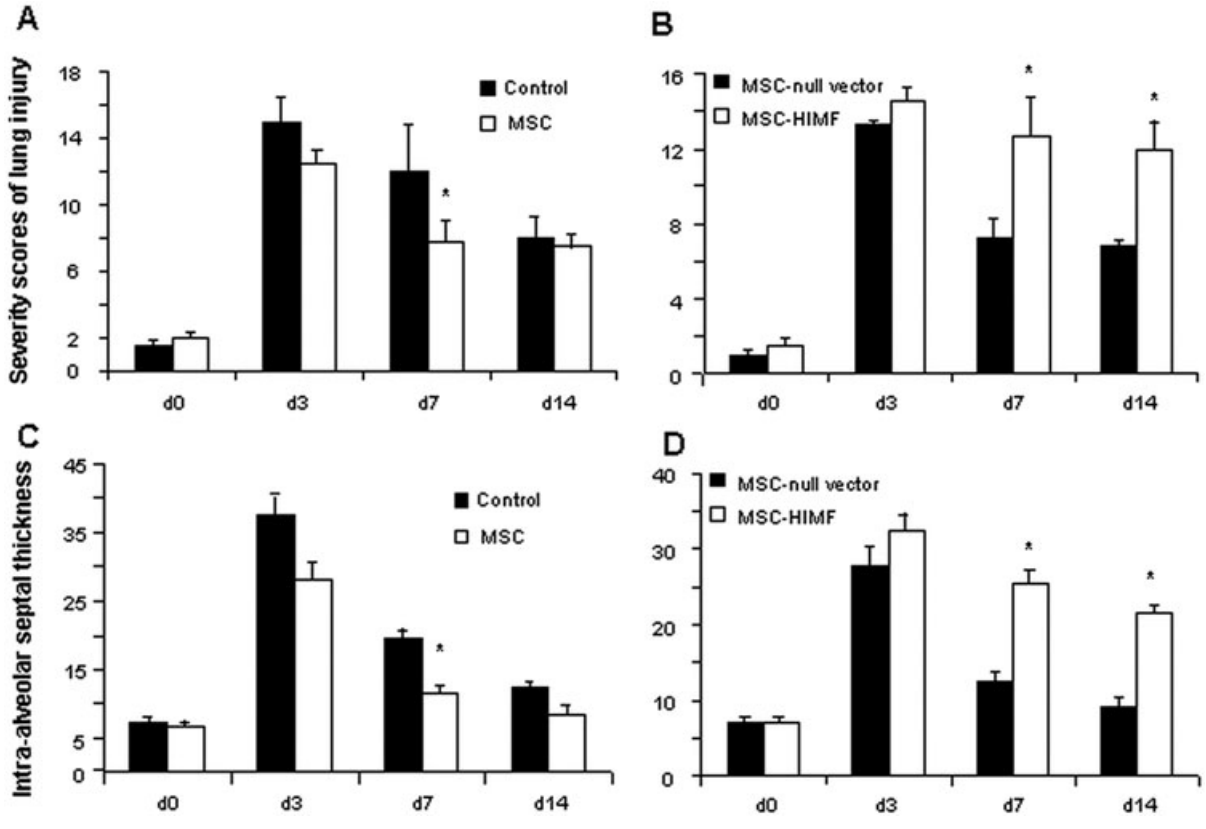

Fig. 4 The histopathology score of lung injury was evaluated after LPS challenge (**A**, **B**). Transplantation of MSCs prevented LPS-induced increase in lung injury score on day 7 compared to treatment with PBS ($^*P < 0.05$), but treatment with MSCs-HIMF had no effect. The scores increased significantly seven days after transplantation of MSCs-HIMF compared with MSCs-null vector ($^*P < 0.05$). Intra-alveolar septal thickness in lung sections of various treatment groups was evaluated (**C**, **D**). In animals treated with MSCs, intra-alveolar septal thickness was significantly reduced compared with controls on day 7 ($^*P < 0.05$), whereas transplantation of MSCs-HIMF resulted in an increase in intra-alveolar septal thickness on day 7 and 14 compared with MSCs-null vector ($^*P < 0.05$). Data are expressed as mean ± S.D.

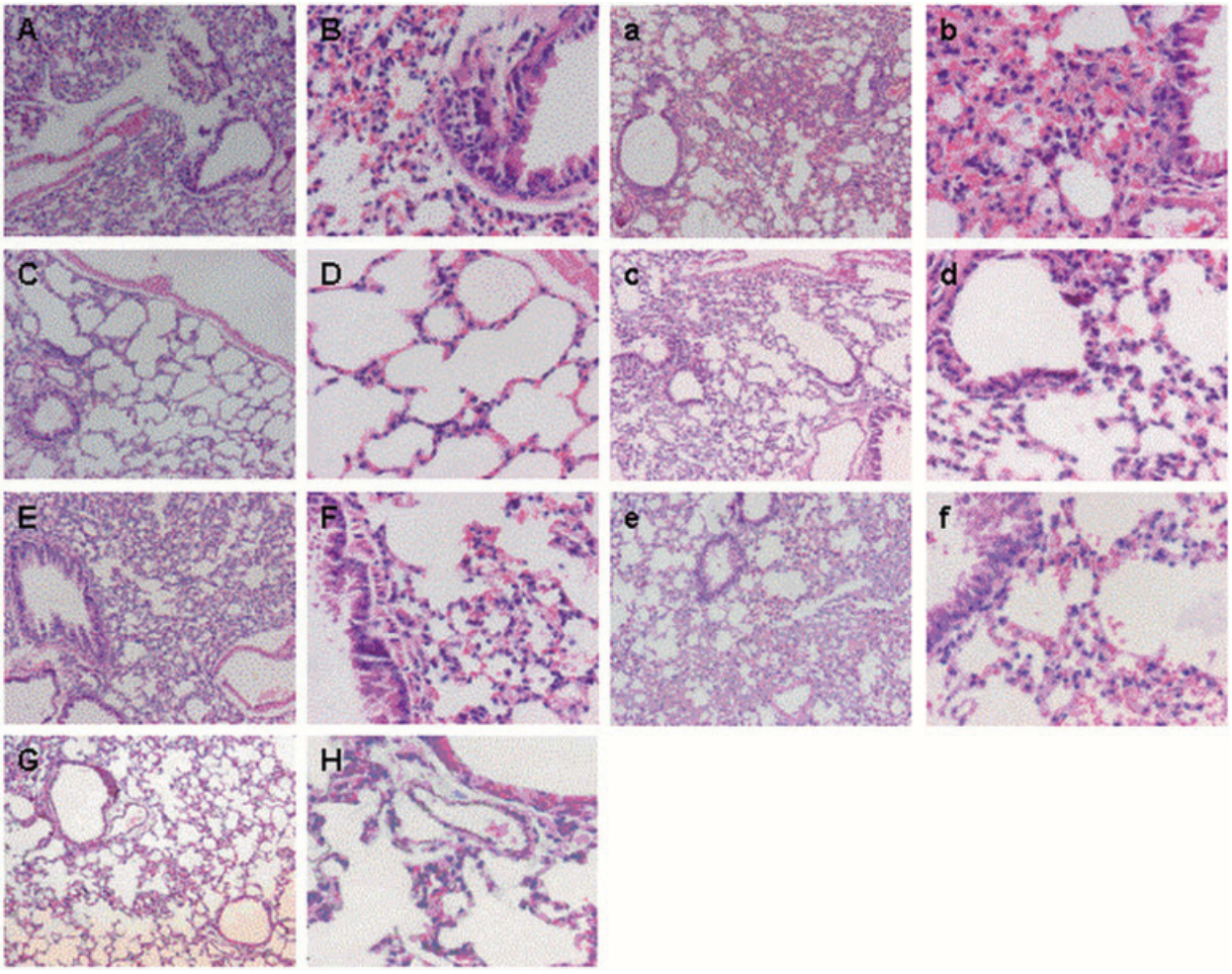

Fig. 5 Histopathology of injured lungs on day 7 (**A–H**) and day 14 (**a–f**). Lung tissue sections were stained with haematoxylin and eosin. Histopathological changes in the lungs are shown at low (×100) and high (×400) magnification. (**A**, **a**) MSCs-HIMF transplantation; (**C**, **c**) MSC transplantation; (**E**, **e**) controls; (**G**) MSC-null vector transplantation.

collagen deposition and fewer α-SMA$^+$ cells after LPS treatment (Fig. 8A–D). Levels of hydroxyproline in the lungs of animals treated with MSCs alone or MSCs-null vector were significantly lower than those treated with PBS 14 days after LPS challenge (Fig. 8E and F, $P < 0.05$).

Discussion

ALI is characterized by extensive damage to the barrier of the lung epithelium and endothelium, neutrophil influx into the lung and an imbalance between pro-inflammatory and anti-inflammatory

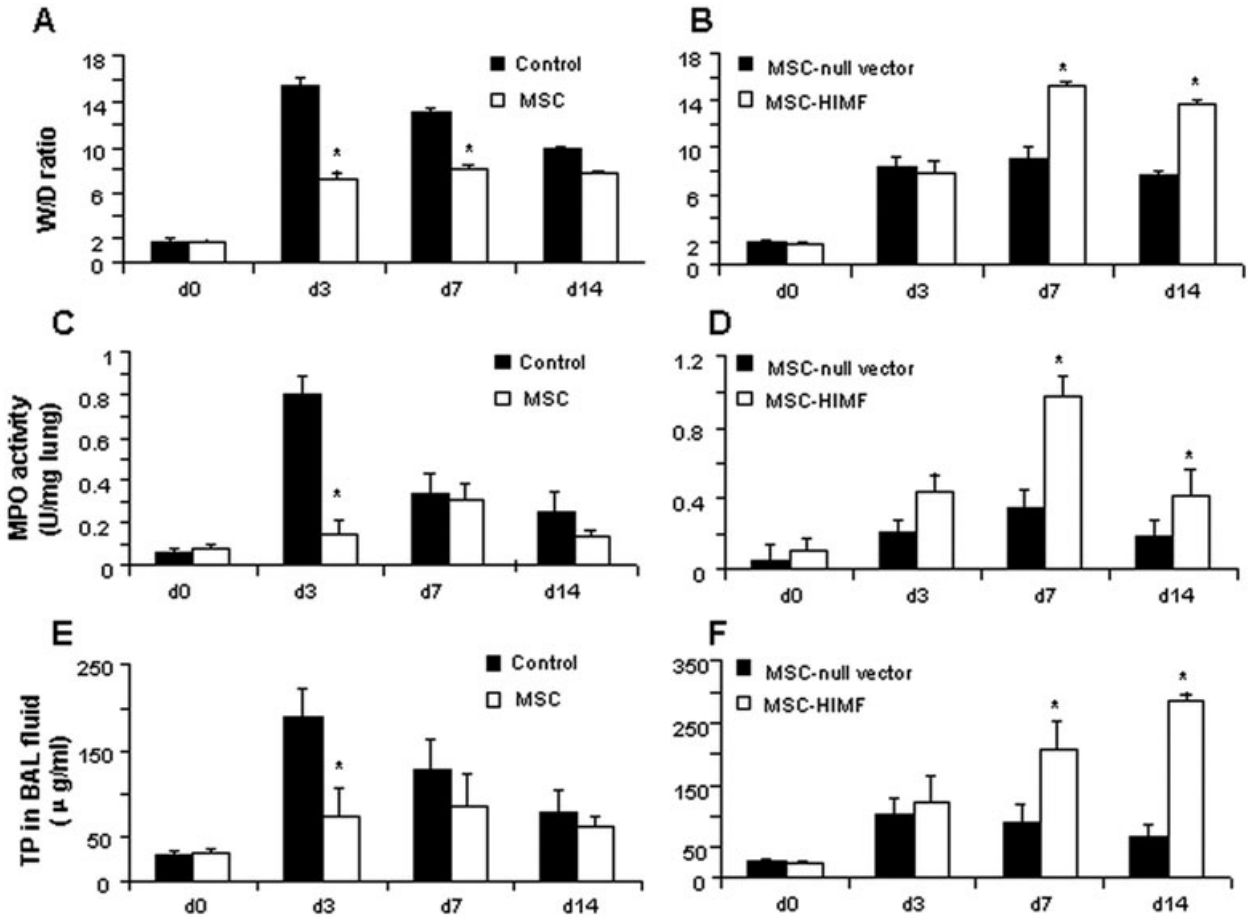

Fig. 6 Biochemical indices of lung injury were examined after LPS challenge. (**A**, **B**) Lung W/D ratio was significantly decreased after transplantation of MSCs compared with controls (*$P < 0.05$), but not after MSCs-HIMF transplantation. (**C**, **D**) MPO activity was significantly reduced in the MSC treated group compared with controls on day 3 (*$P < 0.05$). In animals transplanted with MSCs-HIMF, MPO activity increased significantly seven and 14 days after LPS (*$P < 0.05$). (**E**, **F**) Total protein levels in BAL fluid were significantly decreased with MSC transplantation on day 3 (*$P < 0.05$), whereas TP levels remained high in animals transplanted with MSCs-HIMF. Data are expressed as mean ± S.D.

mediators. LPS, a glycolipid of the outermost membrane of gram-negative bacteria, was used in this study to induce ALI and sub-ALI, as measured by hyper-production of the pro-inflammatory factor TNF-α, neutrophil infiltrates, alveolar congestion, haemorrhage and thickness of the alveolar wall. This was followed by an analysis of the therapeutic potential of MSC treatment, with or without HIMF expression. MSCs are widely used as therapeutic tools in cell and gene therapy due to their pluripotency, easy isolation and culture, and host compatibility [11, 15]. HIV-1-based lentiviral vectors are an efficient method for transduction of MSCs, with transgene expression sustained for up to 5 months [16].

A recent study demonstrated that intravenous injection of MSCs alone could reduce systemic and pulmonary cytokine levels in animals with abdominal sepsis, prevent the occurrence of ALI and organ dysfunction, as well as promote phagocytosis and bacterial clearance [17]. It was noticed that circulating stem-progenitor cells were elevated along with mobilizing cytokines in neonatal respiratory distress syndrome [18]. This suggests that the body mobilizes its own stem-progenitor cells when ALI/ARDS initially occurs, followed by a decompensation in stem cell function if the disease becomes more serious. This could potentially explain why the transplantation of stem cells into the lung could ameliorate LPS-induced ALI and bleomycin and asbestos-induced chronic lung injury, although the exact mechanism by which stem cells function in this context remains unclear. Previous studies suggested that the use of MSCs may support the injured lung directly or indirectly *via* stimulation of resident cells in the lung, and that MSC transplantation was a potentially therapeutic strategy for lung disease [19]. In the present study, BM-derived MSCs were cultured as described previously [20]. We found that systemic transplantation of MSCs could reduce LPS-induced lung injury, leucocyte recruitment, over-production of inflammatory

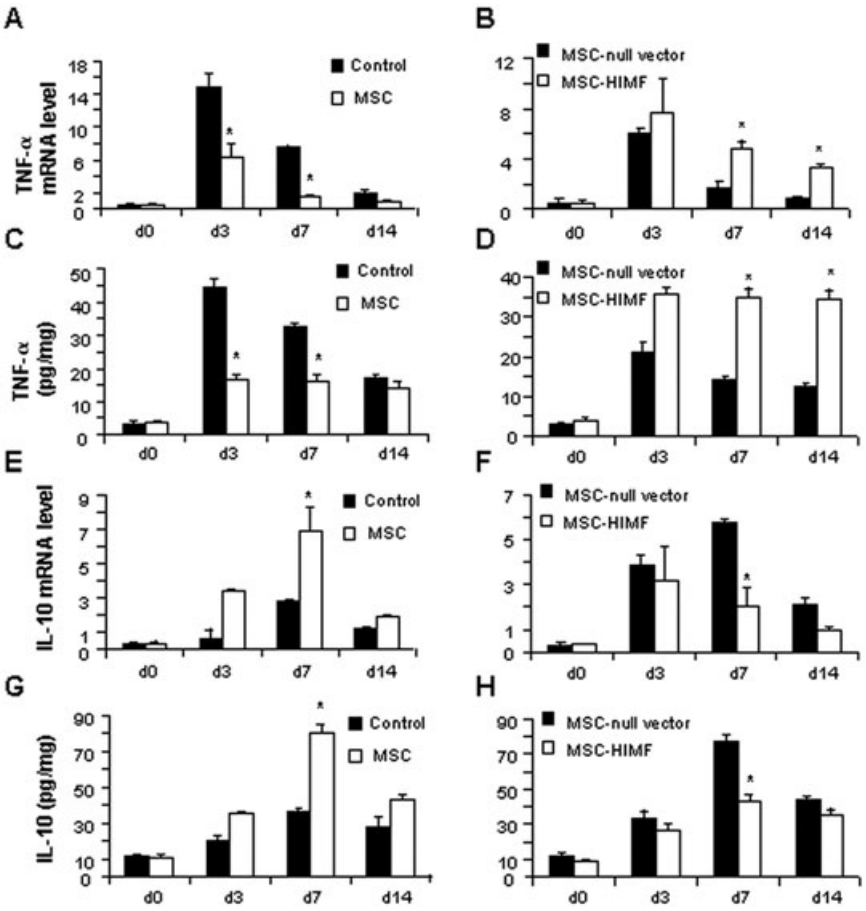

Fig. 7 Pro- and anti-inflammatory factor expression in different groups. (**A**, **B**) Compared to controls, TNF-α mRNA expression was significantly decreased in lung homogenates on days 3 and 7 after MSC transplantation ($^*P < 0.05$), whereas in animals transplanted with MSCs-HIMF, TNF-α mRNA levels increased significantly seven and 14 days after LPS ($^*P < 0.05$). (**C**, **D**) Similar results were observed for TNF-α levels in lung homogenates. (**E**, **F**) Lung levels of IL-10 mRNA in animals treated with MSCs or MSCs-null vector were significantly increased seven days after LPS treatment compared with those treated with PBS ($^*P < 0.05$), but not in animals treated with MSCs-HIMF. (**G**, **H**) Similar results were obtained for IL-10 levels in lung homogenates. Data are expressed as mean ± S.D.

mediators and development of lung fibrosis. However, we found that such therapeutic effects could not ameliorate LPS-induced lung injury completely, possibly due to the quantity of cells and frequency of MSC transplantation. Our data indicate that a single transplantation of MSCs may have therapeutic effects within 1 week of transplantation. The therapeutic effects on LPS-induced lung injury were noticed in animals treated with MSCs alone or MSCs transfected with an empty vector; however, MSCs transfected with a vector expressing HIMF had no additional therapeutic effects.

HIMF, a hypoxia-driven proliferation-promoting cytokine, plays complex roles in lung injury, such as promotion of vascular adhesion molecule-1 and vascular endothelial growth factor expression and increased inflammatory cell infiltration of the lung parenchyma, which worsens lung injury [21]. Alternatively, HIMF can exert anti-apoptotic functions by regulating surfactant protein C production and improving ALI [9]. We did not observe significant improvement after transplant of MSCs-HIMF in LPS-induced lung inflammation, tissue injury and repair. Not only did treatment with HIMF-transfected MSCs fail to result in significant improvement, but also exacerbated epithelial injury. Specifically, we observed increased intra-alveolar septal thickness and pulmonary vascular leak, and worsened lung tissue oedema and fibrosis in MSCs-HIMF-treated animals compared to treatment with MSCs alone or MSCs-null vector after LPS challenge. Therefore, we conclude that MSCs-HIMF transplantation contributed to increased pro-inflammatory factor expression and lung injury, and that HIMF may serve as an important pro-inflammatory but not anti-inflammatory mediator in ALI. We found that LPS initiated the development of fibrotic proliferation after 5 days, as characterized by the increased proliferation of fibroblasts and the *de novo* appearance of myofibroblasts with a distinct α-SMA-expressing phenotype [22]. HIMF was found to stimulate type I collagen and α-SMA expression in lung fibroblasts [23]. The results from the present study demonstrate that the addition of HIMF could increase tissue levels of hydroxyproline and α-SMA expression in the lung 14 days after LPS challenge. It seems that HIMF promotes consistent expression of pro-inflammatory factors, development of chronic lung injury and progressive fibrosis.

In conclusion, MSC transplantation had therapeutic effects on LPS-induced overproduction of inflammatory mediators, leucocyte influx and lung injury. However, the combination of HIMF with MSC therapy failed to show any additive effects on the reduction of LPS-induced lung inflammation and injury. Thus, our data indicate that

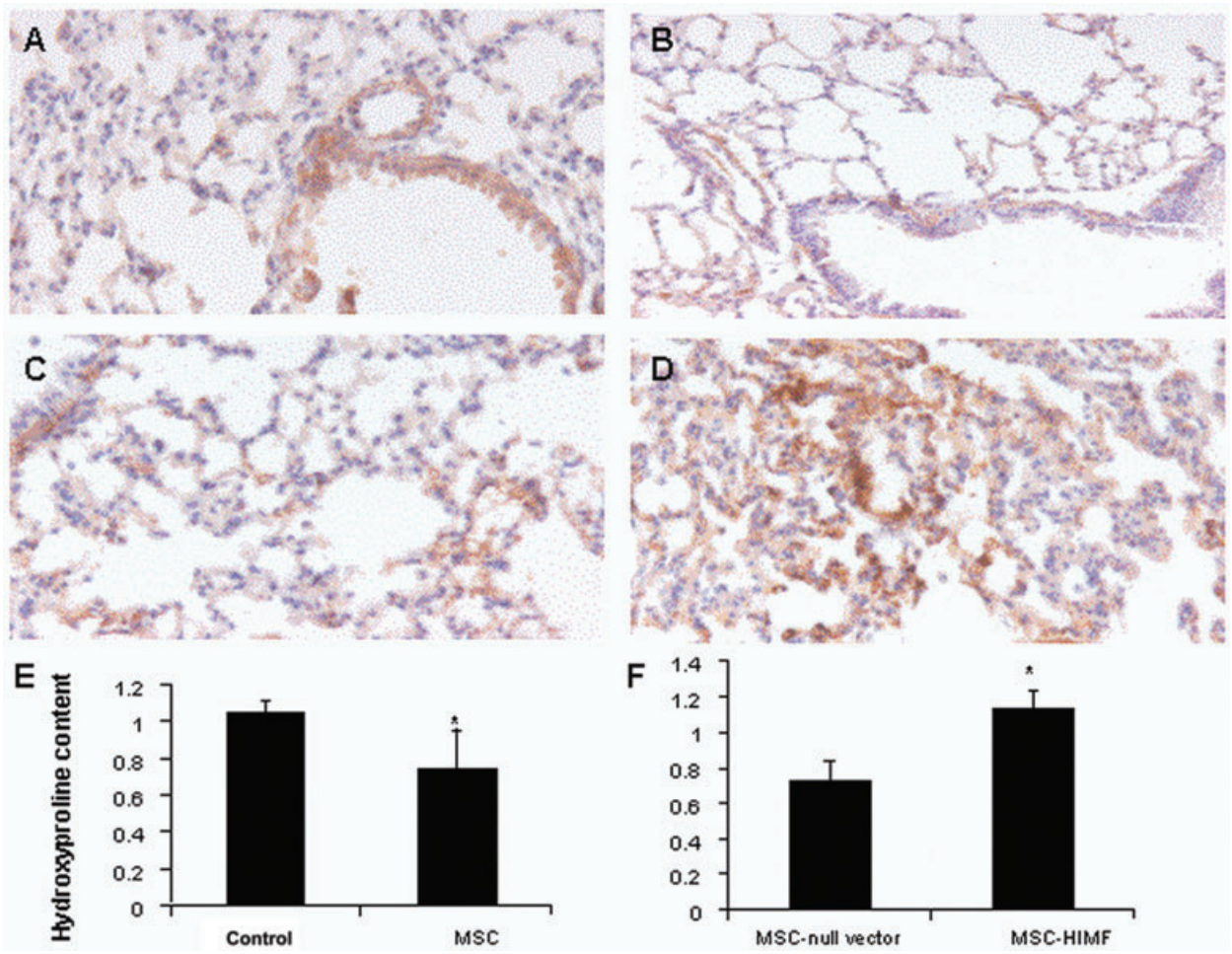

Fig. 8 α-SMA expression and hydroxyproline content on day 14. In lungs of control-treated animals (**A**), those treated with MSCs (**B**) and those treated with MSCs-null vector (**C**), α-SMA$^+$ cells were distributed in a scattered pattern. In the MSCs-HIMF-treated group (**D**), α-SMA$^+$ cells had significantly increased with collagen deposition. (**E**, **F**) Transplantation of MSCs and MSCs-null vector resulted in significantly decreased hydroxyproline levels compared with controls ($^*P < 0.05$). This was not the case for MSCs-HIMF-treated animals. Data are expressed as mean $\pm$ S.D.

MSC transplantation has potential as an alternative therapy for both ALI and chronic lung injury independent of the addition of HIMF.

Acknowledgements

We thank Yaoqiong Cao (Genechem Co. Ltd.) for technical assistance. This study was subsidized by grants from the State Natural Sciences Foundation project (No. 30770930), Shanghai Leading Talent Projects (No. 036, 2010), Program of Shanghai Subject Chief Scientist (No. 07XD14012) and Shanghai Rising Star Program (No. 09QA1404900).

Conflict of interest

The authors confirm that there are no conflicts of interest.

References

1. **Brigham KL, Meyrick B.** Endotoxin and lung injury. *Am Rev Respir Dis.* 1986; 133: 913–27.
2. **Jeyaseelan S, Chu HW, Young SK, *et al.*** Transcriptional profiling of lipopolysaccharide-induced acute lung injury. *Infect Immun.* 2004; 72: 7247–56.
3. **Lee SH, Jang AS, Kim YE, *et al.*** Modulation of cytokine and nitric oxide by mesenchymal stem cell transfer in lung injury/fibrosis. *Respir Res.* 2010; 11: 16–30.
4. **Wang D, Morales JE, Calame DG, *et al.*** Transplantation of human embryonic stem

cell-derived alveolar epithelial type II cells abrogates acute lung injury in mice. *Mol Ther*. 2010; 18: 625–34.

5. **Wary KK, Vogel SM, Garrean S, *et al.*** Requirement of alpha(4)beta(1) and alpha(5)beta(1) integrin expression in bone-marrow-derived progenitor cells in preventing endotoxin-induced lung vascular injury and edema in mice. *Stem Cells*. 2009; 27: 3112–20.
6. **Wagner KF, Hellberg AK, Balenger S, *et al.*** Hypoxia-induced mitogenic factor has antiapoptotic action and is upregulated in the developing lung: coexpression with hypoxia-inducible factor-2 alpha. *Am J Respir Cell Mol Biol*. 2004; 31: 276–82.
7. **Renigunta A, Hild C, Rose F, *et al.*** Human relmbeta is a mitogenic factor in lung cells and induced in hypoxia. *FEBS Lett*. 2006; 580: 900–3.
8. **Teng X, Li D, Champion HC, *et al.*** FIZZ1/RELM alpha, a novel hypoxia-induced mitogenic factor in lung with vasoconstrictive and angiogenic properties. *Circ Res*. 2003; 92: 1065–7.
9. **Tong Q, Zheng L, Dodd-o J, *et al.*** Hypoxia-induced mitogenic factor modulates surfactant protein B and C expression in mouse lung. *Am J Respir Cell Mol Biol*. 2006; 34: 28–38.
10. **Yamaji-Kegan K, Su Q, Angelini DJ, *et al.*** Hypoxia-induced mitogenic factor has proangiogenic and proinflammatory effects in the lung *via* VEGF and VEGF receptor-2. *Am J Physiol Lung Cell Mol Physiol*. 2006; 291: L1159–68.
11. **Gupta N, Su X, Popov B, *et al.*** Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol*. 2007; 179: 1855–63.
12. **Wagner EM, Sánchez J, McClintock JY, *et al.*** Inflammation and ischemia-induced lung angiogenesis. *Am J Physiol Lung Cell Mol Physiol*. 2008; 294: L351–7.
13. **Takao Y, Mikawa K, Nishina K, *et al.*** Attenuation of acute lung injury with propofol in endotoxemia. *Anesth Analg*. 2005; 100: 810–6.
14. **Meirelles Lda S, Nardi NB.** Murine marrow-derived mesenchymal stem cell: isolation, *in vitro* expansion, and characterization. *Br J Haematol*. 2003; 123: 702–11.
15. **Grove JE, Lutzko C, Priller J, *et al.*** Marrow-derived cells as vehicles for delivery of gene therapy to pulmonary epithelium. *Am J Respir Cell Mol Biol*. 2002; 27: 645–51.
16. **Zhang XY, La Russa VF, Bao L, *et al.*** Lentiviral vectors for sustained transgene expression in human bone marrow-derived stromal cells. *Mol Ther*. 2002; 5: 555–65.
17. **Mei SH, Haitsma JJ, Dos Santos CC, *et al.*** Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med*. 2010; 182: 1047–57.
18. **Qi Y, Qian L, Sun B, *et al.*** Circulating CD34(+) cells are elevated in neonates with respiratory distress syndrome. *Inflamm Res*. 2010; 59: 889–95.
19. **Brody AR, Salazar KD, Lankford SM.** Mesenchymal stem cells modulate lung injury. *Proc Am Thorac Soc*. 2010; 7: 130–3.
20. **Dominici M, Le Blanc K, Mueller I, *et al.*** Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006; 8: 315–7.
21. **Tong Q, Zheng L, Lin L, *et al.*** Hypoxia-induced mitogenic factor promotes vascular adhesion molecule-1 expression *via* the PI-3K/Akt-NF-kappaB signaling pathway. *Am J Respir Cell Mol Biol*. 2006; 35: 444–56.
22. **Zhang K, Rekhter MD, Gordon D, *et al.*** Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. *Am J Pathol*. 1994; 145: 114–25.
23. **Liu T, Dhanasekaran SM, Jin H, *et al.*** FIZZ1 stimulation of myofibroblasts differentiation. *Am J Pathol*. 2004; 164: 1315–26.