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Engraftment of bone marrow stromal cells in lipopolysaccharide-injured mouse lungs

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Abstract: **Objective** To explore a feasibility of engraftment of systemically transplanted bone marrow stromal cells (BMSCs) and differentiation into lung epithelial cells in lipopolysaccharides (LPS)-injured lungs. **Methods** BMSCs were isolated from bone marrow of transgenic green fluorescent protein (GFP) C57BL/6J mice and systemically administered to bone marrow-suppressed wild-type C57BL/6J mice. A mouse model of lung injury was prepared by intratracheal instillation of LPS. Recipients were assigned to four groups: intratracheal PBS + BMSCs transplantation (CM), intratracheal LPS + BMSCs transplantation (LM), intratracheal PBS + irradiation + BMSCs transplantation (CIM) and intratracheal LPS + irradiation + BMSCs transplantation (LIM). BMSCs engraftment in recipient lungs was determined by immunofluorescent staining 14 days after BMSCs administration. Alveolar epithelial type II cells were isolated from recipient lungs and the rate of GFP positive cells was measured by flow cytometry. Expression of surfactant protein (SP)-A, SP-C and aquaporin (AQP)-5 mRNA in the lungs was evaluated by real-time PCR. **Results** GFP and cytokeratin positive cells were observed in lung parenchyma of the CIM and the LIM groups, but not in the CM and the LM groups. The LIM group had more positive cells than the CIM group. The rates of GFP positive cells were higher in the CIM (11.10 ± 3.19%) and the LIM groups (14.40 ± 2.40%) than those in the CM and the LM groups (2.82 ± 1.03% and 3.81 ± 0.93%, respectively; $P < 0.05$). The LIM group had higher mRNA expression of SP-C than the CM group (2.09 ± 0.18 vs 1.38 ± 0.30; $P < 0.05$). **Conclusions** Donor derived BMSCs can engraft in LPS-injured lungs and differentiate into lung epithelial cells, suggesting BMSCs transplantation might contribute to lung repair. [**Chin J Contemp Pediatr**, **2009**, **11** (**5**):**321 – 327**]

Key words: Engraftment; Lung; Lipopolysaccharide; Bone marrow stromal cell; Mice

骨髓基质细胞在脂多糖诱导的肺损伤小鼠肺组织的定植研究

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[**摘　要**] **目的**　探讨静脉输注骨髓基质细胞(BMSCs)在脂多糖(LPS)诱导的肺损伤小鼠肺组织定植并向肺泡上皮细胞分化的可能性。**方法**　绿色荧光蛋白(GFP)转基因 C57BL/6J 小鼠作为 BMSCs 移植供体,同种野生型小鼠为移植受体。气道滴入 LPS 诱导肺损伤。受体分为以下几组:(1) PBS + BMSCs 移植(CM);(2) LPS + BMSCs 移植(LM);(3) PBS + 全身放射 + BMSCs 移植(CIM);(4) LPS + 全身放射 + BMSCs 移植(LIM)。移植 14 d 后,以免疫荧光双标染色检测 BMSCs 在受体肺组织的定植情况,流式细胞仪检测体外培养的肺泡 II 型上皮细胞(AEC II)GFP 阳性率,荧光定量 PCR 法检测肺组织表面活性物质蛋白(SP)-A、SP-C 和水通道蛋白(AQP)-5 mRNA 的表达。**结果**　移植后 14 d,免疫荧光双标染色可见 CIM 和 LIM 组有少量肺泡上皮细胞呈 GFP 和角蛋白双染阳性,而且 LIM 组较 CIM 组有较多阳性细胞。与 CM 和 LM 组(分别为 2.82 ± 1.03% 和 3.81 ± 0.93%)比较,CIM 和 LIM 组的 AEC II GFP 阳性率更高(分别为 11.10 ± 3.19% 和 14.40 ± 2.40%;$P < 0.05$)。LIM 组 SP-C mRNA 表达较 CM 组增高(2.09 ± 0.18 vs 1.38 ± 0.30;$P < 0.05$)。**结论**　供体来源的 BMSCs 能在 LPS 诱导的受损肺组织定植分化,提示静脉输注 BMSCs 可能有助于肺损伤的修复。[**中国当代儿科杂志,2009,11(5):321 – 327**]

[**关　键　词**]　定植;肺;脂多糖;骨髓基质细胞;小鼠

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Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), is a significant cause of morbidity and mortality in critically ill patients[1-2], especially when infection is the main etiology. Pneumonia and sepsis are two leading causes of ALI/ARDS, and ALI/ARDS is generally regarded as an inflammatory injury of the lungs based on its pathological and physiological changes[1]. Inflammatory stimuli from microbial pathogens, such as endotoxin (lipopolysaccharides, LPS), are well recognized for their ability to induce systemic and pulmonary inflammation. One of the histological characteristics of ALI/ARDS is severe disruption of the alveolar-capillary membrane barrier as a result of alveolar epithelial and endothelial lesions[1]. Current strategies of anti-inflammatory have not been successful in preventing these lesions as either limited therapeutic effects or specific therapy potential local or systematical adverse effects[3]. ARDS-associated mortality remains as high as 30%-70% in adult and pediatric patients[2,4]. Looking for more effective therapy as complementary to the standard care is of clinical value.

Experimental administration of LPS, either systemically or intratracheally, is commonly used to induce pulmonary inflammation in animal models of ALI[5-6]. As a most important target as well as promotor, alveolar epithelial cells are engaged in reparation of injured lung cells. Recent studies suggest that bone marrow derived stem cells contribute to injured alveolar epithelial and capillary endothelial repair[7]. Bone marrow stromal cells (BMSCs) are a group of stem cells in bone marrow that are distinct from hematopoietic stem cells. BMSCs differentiate into osteoblasts, chondroblasts, adipocytes, and hematopoietic supporting stroma[8-9]. Recent reports suggest that BMSCs may also differentiate into nonstromal tissues, including lung epithelial cells[10-13]. These data provide a strong rationale to explore the potential use of BMSCs for the treatment of intractable lung injury. Therefore, the aim of this study was to explore a feasibility of engraftment of systemically transplanted BMSCs in LPS-injured lungs *in vivo* and identify its differentiation into lung epithelial cells in a mouse model of ALI.

Methods

Animals

Transgenic green fluorescent protein (GFP) C57BL/6J male mice (4-6 weeks) were obtained for donors from the Cardiothoracic Surgery Laboratory, Changhai Hospital of Second Military Medical University in Shanghai. Wild-type C57BL/6J female mice (8-10 weeks) were obtained for recipients from the Experimental Animal Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Science. Protocols for the animal studies were approved by the Scientific and Ethics Committees at Children's Hospital of Fudan University.

Induction of lung injury

Induction of lung injury was performed as previously described[14]. Briefly, the recipient mice were anesthetized by intraperitoneal injection of 4% chloral hydrate followed by an intratracheal instillation of LPS (1 mg/kg in PBS) via a canula. The control mice received only PBS following the same procedure. LPS from *Escherichia coli* serotype 055:B5 was provided by Sigma-Aldrich (St. Louis, MO).

Isolation and culture of BMSCs

BMSCs were obtained from donor mice, and were isolated, as previously described[15]. Briefly, the bone marrow was harvested from the tibias and femurs of mice. Bone marrow was mechanically dissociated and the cells were washed, suspended in DMEM/F12 (Gibco BRL, Grand Island, NY) with 15% fetal bovine serum (Hyclone, Logan, UT) containing penicillin-streptomycin in culture flasks. After 48 hours, nonadherent cells were discarded, and the adherent fraction of cells was grown in DMEM/F12 culture solution changed every 48 hours thereafter. After 10-12 days, the adherent cells were harvested with 0.25% trypsin and 1 mM EDTA, and suspended in PBS at 2×10^6 cells/0.2 mL. BMSCs were labeled with murine antibody of CD59, CD90.1 (Chemicon, Temecula, CA) and CD34 (Biolegend, San Diego, CA) (all dilution 1:200) as primary antibodies, followed by rhodamine-labeled goat anti-mouse IgG (1:50) (KPL, Kitchener, Ontario, Canada) as a secondary antibody. Osteogenic[16] and neurogenic[17-18] differentiation assays of BMSCs were performed as described.

Lethal irradiation and BMSCs transplantation

The recipient mice were assigned to four groups: intratracheal PBS + BMSCs transplantation (CM), intratracheal LPS + BMSCs transplantation (LM), intratracheal PBS + irradiation + BMSCs transplantation (CIM) and intratracheal LPS + irradiation + BMSCs transplantation (LIM). Three control groups included as follows: intratracheal LPS + intravenous sterile saline (L), intratracheal PBS + irradiation + intravenous sterile saline (I), and LPS + irradiation + intravenous sterile saline (LI). The irradiation was performed using doses of 9 Gy ^{137}Cs (0.83 Gy/min) 4 hours after intratracheal instillation of LPS/PBS. Within 24 hours after irradiation, BMSCs (2×10^6 cells/0.2 mL) were injec-

ted intravenously through the tail vein. The mice were then maintained under sterile conditions for 14 days.

Histopathology and immunofluorescence of the lungs

Lung tissue was perfused free of blood by saline perfusion via right ventricle to pulmonary artery. Thereafter the trachea was ligated, and the lungs were fixed en bloc in 4% paraformaldehyde for 12 hours. The fixed lungs were embedded in paraffin and sectioned by standard methods for staining with hematoxylin and eosin. The parallel animals were sacrificed, and then the left lung was excised for RNA extraction. The right lung was fixed in 4% paraformaldehyde for 12 hours followed by embedding in optimal tissue compound for frozen tissue sectioning. Five-micrometer-thick frozen sections were processed for dual-color immunofluorescent staining. Alveolar epithelial cells from donor BMSCs were identified by incubating with murine anti-cytokeratin 5&8 and rabbit anti-GFP antibodies (both dilution 1∶200, Chemicon, Temecula, CA), followed by secondary antibody, rhodamine-labeled goat anti-mouse and FITC-labeled goat anti-rabbit IgG (both dilution 1∶50). Nuclear was counterstained with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Labs, Burlingame, CA). Immunostaining cells were observed under Olympus BH2 fluorescent microscope and imaged with LEICA software. The lung tissues of GFP transgenic mice were used for positive control.

Flow cytometry analysis

Alveolar epithelial type II cells were isolated from recipient mice ($n=4$ each group) by a modification of the method of Richards et al [19]. Saline-perfused lung samples free of blood were enzymatically digested for 30 min at 37℃ by intratracheal infusion of 0.08% trypsin (Gibco BRL, Grand Island, NY) and 1% collagenase (Sigma-Aldrich, St. Louis, MO) and suspended in trypsin solution. Trachea and bronchi were removed, lungs were transferred to containers containing calf serum with 0.01% DNAse I and minced finely. The cell suspension was then sequentially filtered through nylon gauzes (150 and 75 μm). The cells were collected by centrifugation at 300 g for 5 min (4℃) and cultured in DMEM with 10% fetal bovine serum. After 48 hours, cells were harvested with trypsin/EDTA and resuspended in PBS at 1×10^6 cells/mL. The isolated cells were then analyzed by fluorescence-activated cell sorter analysis gating on bright green cells with a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA).

Quantitative real-time PCR

Total RNA was extracted from left lung tissue using TRIzole reagent and 2 μg total RNA was reversely transcribed into cDNA. The following primers for RT-PCR were designed using Primer Express software (ABI): SP-A forward, TGGGAAATGGAATGATAAGG, and reverse, GTGGGAGATGGCGTAACTAA; SP-C forward, GTCCTCGTTGTCGTGGTGAT, and reverse, AAGGTAGCGATGGTGTCTGC; AQP5 forward, GGCCGTGGTGGTGGAGTTAA, and reverse, CCGACAAGCCAATGGATAAGG; β-actin (internal control) forward, AACCCTAAGGCCAACAGTGAAAAG; and reverse, TCATGAGGTAGTCTGTGAGGT. Quantitative RT-PCR was performed using the real-time PCR (RT-PCR) with the SYBR Green reporter on an ABI PRISM 7 000 PCR instrument (PE Applied Biosystems, Tustin, CA). The amplification was carried out in a 25 μL reaction volume containing 12.5 μL SYBR Green I mastermix (TOYOBO, Osaka, Japan). PCR conditions were: initial 20-min denaturation at 95℃, followed by 40 cycles amplification (SP-A:95℃ ×30 s, 55℃ ×30 s, 72℃ ×30 s; SP-C:95℃ ×30 s, 58℃ ×30 s, 72℃ ×30 s; AQP5: 95℃ ×30 s, 60℃ ×1 min; β-actin:95℃ ×30 s, 58℃ ×30 s, 72℃ ×30 s). Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. Copy numbers of the gene in samples were determined from the standard curve. RT-PCR products were run on 2% agarose gels to confirm that correct molecular sizes were present. Eight samples were attained from each group and each sample was tested in duplicate with quantitative RT-PCR. The relative quantities of mRNA were presented as common logarithm of the copy numbers of targeted gene/copy numbers of β-actin($\times10^5$).

Statistical analysis

All data are presented as mean ± standard deviation ($\bar{x}\pm s$). Variables were subjected to ANOVA for differences among all groups, followed by a post-hoc Bonferroni test for difference comparisons between two groups. A P value <0.05 was regarded as statistically significant.

Results

Characterization of BMSCs

BMSCs of primary culture (Figure 1A) were uniformly positive for the stem cell surface marker of CD59 and CD 90.1 by immunofluorescent stainings (Figures 1B and 1C), and were negative for CD34 (Figure 1D). Murine BMSCs were demonstrated to differentiate into osteocytes (Figure 1E) and neurons (Figure 1F).

BMSCs engraftment and differentiation in injured lungs

Alveolar epithelial cells from donor BMSCs were

marked with dual immufluorescent staining of cytokeratin 5&8 and GFP. A few donor derived cells (GFP$^+$ cytokeratin$^+$ cells) were detected in the parenchyma of irradiated and BMSCs transplanted recipient lungs (the CIM and the LIM groups), and were morphologically consistent with alveolar epithelial cells, but not detected in lungs of the CM and the LM groups (Figure 2). Moreover, there were more positive cells in the lungs from the LIM group than from the CIM group.

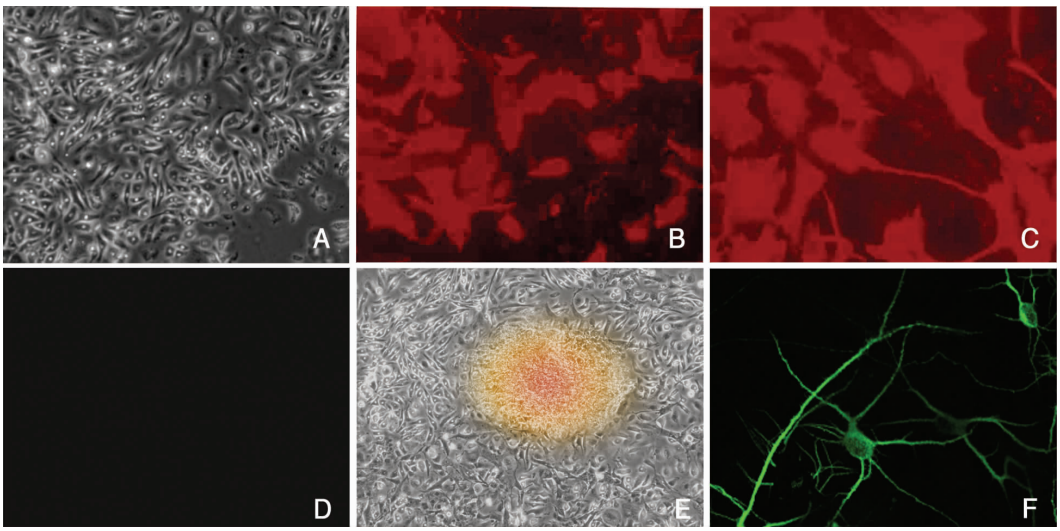

Figure 1 Characterization of BMSCs isolated from bone marrow of C57BL/6J mice. **A**: Undifferentitated BMSCs on day 10 after primary culture (×200); **B**: Immunostaining with murine CD59 immunofluorescent antibodies for surface marker expression on cultured BMSCs, identified by red staining (×400); **C**: Immunostaining with murine CD90.1 immunofluorescent antibodies for surface marker expression on cultured BMSCs, identified by red staining (×400); **D**: Immunostaining with murine CD34 immunofluorescent antibodies for surface marker expression on cultured BMSCs, identified by negative staining (×400); **E**: Staining with alizarin red showed that BMSCs were differentiated into osteocytes (×200); **F**: Immunostaining with rabbit MAP-2 immunofluorescent antibodies showed that BMSCs were differentiated into neurons, identified by green staining (×400).

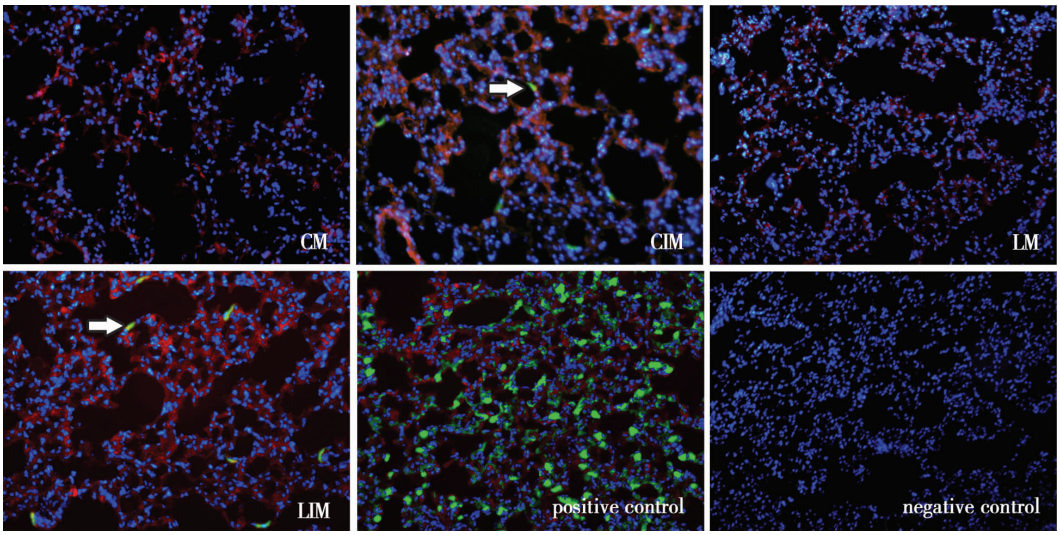

Figure 2 BMSCs engraftment and differentiation in recipient lungs (×200) Recipient murine lungs were immunostained for cytokeratin 5&8 (red) and GFP (green). GFP$^+$ cytokeratin$^+$ cells were observed in the alveolar walls (light green, arrows) in the CIM and the LIM groups.

Freshly isolated alveolar epithelial type II cells were identified by lamellar bodies under an electromicroscope (Figure 3). GFP positive cells were detected in preparations of type II epithelial cells isolated from recipient mice 14 days after BMSCs transplantation (Figure 4). The rates of GFP positive cells were higher in both the CIM and the LIM groups (11.10 ± 3.19% and 14.40 ± 2.40% respectively) than those in the CM and the LM groups (2.82 ± 1.03% and 3.81 ± 0.93% respectively; $P < 0.05$). These results confirmed that donor BMSCs could engraft and differentiate in injured lungs of recipient mice.

Effect of BMSCs transplantation on the repair of LPS-injured lungs

The intratracheal exposure to LPS resulted in the development of lung inflammation as evidenced by prominent edema, infiltration of neutrophils, and even small airway epithelial desquamation 24 hours after intratracheal LPS (Figure 5B). The mice in the L, the I and the LI groups died before the experiment endpoint (14 days) or had obvious pathologic changes at 14 days (Figure 5C), but no abnormalities were observed in the lungs of the CM, the LM, the CIM and the LIM groups at 14 days (Figure 5D).

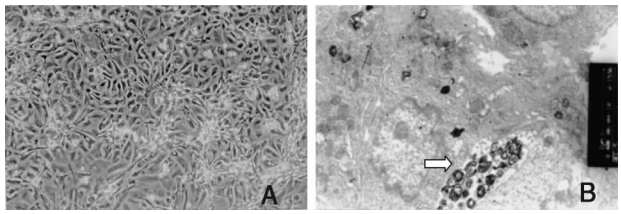

Figure 3 Alveolar epithelial type II cells isolated from the lungs of recipients **A**: Alveolar type II cells on day 2 after primary culture under an inverted microscope (×200); **B**: The electron micrograph showed the characteristic lamellar bodies of alveolar type II cells (arrow).

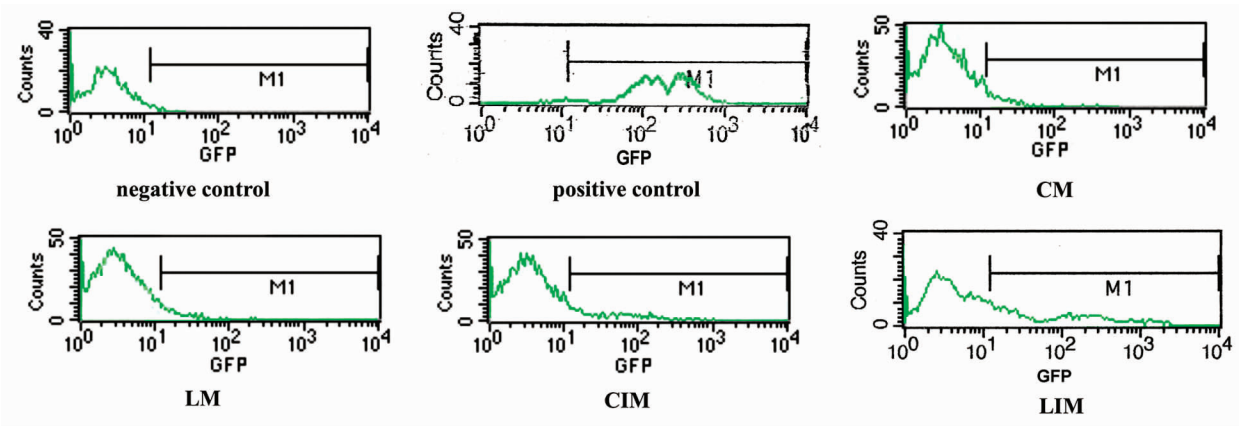

Figure 4 Flow cytometry analysis of GFP positive cells The rates of GFP positive cells in preparations of type II epithelial cells isolated from recipient mice 14 days after BMSCs transplantation in both the CIM and the LIM groups were higher than those in the CM and the LM groups.

Effect of BMSCs transplantation on the mRNA expression of SP-A, SP-C and AQP5 in recipient lungs

The effect of BMSCs transplantation on the function of alveolar epithelial cells of recipients was evaluated by determining the mRNA expression of specific markers, SP-A and SP-C for type II cells and AQP5 for type I cells. SP-C mRNA expression in the lungs in the LIM group was higher than that in the CM group ($P < 0.05$), but no significant differences were found in mRNA expression of SPA and AQP5 among all groups (Table 1).

Table 1 mRNA expression of SP-A, SP-C and AQP5 of recipient lungs ($n = 8$, $\bar{x} \pm s$)

Group	SP-A	SP-C	AQP5
CM	2.24 ± 1.07	1.38 ± 0.30	1.07 ± 0.28
LM	2.24 ± 0.24	1.51 ± 0.54	0.93 ± 0.25
CIM	2.36 ± 0.28	1.83 ± 0.16	0.99 ± 0.21
LIM	2.37 ± 0.22	2.09 ± 0.18[a]	1.13 ± 0.24

a: $P < 0.05$, vs the CM group.

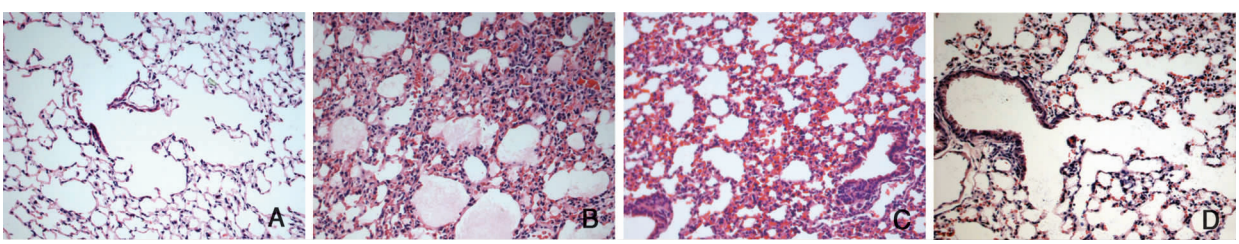

Figure 5 Lung histopathology by light microscopy (hematoxylin and eosin staining, × 200) **A**: Normal control; **B**: Lung inflammation as evidenced by prominent edema, infiltration of neutrophils, and even small airway epithelial desquamation 24 hours after intratracheal LPS; **C**: Diffusive alveolar damage represented by alveolar bleeding, atelectasis, bronchiolar epithelial desquamation, and leukocyte sequestration were observed in lungs of the L group at 14 days; **D**: No abnormalities were observed in lungs of the LM group at 14 days.

Discussion

This study examined whether transplanted BMSCs can migrate into injuried lungs and differentiate into alveolar epithelial cells, and repair injured alveolar epithelial cells. For this purpose, BMSCs were isolated from the bone marrow of transgenic GFP C57BL/6J male mice and systemically administered to wild-type C57BL/6J female mice with bone marrow suppression by lethal irradiation. The donor BMSCs engraftment and differentiation were then determined in the LPS-injured lungs of recipients 14 days after BMSCs transplantation by immunofluorescent staining. This study showed that GFP^+ and $cytokeratin^+$ cells were observed in the alveolar walls in bone marrow suppressed mice on day 14 after BMSCs transplantation. To further quantify the levels of donor derived cells, alveolar epithelial type II cells were isolated from the lungs of recipients and the percentage of cells with positive GFP were measured by flow cytometry analysis. The data showed that GFP positive cells increased significantly in preparations of type II epithelial cells isolated from bone marrow-suppressed recipient mice. These results strongly suggested that donor derived BMSCs can migrate, engraft and differentiate into epithelial cells in injured lungs of recipient mice. Such changes were not found in lungs of mice without irradiation. This suggests that systemically administered BMSCs might home to bone marrow, then migrate to injured tissue, for proliferation and differentiation.

This study further investigated the contribution of BMSCs to damaged lung tissue. Although no differences were observed in lung pathologic changes 14 days after LPS administration among different treated mice, the data did show that more donor derived cells were detected in the irradiated and LPS-injured lungs (the LIM group) than those in the CIM group. Moreover, the result of flow cytometry analysis also showed an increased percentage of GFP-positive type II epithelial cells in the LIM group (about 10%) compared with that in the CIM group. Previous studies reported a percentage of less than 5%[20-21]. The study of Yamada et al[11] showed that the suppression of bone marrow by sublethal irradiation before intrapulmonary LPS led to disruption of tissue structure and emphysema-like changes, and the administration of bone marrow-derived progenitor cells could accumulate within the damaged lungs, differentiate into endothelial and epithelial cells and thus prevent these pathogenic changes. Mei et al[22] reported the administration of bone marrow-derived mesenchymal stem cells significantly reduced LPS-induced pulmonary inflammation, as reflected by reductions in inflammatory cells and proinflammatory cytokines. These data suggest that the bone marrow-derived progenitor cells are important and required for lung repair after LPS-induced lung injury.

There are controversial findings as to how long donor bone marrow-derived cells may differentiate into alveolar epithelial cells in recipient lungs[12,23,24]. Kotton et al[12] reported that by intravenous delivering lacZ-labeled cells into wild-type recipient mice after bleomycin-induced lung injury, marrow-derived cells engrafted in injured recipient lung parenchyma as cells with the morphological and molecular phenotype of type I pneumocytes of the alveolar epithelium 5 days after cell transplantation. Rojas and colleagues[23] found that donor bone marrow derived stem cells could home to the injured lungs of bleomycin-administered mice and differentiate into type I and type II pneumocytes after 14 days. This study also found that donor derived alveolar epithelial cells (GFP^+ $cytokeratin^+$ cells) were not observed in recipient lungs 7 days after BMSCs transplantation (data not shown), but were observed on day 14.

The findings of this study demonstrated the possibility of the engraftment of BMSCs in damaged lungs after intravenous administration. But in this study, because most mice without BMSCs administration died before the experiment endpoint after lethal irradiation including the mice of the LI group, the lung pathologic changes of these animals were not acquired on the endpoint as the control of the LIM group. Other indexes reflecting injury and repair were not measured in this study, for example the changes of collagen deposition, proinflammatory and anti-inflammatory factors. Therefore, this study has limitations as to whether donor derived BMSCs can alleviate LPS-induced lung injury and contribute to proper repair following lung injury. Furthermore, this study did not investigate the functions of type II pneumocytes derived from donor cells, for ex-

ample, the involvement of phospholipids metabolism, secretion of lamellar body, and differentiation into type I pneumocytes as lung progenitor cells. Further studies are needed to be performed in these fields so that the roles of bone marrow derived cells in lung repair may be revealed, which may enable explanation of mechanism of BMSCs in future clinical application.

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