



Bone marrow mesenchymal stem cells protect lungs from smoke inhalation injury by differentiating into alveolar epithelial cells via Notch signaling

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To examine the protective effect of transplanting bone marrow mesenchymal stem cells (BMSCs) in treating lung injury induced by smoke exposure and to investigate the underlying mechanisms of this protection. SD rats were randomly divided into four groups: normal group, normal + BMSC^{GFP} group, smoke group, and smoke + BMSC^{GFP} group. To detect lung injury, we measured arterial blood gas, the wet-to-dry weight ratio, and levels of interleukin-1 β , tumor necrosis factor- α , interleukin-10, and interleukin-13 in bronchoalveolar lavage fluid and lung tissues. We also conducted histopathology examinations. The protein markers of alveolar epithelial cells were measured to determine the BMSC differentiation. The protein levels of Notch1, Jagged-1, and Hes-1 also were detected. In the present study, BMSC transplantation significantly decreased the wet-dry weight ratio of the lung, reduced the production of inflammatory mediators, and alleviated lung injury simply through differentiating into alveolar type II cells and alveolar type I cells. Western blot analysis confirmed that the protein expression of Notch-1, Jagged-1, and Hes-1 increased significantly after systemic BMSC transplantation. No significant difference was observed between the normal group and the normal + BMSC^{GFP} group. Our findings indicate that systemic transplantation of BMSCs alleviated lung injury induced by smoke exposure, which may be associated with BMSCs' ability to differentiate into alveolar-type cells via the Notch signaling pathway.

Keywords. Alveolar epithelial cells; bone marrow mesenchymal stem cells; differentiation; lung injury; Notch signaling; smoke inhalation

1. Introduction

Smoke inhalation injury is an acute lung injury (ALI) and leading cause of acute respiratory failure in patients with critical burn trauma (David *et al.* 2009). It is an important contributor to mortality in people exposed to fire (Toon *et al.* 2010). More than 30% of burn patients may have comorbid inhalation injuries, which increases burn-related mortality by 20%, and it is responsible for 30%–90% of deaths caused by smoke inhalation and burns (Ballardcroft *et al.* 2010). During smoke inhalation, the human respiratory system undergoes a series of pathophysiological changes, such as thickening of the alveolar walls, protein exudation from the alveolar spaces and septa, accumulation of inflammatory cells and cytokines, and pulmonary edema.

Bone marrow mesenchymal stem cells (BMSCs) are multipotent adult stem cells that are mainly found in the

bone marrow. BMSCs can differentiate into multiple lineages (Yu *et al.* 2011). Many animal models have shown that BMSCs have a remarkable ability to localize to injured or inflammatory sites, where they exert nonimmunogenic and immunosuppressive characteristics (Jones and McTaggart 2008; Reagan and Kaplan 2011). These properties make BMSCs an attractive option for cell-based treatment of various disorders, including respiratory disease (Skrahin *et al.* 2014; Wilson *et al.* 2015). BMSCs home to injured lung tissues in inflammatory lung diseases, including asthma, pulmonary hypertension, chronic obstructive pulmonary disease, and ALI, where they help resist lung injury by differentiating into multiple functional cells (Gupta *et al.* 2007; Iyer *et al.* 2009; D'Agostino *et al.* 2010). However, research about the therapeutic effects of BMSC transplantation on smoke inhalation-induced ALI is lacking.

The current study aimed to investigate whether BMSC treatment can ameliorate ALI caused by smoke inhalation and to determine the underlying mechanisms of this process. Using a rat model, we focused on BMSC differentiation into alveolar epithelial cells. Understanding the role of BMSCs in the lung injury response to smoke exposure contributes to understanding their therapeutic effects in ALI.

2. Materials and methods

2.1 Isolation and culture of BMSCs

BMSCs were isolated from the femur and tibia of male SD rats, as previously described (Gao *et al.* 2015). Briefly, the ends of the bones were cut, and the marrow was extruded with medium using a needle and syringe. The cell suspensions were plated on 25 cm² plastic flasks and supplemented with Dulbecco's Modified Eagle's medium with 10% fetal bovine serum and 1% glutamine (Cyagen Biosciences, Santa Clara, CA, USA). The cells were then incubated at 37°C in 5% CO₂. After incubation for 24 h, the non-adherent cells were removed by replacing the medium. The medium was replaced every 3 days. When the adherent cells grew to approximately 90% confluency, they were trypsinized and passaged at 1:2 ratio. All experiments described in the present study were performed using cells from passages 5–8.

2.2 Fluorescence-activated cell sorting analysis

BMSCs were harvested with trypsin containing 0.25% EDTA, washed with phosphate-buffered saline, and resuspended in phosphate-buffered saline supplemented with 2% fetal bovine serum. Then, the cells were stained with antibodies against CD29, CD90, CD34, and CD45 (BD Biosciences, Mountain View, CA, USA) for 30 min at 4°C and then analyzed on a FACS Caliber Flow cytometer (BD Biosciences).

2.3 Differentiation potential of BMSCs

To determine the differentiation potential of BMSCs, we cultured the cells in osteogenic or adipogenic differentiation medium, as previously described (Song *et al.* 2016). The adipose cells were stained for visualization with Oil Red O. The osteogenic cells were fixed with formaldehyde solution and stained with Alizarin Red. The stained cells were visualized by a light microscope.

2.4 Lentiviral vector construction and infection

Lentiviral vectors carrying a green fluorescent protein (GFP) reporter gene were constructed and purified by GeneChem

Co., Ltd. (Shanghai, China). Infection was induced, as previously described (Song *et al.* 2016). Briefly, the BMSCs were seeded into 25 cm² flasks containing appropriate growth medium and grown to 20%–30% confluence. Then, the GFP lentivirus vectors were added at a multiplicity of infection (MOI) of 10. The cells were incubated with the viruses for 10 h, after which fresh medium was added to each flask. Cells then were incubated for another 48 to 72 h. The infection efficiency was about 80%. The cells were then passaged at a 1:2 ratio, named as BMSCs^{GFP}.

2.5 Animals

Male Sprague–Dawley (SD) rats, aged 6 to 8 weeks, were purchased from the experimental animal center of the Fourth Military Medical University. All animals used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The study protocol was approved by the Laboratory Animal Ethics Committee of the Fourth Military Medical University.

2.6 Rat model of smoke inhalation

Models of smoke inhalation were established using a previously described device that was constructed in-house (Song *et al.* 2016). To accomplish this, the rats were exposed to combustion smoke generated by smoldering wood shavings in a smoke-generating container that was connected to a 20 L transparent exposure chamber. The rats were subjected to the smoke for 24 h. Smoke inhalation was assessed by blood gas analysis, measurement of the wet/dry weight ratio of lung tissue, and lung histopathology.

2.7 Bioluminescent imaging of rats injected with BMSCs^{GFP}

The SD rats were randomly divided into four groups: normal, normal + BMSC^{GFP}, smoke, and smoke + BMSC^{GFP}. Each group included 20 rats. At 24 h after smoke inhalation, 100 µL aliquots of BMSC^{GFP} (2×10^6 cells) were injected into the tail veins of rats in the normal + BMSC^{GFP} and smoke + BMSC^{GFP} groups. At 4 h, 12 h, and 24 h after injection, bioluminescent imaging was used to visualize the localization of the BMSC^{GFP}, as previously described (Kidd *et al.* 2009).

2.8 Arterial blood gas analysis

The rats were sacrificed by aortic puncture at 3 days after BMSC^{GFP} administration. The blood samples were immediately analyzed for arterial blood gas. Arterial oxygen

tension (PaO₂) and arterial carbon dioxide tension (PaCO₂) were measured with a blood gas analyzer (Copenhagen, Denmark).

2.9 Analysis of wet/dry weight ratio of lung tissue

The rats were killed 3 days after BMSC^{GFP} administration, and their left lungs were isolated to analyze the wet/dry weight ratio. After blotting of blood and other contaminants, the wet weights of the lung tissue samples were measured. Then, the lungs were dried in an oven at 70°C for 72 h. Dry lung weights then were measured and wet/dry weight ratios calculated.

2.10 Histology

The rats were killed 3 days after BMSC^{GFP} administration, and their right lungs were isolated and fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned into 5-mm-thick slices. Then, the samples were stained with hematoxylin and eosin and observed under an optical microscope.

2.11 Analysis of inflammatory factors

Bronchoalveolar lavage fluid was pooled and centrifuged, as previously described (Song *et al.* 2016). Interleukin (IL)-6, tumor necrosis factor (TNF)- α , IL-10, and IL-13 levels in the supernatant of bronchoalveolar lavage fluid and lung tissues were analyzed using ELISA kits (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) following the manufacturer's instructions.

2.12 Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using 1 ml of Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and then reverse-transcribed into cDNA using Primescript RT Reagent (Takara, Dalian, China). Quantitative reverse transcription PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Shanghai, China). PCR programs were carried out as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension for 5 min at 72°C. GAPDH expression was used as an endogenous control. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.13 Western blot

Total protein was extracted using 1 ml of cell lysis buffer (Cell Signaling Technology, Shanghai, China). Protein

concentrations were measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated using sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk, the membranes were incubated with primary antibodies overnight at 4°C. After washing thrice with Tris-buffered saline, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies at room temperature for 1 h. The protein band was detected using an electrochemiluminescence reagent (Millipore). The relative intensities of protein bands were quantified using Image J software. β -actin was used as the internal control.

2.14 Statistical analysis

All data were processed using GraphPad Prism 5 software. The data are shown as the mean \pm standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by the Bonferroni test. $P < .05$ was considered significant.

3. Results

3.1 Identification of bone marrow mesenchymal stem cells

The isolated BMSCs were observed under a microscope. A morphologically homogeneous population of spindle-shaped and fibroblast-like cells was observed on the 6th day (figure 1A). Fluorescence-activated cell sorting analysis of the cell surface phenotype indicated that the cells were uniformly positive for CD29 and CD90, but negative for CD34 and CD45 (figure 1B), which defines a mesenchymal stem cell. Additionally, the cells had the potential for differentiation along the osteogenic and adipogenic lineages, as determined by staining with Alizaran Red and Oil Red O, respectively, following 3 weeks of culturing in differentiation medium. These results indicated multipotency of the cells, thus verifying their identity as mesenchymal stem cells (figure 1C and D).

3.2 Transplanted BMSCs homed to lung tissues and attenuated lung injury in rats exposed to smoke inhalation

BMSCs infected with lentivirus containing GFP were injected into the tail veins of SD rats with or without smoke inhalation. A live-animal bioluminescent signal was then used to monitor BMSC^{GFP} localization patterns. As shown in

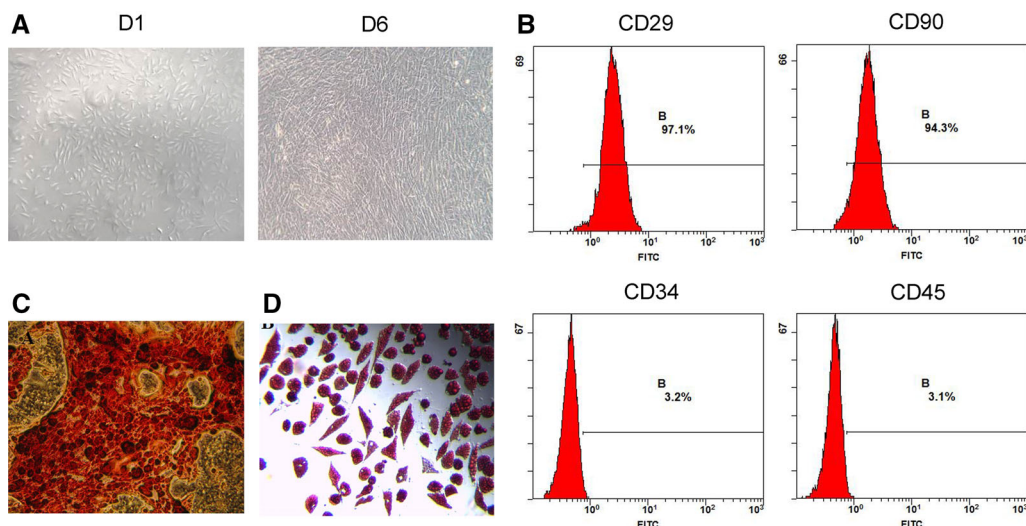


Figure 1. Morphology and characterization of BMSCs phenotype. (A) Morphology of BMSCs on the first and sixth days. (B) Flow cytometry analysis of the cell surface phenotype. Multilineage differentiation capacities of BMSCs, including (C) osteogenic differentiation stained with Alizarin Red and (D) adipogenic differentiation stained with Oil Red O, as observed with a microscope.

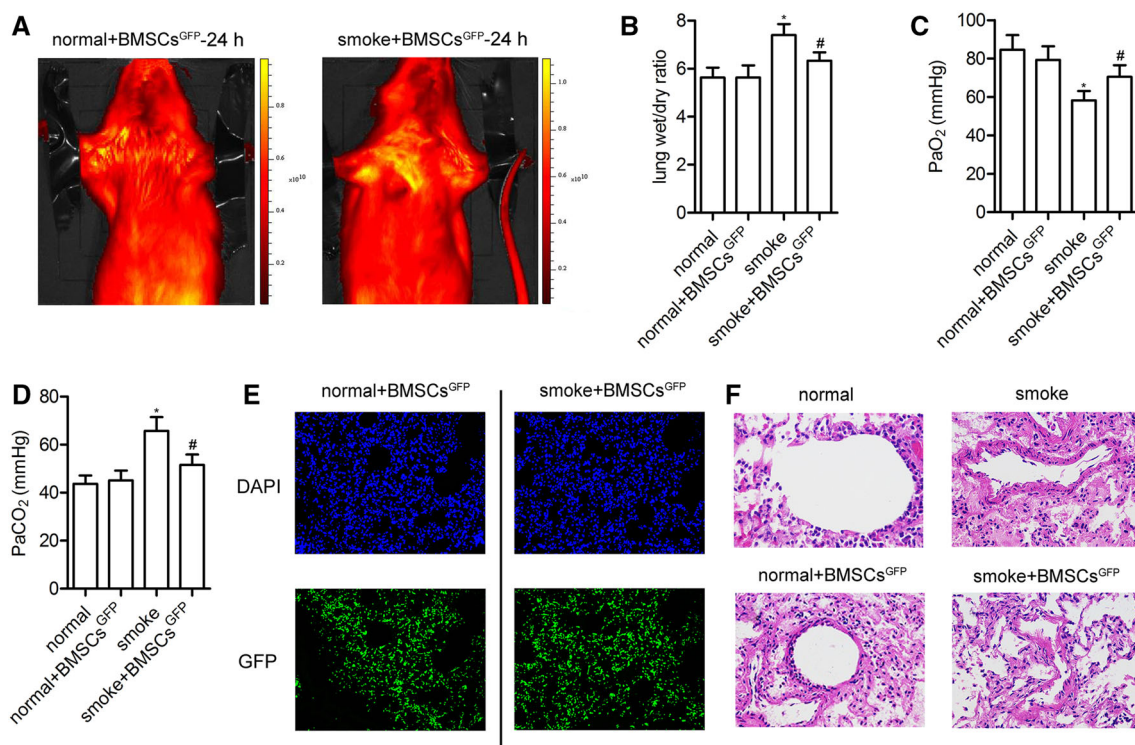


Figure 2. BMSCs attenuated lung injury by homing to the lung tissue exposed to smoke inhalation. (A) BMSCs infected with lentivirus containing GFP were injected into the tail veins of rats with or without smoke inhalation injury and imaged at 24 h after injection. (B) The ratio of wet to dry lung was measured. Normal group, untreated rats; normal + BMSC^{GFP} group, normal rats injected with BMSC^{GFP}; smoke group, rats exposed to smoke inhalation; smoke + BMSC^{GFP} group, rats exposed to smoke inhalation and injected with BMSC^{GFP}. PaO₂ (C) and PaCO₂ (D) levels were measured. **P* < .05, compared with the normal group, #*P* < .05, compared with the smoke group. (E) Representative fluorescence microscopic images of lung sections. (F) Representative histological images of lung sections (*n* = 6).

figure 2A, compared with the normal + BMSC^{GFP} group, stronger bioluminescent signals were produced in the smoke + BMSC^{GFP} group at 24 h. Three days after injecting

BMSCs into the rats, we evaluated the effects of the cells by measuring arterial blood gas and lung wet/dry weight ratios. We also evaluated pathological changes in the lung tissues.

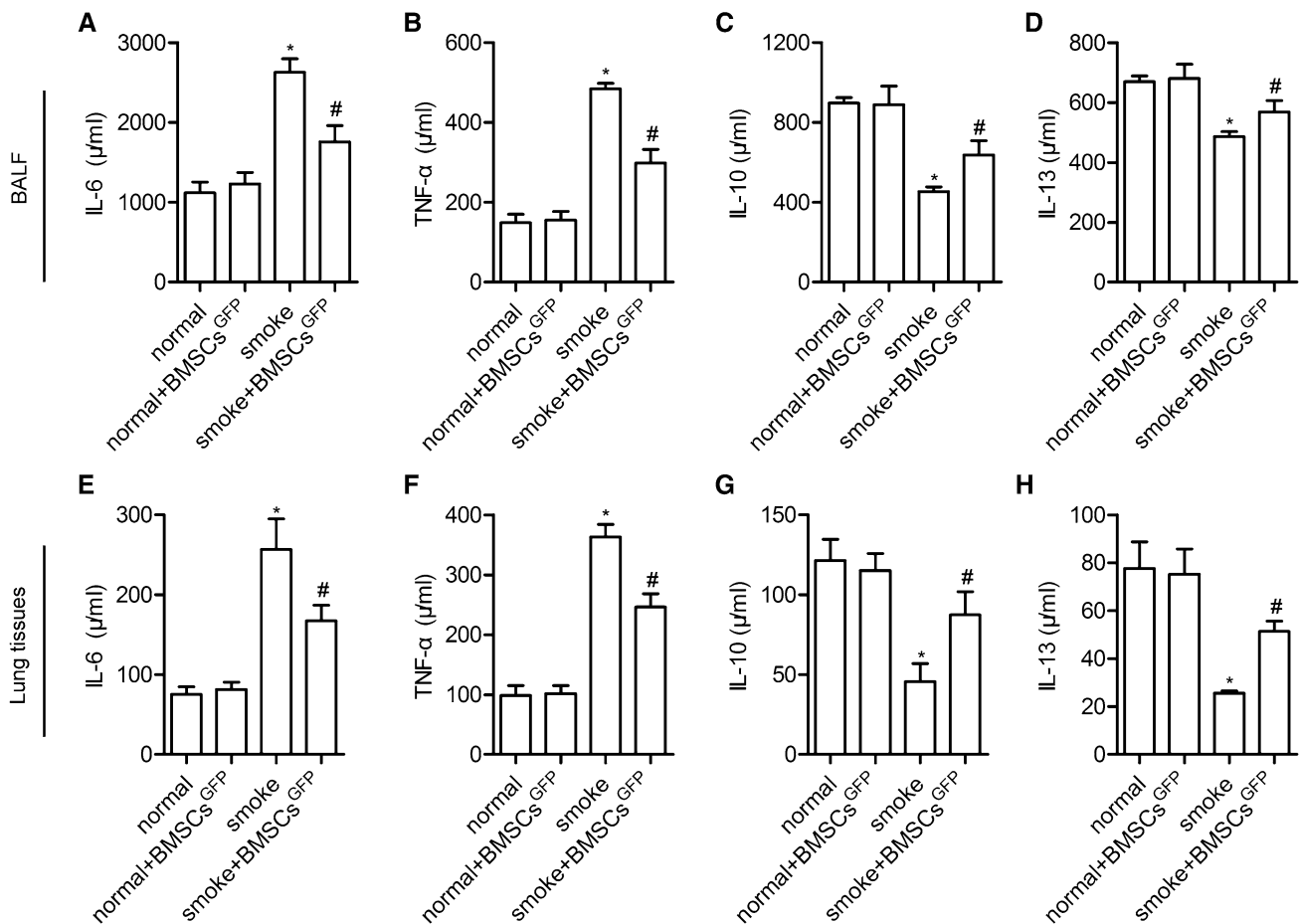


Figure 3. Transplanted BMSCs regulated the production of inflammatory cytokines in rats exposed to smoke inhalation. Levels of (A) interleukin (IL)-6, (B) tumor necrosis factor α , (C) IL-10, and (D) IL-13 in the bronchoalveolar lavage fluid were examined by ELISA assay. Levels of (E) IL-6, (F) TNF- α , (G) IL-10, and (H) IL-13 in the lung tissues were examined by ELISA assay. * $P < .05$, compared with the normal group, # $P < .05$, compared with the smoke group.

The lung wet/dry ratios in the smoke + BMSC^{GFP} group were significantly lower than those in the smoke group (figure 2B), indicating that the administration of BMSCs decreased lung edema induced by smoke inhalation. As shown in figure 2C and D, compared with the normal group, the PaO₂ level decreased and PaCO₂ level increased in the smoke group. Compared with the smoke group, the PaO₂ level increased and PaCO₂ level decreased in the smoke + BMSC^{GFP} group.

As shown in figure 2E, the fluorescence microscopic data visualized GFP positive cells in the lung tissues of BMSCs-treated rats. Histopathology results showed that exposure to smoke led to serious pathological changes in the lungs, including narrowed alveolar space, thickened alveolar walls, and inflammatory cell infiltration, all of which was alleviated by administering BMSCs (figure 2F). No obvious differences were observed between the normal and normal + BMSC^{GFP} groups. These results indicate that BMSC transplantation protected rats from lung injuries produced by smoke inhalation.

3.3 Transplanted BMSCs regulated production of inflammatory cytokines in rats exposed to smoke inhalation

We measured the production of inflammatory cytokines to determine the effects of BMSC injection on lung inflammation induced by smoke inhalation. As shown in figure 3A–D, smoke inhalation induced elevations in IL-6 and TNF- α and reductions in IL-10 and IL-13 in the bronchoalveolar lavage fluid. BMSC^{GFP} treatment significantly decreased levels of IL-6 and TNF- α , but increased IL-10 and IL-13, compared with the smoke group.

The changes in inflammatory cytokines in the lung tissues were consistent with that in the bronchoalveolar lavage fluid. Elevated levels of IL-6 and TNF- α in the lung tissues that were exposed to smoke inhalation were markedly suppressed in the smoke + BMSC^{GFP} group. The reduced levels of IL-10 and IL-13 in the lung tissues of rats in the smoke group were significantly increased by treatment with BMSC^{GFP} (figure 3E–H). No significant differences were

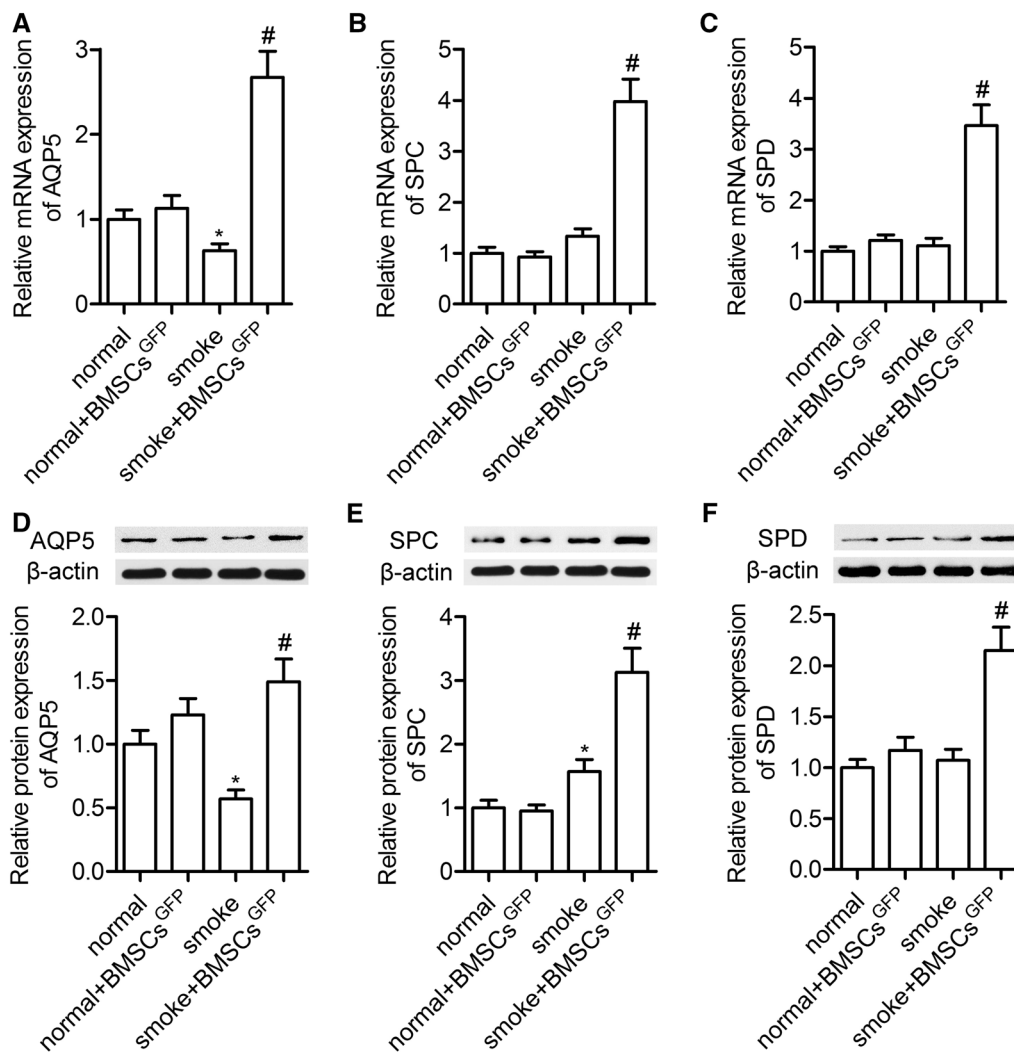


Figure 4. Transplanted BMSCs differentiated into alveolar epithelial cells in lung tissues of rats exposed to smoke inhalation. The mRNA expression levels of (A) AQP5, (B) SPC, and (C) SPD in the lung tissues were measured by RT-PCR assay. The protein expression levels of (D) AQP5, (E) SPC, and (F) SPD in the lung tissues were measured by western blot assay. * $P < .05$, compared with the normal group, # $P < .05$, compared with the smoke group.

observed in inflammatory cytokines between the normal group and the normal + BMSC^{GFP} group.

3.4 Transplanted BMSCs differentiated into alveolar epithelial cells in lung tissues of rats exposed to smoke inhalation

The mRNA and protein levels of AQP5 (a marker of alveolar type I cells) and of SPC and SPD (markers of alveolar type II cells) were evaluated by qRT-PCR and western blot to assess BMSC differentiation. Compared with rats in the normal group, the mRNA and protein levels of AQP5 in rats in the smoke group were significantly downregulated, and the protein expression level of SPC in rats in the smoke group was significantly upregulated (figure 4A, D, E). These results suggest that smoke inhalation induced a disturbance in alveolar epithelial cells. However, we observed no changes in the

mRNA levels of SPC and SPD or the protein level of SPD after smoke inhalation (figure 4B, C, F). Compared with rats in the smoke group, the mRNA and protein expression levels of AQP5, SPC and SPD were markedly upregulated in rats in the smoke + BMSC^{GFP} group (figure 4A–F). This result suggests that BMSCs homed to the injured lung tissues and differentiated into both alveolar type I and alveolar type II cells. No significant difference was observed between rats in the normal group and the normal + BMSC^{GFP} group.

3.5 Transplanted BMSCs upregulated Notch-1 expression in lung tissues of rats exposed to smoke inhalation

The mRNA and protein levels of Notch-1, Jagged-1, and Hes-1 were evaluated by qRT-PCR and western blot to examine whether Notch signaling participated in the

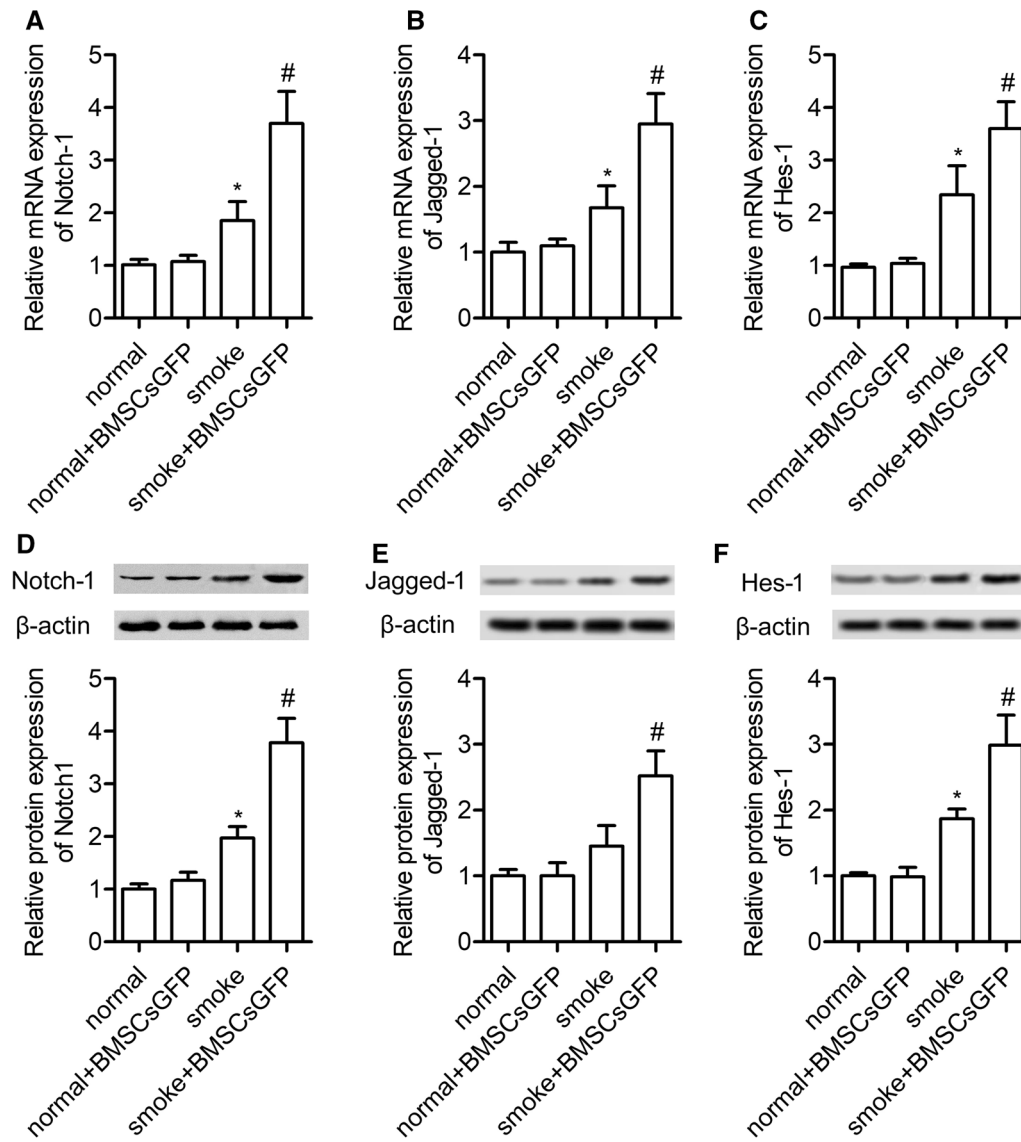


Figure 5. Transplantation with BMSCs enhanced Notch signaling in the lung tissues of rats exposed to smoke inhalation. The mRNA expression levels of (A) Notch-1, (B) Jagged-1, and (C) Hes-1 in the lung tissues were measured by RT-PCR assay. The protein expression levels of (D) Notch-1, (E) Jagged-1, and (F) Hes-1 in the lung tissues were measured by western blot assay. * $P < .05$, compared with the normal group, # $P < .05$, compared with the smoke group.

protective roles observed in rats treated with BMSC^{GFP}. As shown in figure 5A–F, compared with rats in the normal group, the mRNA and protein levels of Notch-1, Jagged-1, and Hes-1 in rats in the smoke group were significantly upregulated, suggesting that smoke inhalation enhanced Notch signaling. Compared with rats in the smoke group, the mRNA and protein levels of Notch-1, Jagged-1, and Hes-1 in rats in the smoke + BMSC^{GFP} group were further upregulated, suggesting that enhanced Notch signaling may be associated with the homing and differentiation ability of BMSCs in response to lung inflammation and lung injury. No significant difference was observed between rats in the normal group and the normal + BMSC^{GFP} group.

4. Discussion

Due to their capacity for multilineage differentiation, BMSCs have been considered one of the most likely sources of endogenous tissue repair. Previous studies of several animal models have confirmed that transplantation with BMSCs significantly reduces lung damage (Wang *et al.* 2012; Zhang *et al.* 2012; Huang *et al.* 2015; Li *et al.* 2016). Systematic or intratracheal administration of BMSCs alleviates pathological impairment, promotes rehabilitation of alveolar epithelium, and inhibits the inflammatory response in several ALI models (Ortiz *et al.* 2003; Gupta *et al.* 2007; Zhao *et al.* 2008; Lee *et al.* 2009). In the present study, we

established a rat model of smoke inhalation that was characterized by systemic inflammation and severe lung injury, including increased pulmonary edema, decreased PaO₂, increased PaCO₂, and serious pathological changes in the lungs. These injuries were significantly attenuated by transplantation with BMSCs, suggesting that BMSCs could protect the lung against smoke inhalation-induced injuries.

BMSCs have potent anti-inflammation activities (Németh *et al.* 2009; Mei *et al.* 2010). Transfusion with BMSCs can inhibit inflammatory and autoimmune diseases (Németh *et al.* 2009; Mei *et al.* 2010). The role of BMSCs in inflammation is complex and involves multiple cell types, growth factors, and signaling pathways. They inhibit inflammation by reducing levels of systemic TNF- α and IL-6 and increasing levels of inhibitory cytokines, such as IL-10 and TGF- β 1 (Krasnodembskaya *et al.* 2012). Consistent with previous studies, we found that smoke inhalation increased IL-6 and TNF- α and decreased IL-10 and IL-13 in the bronchoalveolar lavage fluid and lung tissues of rats. Rats in the BMSC^{GFP} treatment group showed significantly decreased levels of IL-6 and TNF- α and increased levels of IL-10 and IL-13, compared with rats in the smoke group.

Recently, the possibility of differentiation of BMSCs into alveolar epithelial cells in vitro and in vivo has been brought forward (Gupta *et al.* 2007; Ma *et al.* 2011). This differentiation ability may help maintain the integrity of alveolar epithelium in ALI. BMSCs can be engrafted in injured lung tissue and induced to differentiate into alveolar type II epithelial cells, which are characterized by their synthesis and secretion of alveolar surfactant to reduce surface tension and inhibit collapse of the alveoli and by their ability to differentiate into the alveolar type I epithelial cells, which serve as progenitor cells for reepithelization of the injured lung tissue (Matthay *et al.* 2005; Rojas *et al.* 2005). Repair and regeneration of injured alveolar type II cells are critical steps in recovery from ALI (Phua *et al.* 2009).

In our present study, we found that BMSCs homed to the injured lung tissues. The expression of AQP5 (a marker of alveolar type I cells) and of SPC and SPD (markers of alveolar type II cells) was markedly upregulated in rats exposed to smoke inhalation and injected with BMSC^{GFP}, compared with the rats exposed to smoke inhalation only. These findings suggest that the homing BMSCs may differentiate into both alveolar type I cells and alveolar type II cells to prevent smoke inhalation-induced lung injuries.

Elucidation of the mechanisms underlying the engraftment and differentiation of MSCs in injured lung tissue could improve the therapeutic effects of BMSC-based treatments. In our study, we found that the canonical Notch signaling pathway participated in the differentiation of BMSCs into alveolar epithelial cells. However, further in vitro and in vivo experiments should be performed to confirm and clarify this modulation.

Taken together, the results demonstrate that smoke inhalation induced impaired arterial blood gas, and induced pulmonary edema, histopathologic changes, and an

inflammatory response in lung tissues. Moreover, these changes were partly ameliorated by BMSC treatment, whereby BMSCs differentiated into alveolar-type cells in the lung tissues. The Notch signaling pathway was involved in this differentiation process. Thus, treatment with BMSC may effectively treat ALI that is induced by smoke inhalation.

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