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Mesenchymal stem cells ameliorate LPS-induced acute lung injury through KGF promoting alveolar fluid clearance of alveolar type II cells

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Abstract. – OBJECTIVE: It has been shown that Mesenchymal stem cells (MSCs) could prevent or alleviate acute lung injury (ALI) when transplanted into lung; however, the mechanisms involved remains elusive. The study aimed to investigate the effect of MSCs in repairing alveolar fluid clearance (AFC) of alveolar type-II (AT-II) cells through paracrine factors.

MATERIALS AND METHODS: Keratinocyte growth factor (KGF) concentration in the supernatant of MSC culture medium was measured, and MSCs in lung after intravenous administration was traced. Next, MSCs transfected with or without KGF SiRNA were injected intravenously into LPS-induced ALI rats. Histological change and wet/dry ratio were determined. AT-II cells were co-cultured with MSCs under different experimental situations to analyze the variation of α1 and β1 subunits of Na^+-K^+-ATPase in AT-II cells.

RESULTS: LPS-induced ALI was characterized by the typical inflammatory infiltrates, interalveolar septal thickening and increased wet/dry ratio. MSC administration significantly reduced the injury, while MSCs with KGF knockdown did no show the same effect. *In vitro* study also confirmed that expressions of α1 and β1 subunit were up-regulated as impaired AT-II cells co-cultured with MSCs. Furthermore, expression of α1 subunit was down-regulated, while β1 subunit showed no apparent change as AT-II cells were co-cultured with MSCs that were transfected with KGF siRNA.

CONCLUSIONS: AFC was impaired by inflammation insult. MSCs-derived KGF reduced the impaired AFC through up-regulated α1 subunit but not β1 subunit, which might provide a novel therapeutic strategy for ALI.

Key Words:
Acute lung injury, Alveolar fluid clearance, Mesenchymal stem cell, Alveolar type II cell, Na^+-K^+-ATPase.

Abbreviations

ALI = acute lung injury; ARDS = acute respiratory distress syndrome; AFC = alveolar fluid clearance; MSC = Mesenchymal stem cell; AT-II = alveolar type-II cell; qPCR = quantitative real time PCR; KGF = keratinocyte growth factor.

Introduction

Acute respiratory distress syndrome (ARDS), the severe stage of acute lung injury (ALI), is associated with high morbidity and mortality in medical practice. Patients with ALI or ARDS eventually died owing to severe pneumonedema and hypoxia[1]. Currently, respiratory support and conservative fluid therapy are considered the main strategies in treating ALI; however, the therapeutic effect remains limited[2,3]. The pulmonary biotherapy appears to have the inherent advantage due to potential large-scale retention when delivering cellular therapy to the lung via a simple intravenous approach. Even more attractively, the retained cells can target the areas of injured lung.

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Mounting evidence from various *in vivo* experimental and preliminary clinical studies suggest that mesenchymal stem cell (MSC) was an attractive cell therapy candidate for ALI[4]. Notwithstanding the multifaceted activity of MSC demonstrated by these studies, its precise role remains incompletely defined. Currently, increasing data suggest that the therapeutic effects of MSCs in organ function are largely mediated through paracrine factors[5-9]. Of particular note are the studies that demonstrated the high keratinocye growth factor (KGF) level of MSC in response to ALI[9].

Impaired alveolar fluid clearance (AFC) of alveolar type II cells (AT-II cells) always leads to higher mortality in ALI patients[11-13]. However, there is little evidence clarifying whether MSCs can beneficially influence restoration of AFC ability and the role of MSC involved. In the present study, we aimed to investigate whether MSCs can provide beneficial effects in an LPS-induced ALI rat model by improving AFC ability, which may be related to their KGF production.

Materials and Methods

All animal procedures were approved by the Animal Care Committee of Chinese PLA General Hospital (Beijing, China).

MSC Culture and Identification

MSCs were isolated from the bone marrow (BM) of the tibiae and femurs of 8-week old male Sprague Dawley (SD) rat (HFK BIOSCIENCE Co., Ltd, Beijing China). As cells reached 80-90% confluence, MSCs were passaged every 3-4 days and used for experiments between the 3rd and 6th passage. MSCs were cultured with Dulbecco's Modified Eagle Medium (DMEM) (Gibco by Life Technologies, Grand Island, NY, USA) containing 1% glutamine, 100 □/ml penicillin, streptomycin and 2% Fetal Bovine Serum (Gibco, Carlsbad, CA, USA) in a humidified incubator (5% CO_2 and 37°C) under sterile conditions. MSCs were identified with phenotypic markers by flow cytometry (BD Biosciences, San Jose, CA, USA). Pohycoerythrin- and fluorescein isothiocyanate-conjugated antibodies containing CD34, CD45, CD29, CD44, CD90 and CD105 were used for detecting the MSC surface markers (BD Biosciences, San Jose, CA, USA). MSCs (5×10^4 cells per well) were cultured with adipogenic or osteogenic induction medium (Cyagen Biosciences, Guangzhou, China) until they reached 90% confluence. After 2 weeks, MSCs were stained with oil red O or alizarin red in culture plate.

Primary Culture of AT-II cells

AT-II cells were isolated from male SD rat according to the previous study[14]. AT-II cells were then cultured with DMEM/F12 containing 2% FBS, 100U/ml penicillin and 100 U/ml streptomycin (Gibco by Life Technologies, Grand Island, NY, USA). Cells were identified by surfactant protein A (SP-A) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and goat anti-rabbit secondary antibody (ZSGB-BIO, Beijign, China) by immunofluorescence technique. Images were taken under confocal microscopy (Leica TCSSP8, Wetzlar, Germany).

Building Co-culture System and AT-II Cell Proliferation Assay

AT-II cells (5×10^5 cells) were seeded into each transwell chamber and placed in a 6-well plate (0.4 m pore size and collagen I-coated insert, Corning, Corning, NY, USA), in which the bottom of each well was covered with MSCs (5×10^5 cells pre well). To induce cell injure, AT-II cells were then exposed to the major proinflammatory cytokines (PC) including TNF-α, IL6 and IL1- (1.7 ng/ml, 87.6 ng/ml and 4.4 ng/ml, respectively) (Peprotech, Rocky Hill, NJ, USA) according to the previous study[15]. Cell proliferation assay was carried out using a CCK8 kit (Beyotime, Jiangsu, China) according to the manufacturers' protocol on the following groups (n = 5 per group): (1) AT-II cells alone without any treatment; (2) PC-exposed AT-II cells; (3) PC-exposed AT-II cells + MSCs. The optical density (OD) was then measured with a microplate reader at the wavelength of 450 nm (Spectra MR Dynex, Technologies, Chantilly, VA, USA). Cell proliferation was continuously monitored for three days after inflammatory insult.

KGF Concentration Detected with ELISA

MSCs were either exposed to PC (TNF-α 1.7 ng/ml, IL6 87.6 ng/ml and IL1-β 4.4 ng/ml) or cultured in normal medium for 1/2h, 6h, 24h and

72h (n = 3 per group). MSCs were starved using serum-free medium for 24h before AT-II cells were exposed to PC. Concentrations of keratinocyte growth factor (KGF) secreted by MSCs (5×10^5 cells) in the supernatant of culture medium were measured with rat KGF specific ELISA kit (RD rat-specific KGF ELISA kit, Bioscience, San Diego, CA, USA).

KGF Knockdown with siRNA

Using lipofectamine 2000 (Invitrogen by Life Technologies, Carlsbad, CA, USA), 5×10^5 cells MSCs were transfected with small interfering RNA (siRNA) to knock down KGF expression according to the manufacturers' instructions. The following KGF siRNA was used: (3'-dT-dTCGCUGUGUGCUCUUCAAUA-5' (siG1312692455)) (Ribobio Co., Ltd, Guangzhou, China). Additionally, mock transfection was performed as well. Pictures of MSCs transfected with cy5-labeled negative siRNA was taken under confocal microscopy. Knockdown efficiency was evaluated by examining the KGF levels in supernatant of MSCs, MSCs transfected with KGF siRNA (MSCs-SiRNA) and MSCs transfected with only lipofectamine (MSCs-lipof) at 48h after transfection.

MSC Tracing Study

Male rats were randomly assigned to five different groups (n=5 per group). ALI rat model were established by intravenous administration of 10 mg/kg lipopolysaccharide (LPS) (*Escherichia coli* 055:B5; Sigma, St. Louis, MO, USA) dissolved in normal saline. MSCs were digested into single cells with 0.25% trypsase and stained with cell membrane dye DiI. DiI-stained MSCs (5×10^5 cells) were then injected via tail vein 4h after LPS administration. Rats were sacrificed to determine the quantity of MSCs retaining in lung after injection at different time points. To eliminate the influences deriving from rat hair and thoracic wall on the emitted light from DiI-stained MSCs, the rat lung was excised for tracing (n = 5 per time point) and quantitation with small animal imaging technology (Bruker *in-vivo* FX Pro imaging system) at 15 min, 2h, 24h and 72h after MSC administration. Lung tissue was then stored in OCT glue immediately. Frozen sections (6 μm in thickness) were made at 15 min, 24h and 72h to determine the distribution of MSCs. Meanwhile, cell nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI).

Pathological Assessment and Wet/Dry Analysis

All 7-8 week old male rats were randomly assigned to six different groups (n=8 per group). Rats were intravenously injected with 10 mg/kg lipopolysaccharide (LPS) followed by phosphate buffered saline (PBS), MSCs, MSCs-SiRNA or MSCs-lipof (5×10^5 cells, 300 μl at final volume) respectively 4h later. Meanwhile, rats without LPS treatment were injected with PBS or MSCs to observe possible inflammatory reaction caused by PBS or MSCs. All rats were sacrificed at 72h. Left lung lobes were harvested, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5- m thick sections which were stained with hematoxylin and eosin (H&E), and images were taken under Leica CTR4000 microscope (Leica, Wetzlar, Germany). Concurrently, right rat lung lobes (n=8 per group) were excised and their wet weights were recorded before further placing them in the incubator at 65°C for 3 days to obtained their dry weights, and the ratio of wet/dry was then determined[16,17].

Detection of α1 and β1 Subunits with Immunofluorescence and Confocal Microscopy

AT-II cells were seeded on the sterile glass slide in culture plate (60 mm × 15 mm) at the density of 1×10^5 cells/cm^2. Immunofluorescence detection for α1 subunit and β1 subunit (Santa Cruz, Heidelberg, Germany) was carried out when cells reached 60% confluence as described previously. The used primary antibodies were mouse anti-Na^+/K^+-ATPase α1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit anti-Na^+/K^+-ATPase β1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), which was incubated with goat anti-mouse or goat anti-rabbit secondary antibody respectively (ZSGB-BIO, Beijing, China). Images were taken under confocal microscopy.

Total Protein Extraction and Western Blot Analysis

To further study the mechanism by which MSCs provided therapeutic benefits on AT-II cells, we developed co-culture system with AT-II cells (5×10^5 cells per well) grown on the Transwell chamber and placed in a 6-well plate, in which the bottom of each well was covered with MSCs (5×10^5 cells per well)[16]. In advance, AT-II cells were firstly exposed to PC (TNF-α 1.7 ng/ml, IL6 87.6 ng/ml and IL1-β 4.4 ng/ml) fol-

lowed by MSC treatment. Therapeutic effects were evaluated at the following situations: (1) AT-II cells with PBS (control group); (2) PC-exposed AT-II cells + PBS; (3) AT-II cells with MSCs; (4) PC-exposed AT-II cells + MSCs; (5) PC-exposed AT-II cells + MSCs-siRNA; (6) PC-exposed AT-II cells + MSCs-lipof.

After co-culture for 72h, total cellular protein was extracted from AT-II cells using 0.2 ml of radioimmunoprecipitation (RIPA) lysate per well, and Western blot assay was carried out according to the protocol. The used primary antibodies were mouse anti-Na^+/K^+-ATPase $\alpha 1$ (1:200 dilution; from Santa Cruz Biotechnology Inc., Dallas, TX, USA), rat anti-β Actin monoclonal antibody (1:800 dilution; ZSGB-BIO, Beijing, China) and rabbit anti-Na^+/K^+-ATPase $\beta 1$ (1:200 dilution; Santa Cruz Biotechnology, Inc.). The polyvinilydene fluoride (PVDF) membrane was incubated with the 1:4000 dilution of goat anti-mouse or goat anti-rabbit secondary antibody (ZSGB-BIO, Beijing, China) respectively for 2 hours. Protein on the PVDF membrane were visualized by enhanced chemiluminescence reagent detection reagents (Applygen, Beijing, China) and quantified with ImageJ2x software. All the protein expressions in different group were normalized to the expression of Actin protein.

Total RNA Extraction and qPCR Analysis

The mRNA levels of $\alpha 1$ or $\beta 1$ subunit were analyzed by two step quantitative real time PCR (qPCR) after co-culture for 72h. Total RNA was isolated from AT-II cells using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions and quality was assessed by the ratio of 260/280. The cDNA was prepared using C1000 Thermal cycler (Bio-Rad, Hercules, CA, USA). The cDNA was amplified in a MicroAmp® Optical 96-Well Reaction Plate using ABI 7500 in Fast Mode. Accumulating cDNA products were monitored by the ABI 7500 sequence detection system and data was stored continuously during the reaction. Primers for $\alpha 1$, $\beta 1$ subunit and rat Actin gene were obtained from Sangon Biotech co., Ltd (Shanghai, China) (Table I).

Statistical Analysis

All experimental groups were carried out at least three times. Results are expressed as the mean ± SD. AT-II Cell proliferation was assessed by analyzing the variance of repeated measurement data. Comparison between more than two groups were made by One-Way ANOVA method with SPSS13.0 software (SPSS Inc., Chicago, IL, USA) and $p < 0.05$ was considered statistically significant.

Results

Characterization of MSCs and AT-II Cells

Rat MSCs isolated from rat bone marrow were adherent and spindle-like cells, which later differentiated into the predominant mesenchymal lineages: adipocytes and osteocytes. MSCs seeded in a culture plate started from a single cell to a colony (Figure 1A to D), which was considered an important character of MSCs. Rat MSCs expressed phenotypic markers of CD29, CD44, CD90 and CD105, but not CD45 and CD34 as indicated in the results from flow cytometry (Figure 1E). Primary culture of rat AT-II cells were round flat adherent cells. Cells were identified by fluorescein isothiocyanate (FITC)-labeled surfactant protein-A (SP-A) (Figure 1F and G).

MSCs Increased AT-II Cell Proliferation

The result of the assay using Cell Counting kit-8 (CCK8 kit) demonstrated that exposure to PC led to decreased cell proliferation of AT-II cells compared with those in the normal culture medium at 1, 2 and 3 day. However, co-culture with MSCs increased proliferation of PC-exposed AT-II cells (Figure 2A and B).

KGF Secretion and Knockdown Efficiency by siRNA

KGF secretion of MSC was detected at 0.5h, 6h, 24h and 72h after inflammatory stimulus. Concentration of KGF was found higher as MSCs were cultured under inflammatory condition in comparison to that under non-inflammatory condition at 0.5h, 6h and 24h, respectively ($p < 0.05$). Simultaneously, KGF secretion approached the highest level in presence of PC at

Table I. The primer sequences used in the experiment.

ATP-α1 F	5'-CTCCTTCTGCCTGACGAACA-3'
ATP-α1 R	5'-GCTGTGATTGGATGGTCTCC-3'
ATP-β1F	5'-ATCAAGCTCAACCGAGTGCT-3'
ATP-β1R	5'-TGGA CAGGTAGGACGTTTGG-3'
Actin F	5'-CTAAGGCCAACCGTGAAAAGA-3'
Actin R	5'-CCAGAGGCATACAGGGACAAC-3'

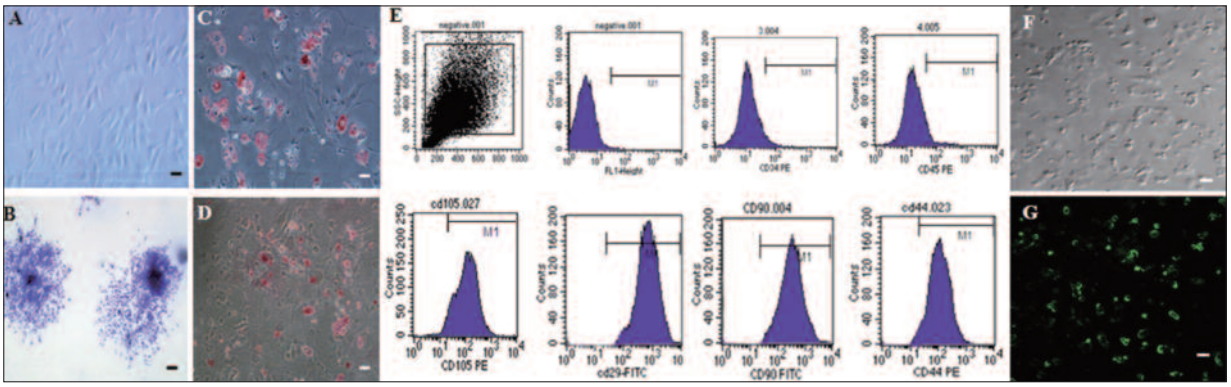

Figure 1. Characterization of mesenchymal stem cells (MSCs) and alveolar type-II cells (AT-II cells). ***A,*** Undifferentiated 5-passage MSCs. ***B,*** MSCs seeded in a culture plate started from a single cell to a colony. ***C,*** Adipogenesis was detected by oil red O. ***D,*** Osteogenesis was detected by alizarin red. ***E,*** Rat MSCs uniformly expressed the surface markers CD29, CD44, CD90 and CD105 and negatively express CD4, CD34 by flow cytometry. ***F, G,*** Isolated AT-II cells like round flat stone was identified by alveolar surfactant protein A (SP-A) stained with green fluorescence by immunofluorescence. (Scale bars: A, C and D =50 μm; B = 200 μm; F, G = 50 μm).

6h (Figure 3A). But we found that MSCs produced less KGF at 72h at inflammatory situation versus at non-inflammatory situation.

Majority of MSCs has been transfected with the cy5-labeled siRNA (red) in cytoplasm surrounding DAPI-stained nuclei (blue) (Figure 3B), which was photographed at 24h after transfection. Efficiency of knockdown was assessed by KGF secretion via ELISA at 48h after transfection. KGF concentration in culture medium of MSCs-siRNA decreased significantly after transfection. Meanwhile, KGF concentration of MSCs-lipof showed no significant difference compared with that of MSCs without transfection (Figure 3C).

MSC Tracing in LPS-injured Lung

MSC after intravenous administration was traced using small animal imaging technology. Signal intensity of DiI-labeled MSCs in rat lungs reached the highest level of 591.27 at 15 min after injection. Signal intensity of injected MSCs decreased gradually after 2h and 24h. Majority of MSCs, which decreased to 14.7%

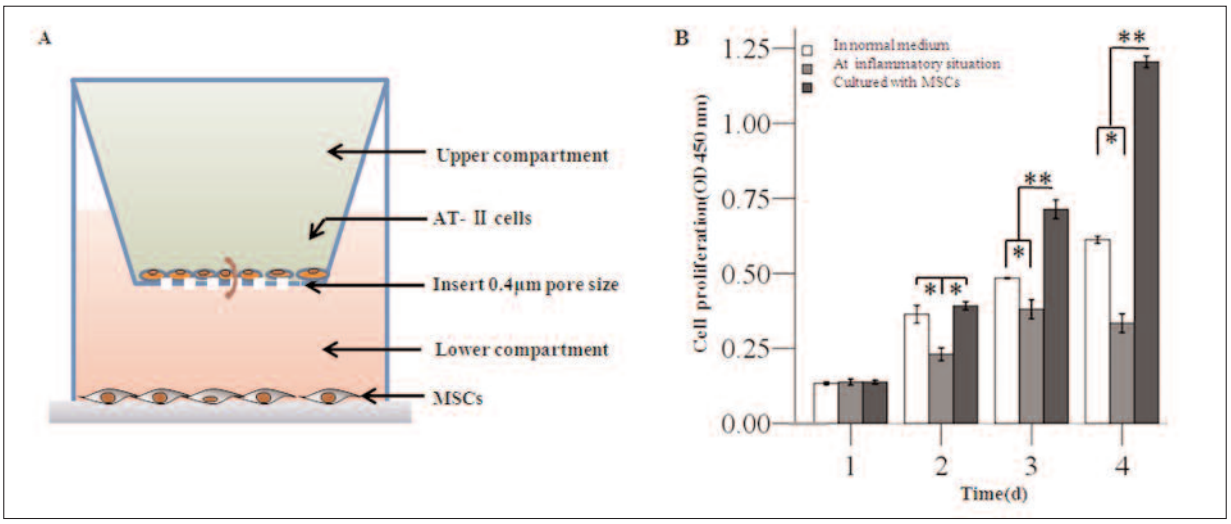

Figure 2. Co-culture with MSCs improved AT- II cell proliferation. ***A,*** AT-II cells were grown on upper compartment and MSCs were cultured in bottom compartment. ***B,*** AT-II cell proliferation was evaluated by cck8 assay. All data were expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$).

of original signal intensity, were lost from the lung at 72h (Figure 4A and B). Meanwhile, the distribution of injected MSCs was further visualized according to images of frozen sections at 15 min, 24h and 72h. Nuclei stained with DAPI (blue) were surrounded by DiI-labeled cytomembrane, which suggested that MSCs were clustered at 15 min and scattered at 24h and 72h in lung tissue according to images of frozen sections (Figure 4C).

Therapeutic Benefits from MSCs on LPS-induced ALI

Therapeutic effects were evaluated by the HE-stained lung histological sections and wet/dry ratio at 72h after LPS injection. The results demonstrated that LPS administration resulted in the marked inflammatory infiltrates and interalveolar septal thickening. Treatment with MSCs following LPS reduced inflammatory infiltrates and septal thickening, which was attenuated by KGF siRNA. However, MSCs-lipof improved the inflammatory reaction as well, which suggested that mock transfection had no side effect on MSCs. Meanwhile, we did not found the inflammatory reaction caused by PBS or MSCs (Figure 5A). Ratio of wet/dry in LPS-exposed rats increased (5.3 ± 0.3) compared with those of the rats received only PBS (4.3 ± 0.3). The ratio decreased (4.4 ± 0.2) when LPS-exposed rats were treated with MSCs, which increased again (4.9 ± 0.5) as further administered with MSCs-siRNA. Treatment with MSCs-lipof decreased the ratio as well. Injection with only MSCs did not lead to increased wet/ dry ratio (Figure 5B).

Effects of MSC on the Expression of α1 and β1 Subunits in AT-II cells

Fluorescence emitted from α1 subunit (red) and β1 subunit (green) were detected on AT-II cell membrane, and cell nuclei were stained with DAPI (Figure 6A). Western blot assay was performed at 72h for the six groups (Figure 6B). As shown, expression of protein α1 or β1 subunit was decreased after exposure to inflammatory cytokines ($p < 0.01$). Co-culture with MSCs increased the protein expression of α1 and β1 subunit in PC-exposed AT-II cells. This increased protein expression of α1 subunit was downregulated as PC-exposed AT-II cells were cultured with MSCs-siRNA, but we did not found the decreased expression of β1 subunit. Meanwhile, expressions of α1 and β1 subunit at mRNA level were evaluated. Exposure to the inflammatory cytokines decreased mRNA level of α1 and β1 subunits, which were increased when treated with MSCs. However, increased mRNA level of α1 subunit was down regulated by MSCs-siRNA. Same change was not observed for the β1 subunit. Expression of α1 or β1 at both mRNA and protein level showed no difference as PC-exposed AT-II cells were treated with MSCs-lipof in comparison to those treating with MSCs (Figure 6C and D).

Discussion

In the present study, we demonstrated that BM-MSC administration restored the impaired AFC in LPS-induced ALI rat, and therapeutic benefits were provided by MSCs-secreted KGF, which was mainly mediated by up-regulating α1 subunit of Na^+/K^+-ATPase in AT-II cells. These findings imply that restoring the impaired AFC may be one key action of MSCs in treating ALI.

We developed a co-culture system for AT-II cells and MSCs as described above. It was found that exposure to the proinflammatory cytokines resulted in decreased AT-II cell proliferation. Of interest, delayed AT-II cell proliferation was restored by co-cultured MSCs, which had no direct contact between them. To identify the potential mechanism by which MSCs provided therapeutic benefits for injured AT-II cells we focused on the paracrine factor secreted by MSCs and its influence on AT-II cells' ability in reabsorbing the residual pulmonary edema fluid during ALI.

KGF, one major paracrine soluble factor secreted by MSCs, was proven to be a potent mitogen stimulating AT-II cells proliferation both *in vivo* and *in vitro*[18,19]. It was reported that KGF administration increased active ion transport by upregulating the expression of α-epithelial sodium channel gene and the activity of Na^+-K^+-ATPase in AT-II cells[20,21]. We, therefore, detected KGF secretion in MSC culture medium, which showed that MSCs produced more KGF under inflammatory condition compared to that at the normal condition. The fact that the KGF levels reached the peak at 12h and 24h after stimulation suggested that MSCs might produce more KGF in response to the inflammatory cytokines at early stage. However, the level of KGF decreased at 72h under inflammatory condition. We speculated that this might be due to the fact that MSCs were starved for 24h using serum-free before inflammatory stimulus.

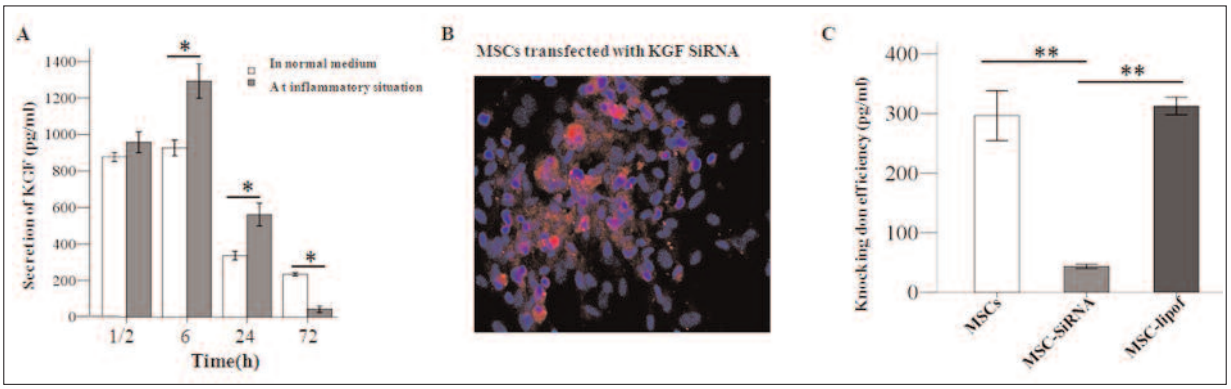

Figure 3. Inflammatory stimulus enhanced KGF secretion of MSCs. ***A,*** Concentration of KGF in MSC culture medium at inflammatory situation or normal situation at was measured by ELISA at different time points. Data were expressed as mean ± SD (pg/ml, n = 3 per group, $*p < 0.05$). ***B,*** Picture of MSCs transfected with cy5-labeled SiRNA (red) and stained with Dapi (blue) was taken by confocal fluorescence microscope. ***C,*** KGF concentration by ELISA was measured to evaluate knocking down efficiency at 48h after transfection with KGF SiRNA. Data were expressed as mean ± SD. (pg/ml, n = 3 per group, $**p < 0.01$).

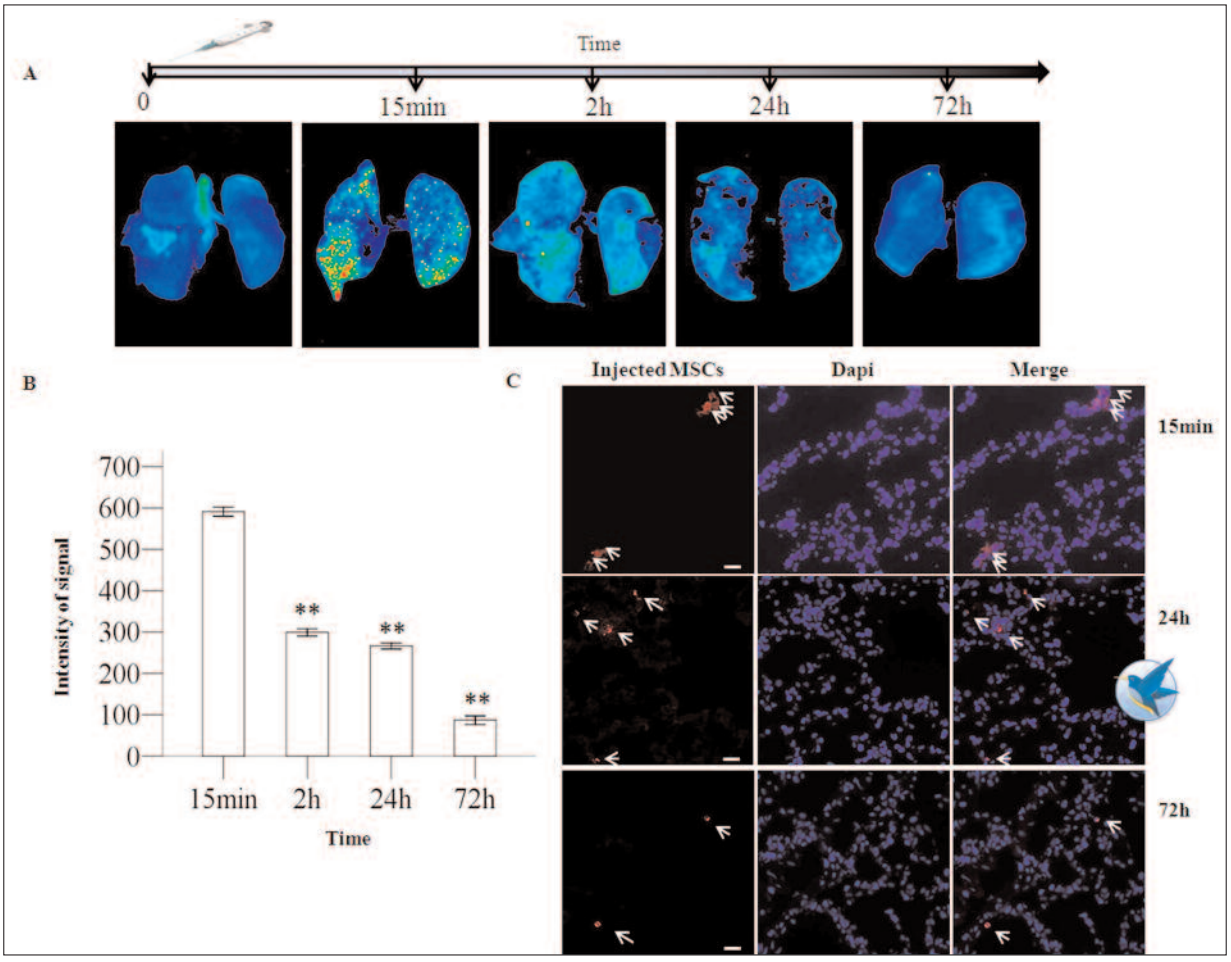

Figure 4. MSC tracing was determined with small animal imaging technology. ***A,*** DIi(red)-labeled MSCs in lung were traced at 0, 15 min, 2h, 24h and 72h after intravenous injection via rat tail vein. ***B,*** Signal intensity was quantified at different time points. Data were expressed as mean ± SD. (n = 5 per time point, $**p < 0.01$). ***C,*** Lung frozen sections (6 μm in thickness) showed that DIi and Dapi stained MSCs got clustered at 15 min and scattered gradually at the following time points. White arrow indicated that injected MSCs in lung tissue. (Scale bars = 50 μm).

According to previous researches, majority of the MSCs accumulated in lung tissue immediately after systemic administration[22,23]. In the present work, DiI-labeled MSCs were detected with adequate amount in lung at 15 min and decreased gradually after injection. Signal intensity decreased significantly at 72h. In addition, images of frozen section demonstrated that MSCs were clustered in lung tissue at 15 min and scattered gradually at the following time points. At 72h, only 14.7% of the injected MSCs could still be detected in lung. Hence, MSC engraftment in lung probably has provided a favorable condition for treating ALI.

In LPS-induced rat ALI model, residual fluid emerging in alveoli and increased ratio of wet/dry implied that AFC of alveolar epithelium, as well as permeability of alveolar-capillary, might be impaired by inflammatory insult. Pulmonary edema and increased wet/dry ratio were ameliorated simultaneously as LPS-exposed rats were treated with MSCs. This effect was attenuated when MSCs were pretreated with KGF siRNA, indicating that KGF might be responsible factor for this therapeutic effect.

We hypothesized that AFC of AT-II cells was impaired by inflammatory insult and restored by MSCs, which was further tested *in vitro*. As

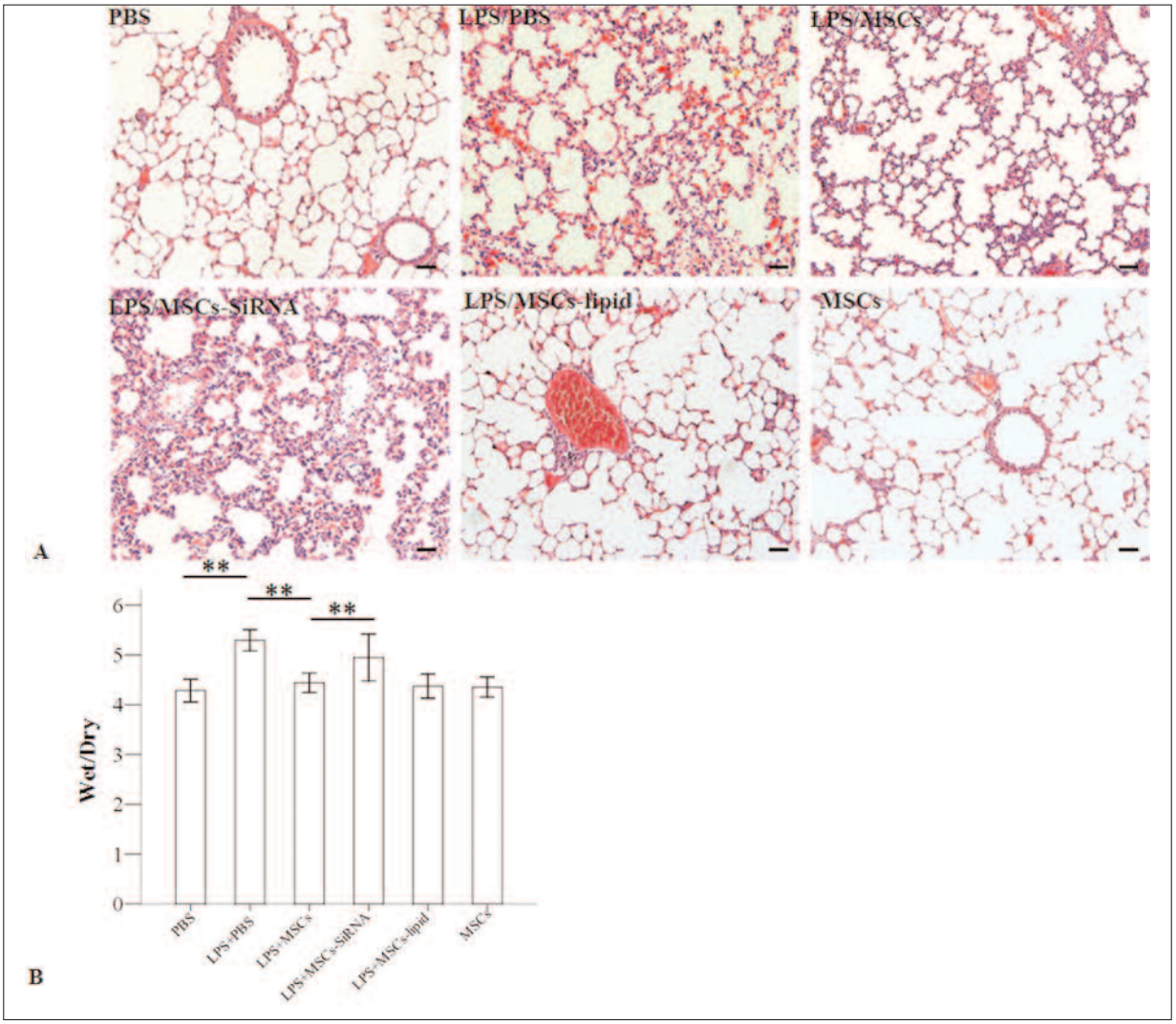

Figure 5. MSC administration attenuated LPS-induced rat ALI. ***A,*** Histological staining with hematoxylin and eosin (HE) was carried out to observe therapeutic effects on ALI from MSCs. MSC administration reduced LPS-induced injury, which were attenuated by MSCs-KGF SiRNA. Injection of phosphate buffered saline (PBS) or MSCs did not cause the inflammatory reaction. (Scale bars =50 μm). ***B,*** Ratio of Wet/dry was evaluated to further confirm therapeutic effects in six groups. Data were expressed as mean ± SD. (n=8 per group, $^{**}p < 0.01$).

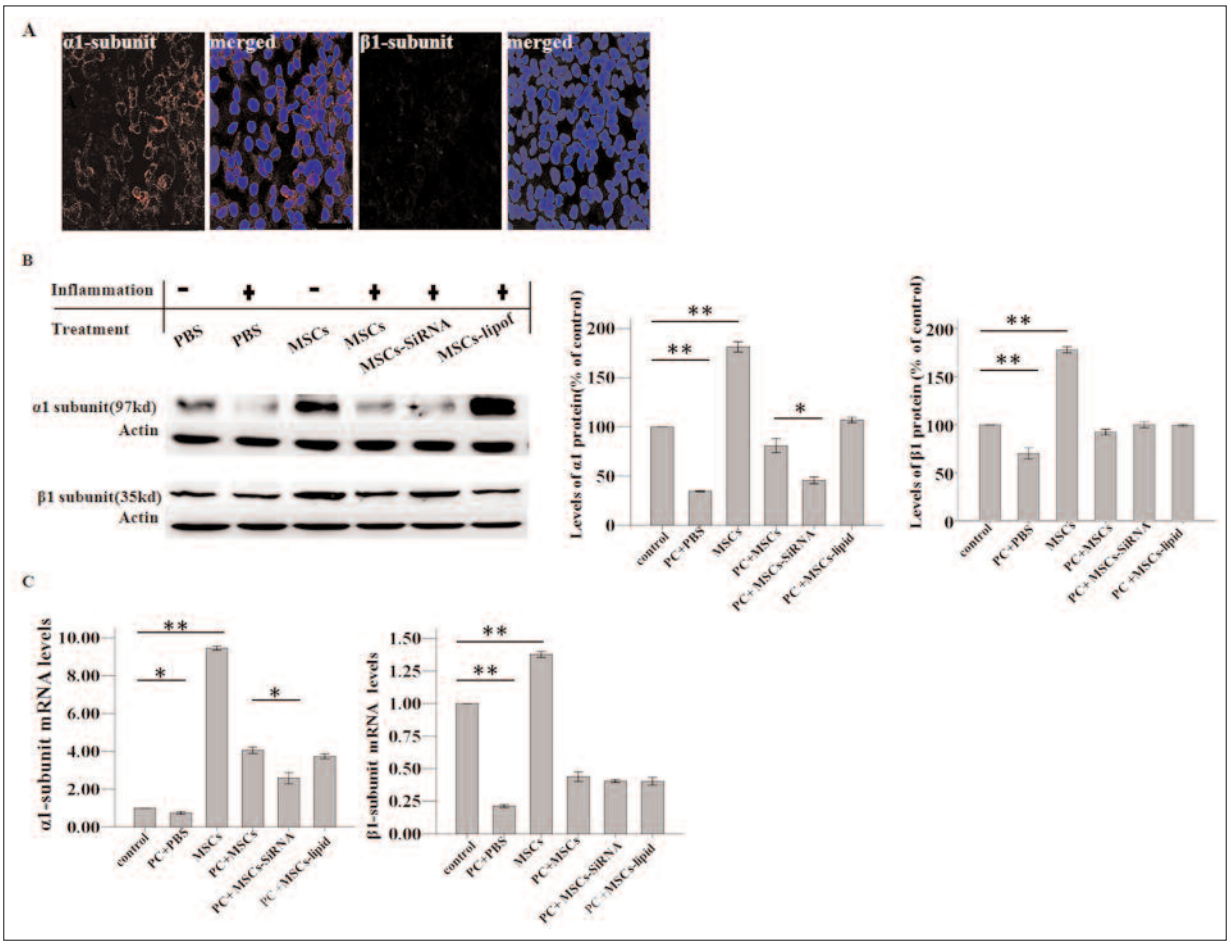

Figure 6. MSC administration increased the expression of α1 and β1 subunit at protein and mRNA level. ***A,*** Cy3-labeled α1 subunit and FITC-labeled β1 subunit by immunofluorescence were detected in cytomembrane of AT-II cells (Scale bars: A, B = 100 μm). ***B, C,*** Protein and mRNA level of α1 or β1 subunit by Western blot and qPCR were performed to observe therapeutic effects on Na^+/K^+-ATPase from MSCs under six different conditions. All experiments were run three times and protein expression of α1 or β1 subunit was normalized to control expression. All data were expressed as mean ± SD. (n=3 per group, $*p < 0.05$, $**p < 0.01$).

known, sodium channel and Na^+-K^+-ATPase are essential in transporting sodium to pulmonary interstitium. Na^+-K^+-ATPase is primarily comprised of α1 and β1 subunits. The α1 subunit functions as catalytic subunit, which pumps Na^+ out and transports K^+ into the cell against their respective concentration gradients by consuming ATP, while β1 is known as a regulatory subunit[24,25]. Our results showed that protein levels of both α1 and β1 subunit were down-regulated as AT-II cells were exposed to the proinflammatory cytokines, while the protein levels of α1 and β1 subunit were increased by MSCs. Expression of α1 subunit was down-regulated by MSCs pretreated with KGF siRNA, but the expression of β1 subunit did not display the same change. Therefore, it suggested that MSCs-secreted KGF might act through increasing α1 subunit expression instead of β1 subunit. Expressions of α1 and β1 subunit were further confirmed at the gene level, which was consistent with their protein expression. Hence, the therapeutic effect of MSC might be highly associated with ameliorating injuried AFC via increasing expression of α1 subunit in AT-II cells.

Additionally, some other potential factors produced by MSC might also contribute to reducing ALI, which deserve further research. Except for AT-II cells, AT-I cells was reported to play a role in clearing pulmonary edema as well[26]. Furthermore, chloride channel and aquaporin in pul-

monary epithelial cell might also participate in transporting pulmonary edema fluid, which also requires further investigation.

Conclusions

We demonstrated that AFC was impaired by inflammation insult, and MSCs-derived KGF reduced the impaired AFC through up-regulated $\alpha 1$ subunit but not $\beta 1$ subunit, which might provide a novel therapeutic strategy for ALI.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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